J Musculoskelet Neuronal Interact 2008; 8(2):154-165

Hylonome

Review Article

Osteotropic effects by the neuropeptides calcitonin gene-related peptide, substance P and vasoactive intestinal peptide

U.H. Lerner and E. Persson

Department of Oral Cell Biology, Umeå University, Umeå, Sweden

Abstract

Immunohistochemical phenotypic characterization of skeletal nerve fibers has demonstrated the expression of a restricted number of neuropeptides, including calcitonin gene-related peptide (CGRP), substance P (SP) and vasoactive intestinal peptide (VIP). According to the neuro-osteological hypothesis, such neuropeptides can be released and exert paracrine biological effects on bone cells present close to the nerve endings expressing these signaling molecules. The existence of such interplay is most convincingly shown by the hypothalamic control of bone formation, in the case of leptin stimulation of hypothalamic nuclei mediated by the sympathetic nervous system and inhibitory β-adrenergic receptors on osteoblasts. In addition to these receptors, osteoblasts and osteoclasts express functional receptors for CGRP, SP and VIP, which can regulate both bone formation and bone resorption. The evidence for these observations is summarized in the present paper.

Keywords: CGRP, SP, VIP, Osteoclasts, Osteoblasts

Introduction

The skeleton is not only a hard framework to which muscles and tendons are attached as a basis for our mobility, and a mineral reservoir susceptible to control by calcium-regulating hormones, it is an organ whose activities are influenced by many endocrine and paracrine signals, including sex hormones, glucocorticoids, cytokines and growth factors. Recently, it has been shown that the adipocyte-derived hormone leptin affects bone formation through hypothalamic and sympathetic nervous system relays[1-3]. Even more sensational, the osteoblast-derived extracellular matrix protein osteocalcin can act in a potential feedback hormonal manner to control energy metabolism[4]. In addition, the hypothalamic neuropeptide Y2 receptors are essential for control of bone metabolism by a peripheral mechanism not yet known[5].

The central nervous control of bone remodeling has been suggested by several authors given the reports during last decades that chemical and surgical denervation results in skeletal phenotypes, not only in loaded bones but also in unloaded parts of the skeleton[6]. Immunohistochemical analyses have shown that the skeleton is more widely innervated than previously thought and several neuropeptides and other nerve signaling molecules have been shown to be expressed by skeletal nerve fibers. Skeletal nerve fibers are present in the periosteum, cortical bone and bone marrow and are particularly rich at the osteochondral junction of the growth plate[7-9]. Hierarchically organized pathways have been described between femoral bone marrow and the brain and the spinal cord by injecting green fluorescent protein and β-galactosidase expressing pseudorabies virus[10]. Further evidence pointing to a role of the nervous system in bone remodeling are the findings that both osteoblasts and osteoclasts express functional receptors for a wide variety of signaling molecules in the peripheral nervous system[6]. During skeletal development, skeletal nerve fibers are particularly abundant in areas of bone formation, and in agreement with this observation, sprouting of nerve fibers has been observed during fracture healing[11]. Using an elegant model of angulated fracture healing, Li et al.[12] have shown that the presence of newly formed nerve fibers is particularly abundant in areas with a high rate of new bone formation. In some areas of the skeleton, certain skeletal nerve fibers are associated

The authors have no conflict of interest.

Corresponding author: Professor Ulf H. Lerner, Oral Cell Biology, Umeå University, 901 87 Umeå, Sweden
E-mail: ulf.lerner@odont.umu.se

Accepted 14 January 2008

Neuropeptide	Osteoblast receptors[a]	Osteoclast receptors[a]	Bone formation	Osteoclast activity	Osteoclast formation
CGRP	+	+	↑[b]	↓[c]	↓[d]
SP	+	+	↑↓[e]	↑[f]	↑[g]
VIP	+	+	↑[h]	↓[i]	↓[j]

[a] The presence of receptors has been indicated by mRNA expression, a rise of cyclic AMP/Ca^{2+}i in individual cells, or immunohistochemistry.
[b]CGRP stimulates osteoblast proliferation and increases bone mass *in vivo*.
[c]CGRP inhibits bone resorption *in vitro* and causes hypocalcemia *in vivo*.
[d]CGRP inhibits osteoclast formation in bone marrow cultures.
[e]SP has been reported to both inhibit and stimulate bone formation *in vitro*.
[f]SP stimulates rabbit osteoclast pit resorption area.
[g]SP potentiates RANKL-induced osteoclast formation in bone marrow macrophages.
[h]VIP stimulates ALP; VIP increases calcium accumulation in bone nodules.
[i]VIP causes an initial, transient, "calcitonin-like" inhibition followed by delayed stimulation of rat osteoclasts; VIP stimulates calcium release from mouse calvariae.
[j]VIP inhibits osteoclast formation in mouse bone marrow cultures.

Table 1. Receptors for CGRP, SP and VIP on bone cells and their effects on osteoblasts and osteoclasts.

with blood vessels but in other areas no such association can be seen. Associations with blood vessels have been shown to be dependent on the phenotype of the nerve fibers. Skeletal pain is a common clinical finding in several conditions, which severely affects mobility, rehabilitation and quality of life for many patients. The presence of sensory nerve fibers expressing calcitonin gene-related peptide (CGRP) and substance P (SP) in the skeleton provides one explanation to the clinical observations, but how these nerve fibers are activated and how the network of such nerve fibers are distributed and regulated in diseases such as osteoporosis, osteoarthritis, rheumatoid arthritis, cancer and fracture healing is essentially unknown.

Besides the possibility that skeletal nerve fibers have important roles in skeletal pain, accumulating evidence suggests that signaling molecules present in skeletal nerve fibers may also have a role in bone remodeling. In the present review, the osteotropic effects by CGRP, SP and vasoactive intestinal peptide (VIP), three neuropeptides expressed in skeletal nerve fibers, are summarized.

Calcitonin gene-related peptide (CGRP)

CGRPs belong to the calcitonin (CT) superfamily of peptides including also CT, amylin, adrenomedullin and the two recently discovered peptides intermedin and calcitonin receptor-stimulating peptide (CRSP)[13]. CGRP exists in two different forms, α-CGRP and β-CGRP. Both peptides consist of 37 amino acids and differ only by three amino acid residues in humans. α-CGRP is encoded by the same gene as calcitonin (CT) and alternate processing of the CT/α-CGRP transcript is the mechanism regulating the cell specific formation of either α-CGRP or CT. α-CGRP, which only has some few amino acid homologies with CT in the amino terminal moiety, is widely expressed in nerves in both the peripheral and central nervous system, including skeletal nerve fibers. CT is almost exclusively expressed in parafollicular C-cells in thyroid glands. β-CGRP is encoded by a gene distinct from the CT/α-CGRP gene and is expressed in nerve fibers in numerous different tissues. It is not known which is the physiological role of α-CGRP versus β-CGRP expression.

Amylin is a 37 amino acid peptide expressed in pancreatic β-cells and is almost identical with CGRP in the amino terminal end. Adrenomedullin is a 52 amino acid peptide expressed in many different cells and tissues, including osteoblasts and macrophages. In contrast to other peptides in the CT family, adrenomedullin does not have a disulphide bridged amino terminal loop but instead an amino terminal linear extension. Intermedin was identified as a peptide with amino acid homologies to the peptides in the CT family using a phylogenetic profiling approach to analyze the GenBank/EBI DataBank. Intermedin has approximately 20% homology with CGRP and is expressed in the gastrointestinal tract and in the pituitary gland. CRSP was identified in porcine brain and thyroid glands and was found to have 60% homology with CGRP although having several structural and pharmacological features distinct from CGRP. So far, three CRSPs have been identified and the expression has been found in porcine, bovine and canine species, but not in humans and rats.

The peptides in the CT superfamily can activate target cells through a complex family of receptor proteins made up by the calcitonin receptor (CTR), calcitonin receptor-like receptor (CRLR) and three receptor activity-modifying proteins (RAMPs)[14]. CTR is a seven transmembrane G-protein coupled receptor, which acts as a ligand binding and signal-

ing transducing receptor when activated by CT or CRSP-1. CTR dimerized with RAMP1-3 can function as a receptor for amylin. CTR is not expressed by macrophages/early osteoclast progenitor cells but is induced during osteoclastogenesis by activation of receptor activator of NF-κB (RANK) by its ligand RANKL and is abundantly expressed in terminally differentiated multinucleated osteoclasts. CTR is not expressed in osteoblasts.

CRLR also belongs to the type II family of G protein-coupled receptors and has, overall, 55% homology with CTR and 80% homology in the transmembrane moiety. It functions as a receptor for either CGRP or adrenomedullin, ligand specificity depending on to which of the RAMPs CRLR heterodimerizes. CRLR mRNA is expressed in primary osteoblasts and osteoblastic cell lines. CRLR mRNA has also been detected in multinucleated osteoclasts[13]. We have found CRLR mRNA and protein to be expressed also in mononucleated osteoclast progenitor cells[15]. Activation of RANK results in a transient enhancement of CRLR mRNA but not of CRLR protein in the osteoclast progenitor cells.

RAMPs are type I single transmembrane proteins with a large extracellular amino terminal domain and a short cytoplasmic moiety[13]. RAMP1 is a 148 amino acid peptide and the three RAMPs have approximately 30% amino acid homology. RAMPs are important for post-translational glycosylation of CRLR and its translocation to the cell membrane. RAMPs were initially found to be able to heterodimerize with CRLR, but later also to CTR and some other cell membrane receptors, including VIP receptors. CRLR/RAMP1 makes up the receptor for CGRP and CRLR/RAMP2 or RAMP3 acts as a receptor for adrenomedullin. CTR can dimerize with all three RAMPs, thus making up receptors for amylin. CRSP1 can activate CTR but CRSP2 and 3 cannot activate CTR, nor CRLR/RAMPs. Intermedin can activate CRLR/RAMP. Primary osteoblasts and osteoblastic cell lines express all the RAMP mRNAs[13]. Multinucleated osteoclasts have been reported to express not only mRNA for CTR and CRLR but also RAMP2 mRNA, but not RAMP 1 or 3 mRNA[16], a finding hard to reconcile with the observation that multinucleated osteoclasts are responsive to CGRP. At variance, we have found all three RAMPs, both at mRNA and protein level, to be expressed in mononucleated osteoclast precursor cells[15]. Stimulation by RANKL transiently decreases RAMP1 mRNA, but does not affect any of the RAMP proteins all of which could be detected also late during osteoclastogenesis in mouse bone marrow macrophages stimulated by M-CSF and RANKL when very many multinucleated osteoclasts were present.

The first evidence that CGRP can affect bone cells was the finding that injection of CGRP into either rats or rabbits resulted in a hypocalcemic response, similar to that caused by CT, studies prompted by the amino terminal homologies between CGRP and CT[17,18]. The observations that CGRP, also similarly to CT, inhibits bone resorption in organ cultured fetal rat long bones and in neonatal mouse calvariae[19-21] supported the view proposed in the initial reports that the hypocalcemic response was due to CGRP-induced inhibition of bone resorption. The inhibitory effect by CGRP on bone resorption is transient, also similar to that caused by CT, a phenomenon called "escape from calcitonin-induced inhibition of bone resorption". This phenomenon is associated with a disappearance of CTR mRNA, which could be the reason for the escape phenomenon. If a similar disappearance of CGRP receptors on osteoclasts is the reason for the "escape from CGRP-induced inhibition of bone resorption" is not known. We have, however, been unable to observe any effects by CGRP on the mRNA expression of CRLR nor RAMP1-3 during M-CSF/RANKL stimulated osteoclastogenesis in mouse bone marrow macrophage cultures, neither in the absence nor in the presence of CGRP, in contrast to the complete inhibition by CT of RANKL-induced expression of CTR mRNA[15]. Interestingly, inhibition of bone resorption in neonatal mouse calvarial bone by cyclic AMP analogues and forskolin is also associated with "escape from inhibition", which suggests that down-regulation of receptors is not the mechanism explaining the escape phenomenon to CT and CGRP. Also amylin can inhibit bone resorption in organ cultures and isolated osteoclasts and also these responses are transient[6]. In contrast, adrenomedullin does not affect bone resorption in organ cultures. The reason for the lack of effect by adrenomedullin is not known, but seems not to be explained by the absence of receptors (CRLR/RAMP2 or 3). Recently we have observed that also intermedin and CRSP inhibit PTH-stimulated bone resorption in neonatal mouse calvariae, a response also associated with the escape phenomenon[22]. The effect on ^{45}Ca release in mouse calvariae by CGRP and by CT, amylin, intermedin and CRSP, can be observed at very early time points (1-3 hours) in bone pre-stimulated by PTH for 24 hours, which indicates that the escape phenomenon is primarily due to effects on multinucleated osteoclasts (Granholm et al. in preparation).

CGRP, similar to CT and amylin, inhibits the bone resorbing activity of isolated rat osteoclasts, effects associated with ceased motility of the osteoclasts. In contrast to CT, however, CGRP does not affect osteoclast retraction, nor does CGRP cause a rise of intracellular Ca^{2+} [23-25]. These observations indicate that CGRP and CT act on different receptors, in agreement with the view that CT acts via CTR and CGRP via CRLR/RAMP1. The effects by CGRP on bone resorption and osteoclast activity are observed at considerably larger concentrations than CT which has led some authors to speculate that CGRP, at high concentrations, also may have an affinity to CTR.

Most studies on the effect by CGRP and CT on hypocalcemia and bone resorption have been directed to studies on their effects on terminally differentiated multinucleated osteoclasts. However, the rate of bone resorption is dependent also on the formation of osteoclasts. Interestingly, it has

been shown that CGRP can inhibit osteoclast formation in co-cultures of mouse alveolar macrophages and mouse calvarial osteoblasts stimulated by 1,25$(OH)_2$-vitamin D_3[26] and in mouse bone marrow cultures stimulated by 1,25$(OH)_2$-vitamin D_3[27,28]. Some earlier studies have reported that CT also has a similar action[29,30]. We have recently reported that the inhibitory effect by CT and CGRP on osteoclastogenesis can be seen in highly purified osteoclast progenitor cells stimulated by M-CSF and RANKL[22,31]. These observations are in line with the finding that mononucleated osteoclast progenitor cells stimulated by RANKL express CTR, CRLR and RAMP1 mRNA and protein[15]. Interestingly, the inhibitory effect on osteoclast formation was not associated with any effects on the expression of a wide variety of molecules known to be induced by M-CSF and RANKL and found to be important for osteoclast differentiation, fusion and activity[31].

Although CGRP and CT have well-established inhibitory effects on bone resorption *in vitro* and *in vivo*, their physiological roles in bone remodeling have been questioned. This has particularly been the case for CT, since thyroidectomy seems to have no effect on mineral metabolism or on bone remodeling. The uncertainty of the role of CT and α-CGRP on bone resorption *in vivo* became even larger when it was found that homozygous knockout of the *CT/α-CGRP* gene did not result in any obvious effects on bone resorption, besides the fact that the *CT/α-CGRP* deleted animals were slightly more responsive to an injection of PTH in terms of hypocalcemia and increased excretion of deoxypyridinoline[32]. Surprisingly, the *CT/α-CGRP*$^{-/-}$ mice did not have any osteoporotic phenotype, as expected, but exhibited enhanced bone mass, because of a robust stimulation of bone formation rate. There is very little evidence to indicate that osteoblasts express CTR and, therefore, the effect by CT on bone formation is not likely due to an effect by the hormone directly on osteoblasts, but by CTR expressing cells outside the skeleton. Since the *CT/α-CGRP* null mice are deficient not only in CT but also in α-CGRP, the possibility exists that the skeletal phenotype is due to lack of α-CGRP rather than CT, a possibility not unlikely since osteoblasts respond to CGRP (see below). However, since *in vitro* and *in vivo* studies have shown CGRP to be a stimulator of bone formation[13], α-CGRP deficiency is not likely to be the cause of the enhanced bone formation rate in *CT/α-CGRP* null mice. The subsequent selective deletion of the *α-CGRP* gene has also further supported this view since these mice display the expected osteopenic phenotype caused by the decreased rate of bone formation[33].

The fact that mice with heterozygous deletion of the *CTR* gene (*CTR*$^{-/-}$ mice die as embryos before skeletogenesis) also display enhanced bone mass due to an increased rate of bone formation[34], further supports the view that CT is not primarily a physiological regulator of bone resorption but rather a hormone controling bone formation. Recently, however, it was shown that the excessive loss of bone mass (24%) observed during lactation in wild-type mice was considerably larger (54%) in *CT/α-CGRP* null mice, indicating that one important physiological role of CT is to protect the maternal skeleton against enhanced bone resorption during lactation[35]. In summary, the role of CGRP and the other members of the CT superfamily peptides on bone resorption is complex and to a large extent still elusive.

Interestingly, inactivation of the *amylin* gene results in mice which display an osteoporotic phenotype due to enhanced bone resorption[34], which is in line with the *in vitro* observations showing that amylin is an inhibitor of osteoclastic activity and formation. It was, however, not anticipated that amylin, which is co-stored with insulin in pancreatic β-cells, could be a physiological regulator or bone resorption. The fact that an identical phenotype could be observed in compound heterozygote mice for *CTR* and *amylin*-deficiency demonstrates that amylin does not act via the CTR, an observation in line with our finding that amylin can enhance cyclic AMP formation in mononucleated osteoclast progenitor cells not expressing the CTR[15]. The *amylin* deficient mice did not display any osteoblastic or bone formation phenotype, in contrast to the *in vivo* and *in vitro* studies showing that amylin is a stimulator of bone formation.

Osteoblastic cell lines and primary osteoblasts from humans, rats and mice have been shown to exhibit functional CGRP receptors linked to enhanced cyclic AMP formation[36-39]. In line with this observation, the rat osteoblastic cell line UMR 106-01, the mouse osteoblastic cell line MC3T3-E1, as well as rat and mouse calvarial osteoblasts have been found to express CRLR mRNA and protein[33,40,41] (Granholm and Lerner, in preparation). Mouse calvarial osteoblasts not only express CRLR mRNA, but also RAMP1, RAMP2 and RAMP3 mRNA. It is not known if CRLR and RAMPs are regulated during osteoblastic differentiation, but the fact that CGRP-induced cyclic AMP formation is related to enhanced expression of alkaline phosphatase[37] suggests such a possibility.

Activation of osteoblastic CGRP receptors results in enhanced osteoblast proliferation *in vitro*[42]. Transgenic mice expressing CGRP under control of the osteocalcin promoter display enhanced bone mass due to increased bone formation[43]. These observations, together with the finding that selective *α-CGRP* null mice exhibit decreased bone mass due to reduced bone formation rate[33], suggest that α-CGRP has a physiological role as a stimulator of bone formation. It is likely that it is α-CGRP expressed in skeletal nerve fibers, which are crucial for its role in bone formation, although this remains to be proven. Sprouting of CGRP-expressing nerve fibers have been demonstrated during fracture healing and suggested to be involved in the anabolic response[12], a view supported by the observation that capsaicin treatment, destroying CGRP-expressing nerve fibers, is associated with decreased bone mass[44]. Mice with deletion of the *α-CGRP* gene still express β-CGRP and it will be important to know the skeletal phenotype of *β-CGRP* knockout mice and also that of *α-CGRP*$^{-/-}$/*β-CGRP*$^{-/-}$ mice to gain more insight into the role of CGRP in bone remodeling.

Substance P

SP is an 11 amino acid peptide which is widely distributed in the peripheral and central nervous system[45,46]. SP belongs to the tachykinin family also including neurokinin A (NKA), NKB, neuropeptide K and neuropeptide γ. The tachykinins act via three distinct receptor subtypes, the neurokinin-1 receptor (NK_1-R), NK_2-R and NK_3-R and SP has the largest affinity for the NK_1-R. SP is synthesized in dorsal root ganglions and is expressed in terminal nerve endings where it is released through axon reflexes. Interestingly, SP is often co-expressed with CGRP in sensory nerves. SP is known to be involved in many physiological and pathophysiological processes including vasodilation, extravasation, smooth muscle contraction, pain transmission and angiogenesis. Release of SP can result in neurogenic inflammation and, as summarized here, SP has also been suggested to be involved in bone remodeling.

SP-positive nerve fibers have been demonstrated in various locations in the skeleton, including the periosteum, bone marrow, epiphyseal growth plate, subchondral bone, ligaments and the synovium, using immunohistochemical analysis[47]. The expression of receptors for SP in osteoblasts is controversial since NK_1-R has been immunolocalized in rat bone osteoblasts[48], but it was not possible to demonstrate NK_1-R in primary human osteoblasts or osteosarcoma cell lines using RT-PCR[49]. Functional receptors for SP have, however, been demonstrated as assessed by enhanced bone colony formation[50]. Interestingly, Goto et al.[51], recently reported that rat calvarial osteoblasts, at an early stage of differentiation, do not express NK_1-R, but that NK_1-R mRNA and protein could be demonstrated when the osteoblasts were more differentiated along the osteoblastic cell line, similar to what has been shown for the $VPAC_1$ receptors[52]. The addition of SP to the rat calvarial osteoblasts enhanced mineralized bone noduli formation and the expression of cbfa1, osteocalcin and type I collagen, effects inhibited by two different NK_1-R antagonists (spantide, FK888). In contrast, Azuma et al.[53] have reported that SP inhibits bone noduli formation in rat calvarial osteoblast cultures and the expression of bone sialoprotein and osteocalcin. Using rat bone marrow stromal cells, it has been shown that SP causes a small stimulation of thymidine incorporation and a strong inhibition of calcium accumulation in bone noduli, associated with a small decrease of alkaline phosphatase activity[54]. Thus, osteoblasts, at least more terminally differentiated cells, seem to express functional receptors for SP, although it is still controversial if activation of these leads to enhanced or decreased bone formation.

In multinucleated rat osteoclasts, NK_1-R has been demonstrated both on the cell membrane and in the cytoplasm[48]. The fact that SP causes an acute rise of intracellular calcium in isolated rabbit osteoclasts shows that functional SP receptors are expressed by terminally differentiated osteoclasts[55]. This view is further supported by the finding that SP enhances the bone resorbing activity of such isolated osteoclasts. SP can also stimulate bone resorption in cultured neonatal mouse calvarial bones[56], which may be due either to enhanced osteoclast formation and/or increased osteoclast activity. If the expression of NK_1-R is regulated during osteoclastogenesis is not known but the fact that SP has been shown to potentiate the osteoclastogenic response to RANKL[57], an effect associated with increased translocation of the NF-κB subunit p65 from the cytosol to the nuclei in osteoclast progenitor cells[57], indicate that also mononuclear osteoclast precursor cells express receptors for SP. If SP also may be involved in osteoclast formation by affecting the RANKL/OPG ratio in bone marrow stromal cells or periosteal osteoblasts is not known, but not unlikely since SP can enhance RANKL and decrease OPG expression in rat synovial cells[58]. Interestingly, experimental spinal cord injury in rats results in an osteoporotic phenotype in proximal tibiae due to enhanced osteoclastic resorption which is associated with a significant increase of SP-immunoreactive nerve fibers[59], in line with the *in vitro* observations that SP can enhance osteoclast activity.

Polyethylene particle-induced local osteolysis in mouse calvarial bones *in vivo*, used as an experimental model of aseptic loosening of joint replacements, results in an increased number of osteoclasts and decreased bone volume. Interestingly, the responses were significantly reduced in *SP* null mice[60], suggesting the possibility that SP may have a role in particle-induced inflammatory bone loss in patients with loose joint prosthesis. The presence of SP immunoreactive nerve fibers in the interface membrane present in aspetically loosened prosthesis in humans[61] is in line with this hypothesis.

Vasoactive intestinal peptide

Vasoactive intestinal peptide (VIP) is a member of the VIP/secretin/glucagon family of neuropeptides, which besides VIP currently also includes pituitary adenylate cyclase-activating peptide (PACAP)-27 and PACAP-38, secretin, glucagon, glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), growth hormone-releasing hormone (GHRH), glucose-dependent insulinotropic polypeptide (GIP), and peptide histidine isoleucine amide (PHI; mouse)/peptide histidine methionine (PHM; human). All members are structurally related through a highly conserved amino terminal end and are products of six different genes. Although GIP has not been identified as a neuropeptide, the family is referred to as a family of neuropeptides. It can also, however, be considered a family of hormones because of the presence and hormonal effects of all family members in the gut and other tissues.

In 1969, the presence of an unidentified, potent vasodilator was demonstrated in porcine lung tissue[62]. It was also found in porcine gut, from where it was isolated as a vasore-

laxant peptide[63]. The isolated peptide was named vasoactive intestinal polypeptide and was later shown to be a 28 amino acid protein related to both secretin and glucagon[64-66]. After the first discovery as a vasodilator, the presence of VIP was demonstrated in the central and peripheral nervous systems, as well as several neural cell lines[67], and it has thereafter been identified as a neurotransmitter and neuromodulator in many tissues. In the brain, VIP is predominantly found in the cerebral cortex but also to a lesser extent in other structures, and it has been shown to regulate neuronal differentiation and survival in an indirect manner primarily mediated by glial cells[68,69]. VIP also affects the function of several other systems, comprising the pulmonary, cardiovascular, gastrointestinal, reproductive, immune, and endocrine systems[70-77]. Besides the neuromodulatory functions in the brain, additional physiological effects of VIP are broncho- and smooth muscle dilation, hormonal secretion, increase of gastric motility, regulation of differentiation and activity of many cell types, and anti-inflammatory and immunosuppressive actions through regulation of immune cell function.

After the first pioneering study demonstrating the presence of the neuropeptide SP in bone by Grönblad and coworkers in 1984, many reports followed on detection in different skeletal sites in several species of several neuropeptides, including VIP. Hohmann and colleagues reported that VIP-containing nerve fibers of post-ganglionic sympathetic origin were localized in the periosteum, associated with vascular elements of bones from different species[78]. In a study of neuropeptide expression in rat bone nerve fibers, Bjurholm et al. detected VIP preferentially in the periosteum and the epiphysis. These VIP-containing nerve fibers of uncertain origin were only occasionally associated with blood vessels[79]. In line with these data, Hill and Elde also demonstrated VIP-containing nerve fibers in rat periosteum. Treatment of rats with guanethidine resulted in a dramatically decreased presence of VIP-containing nerves in the bone tissue, indicating a sympathetic origin of these nerves[8].

During the 1990s, three different subtypes of VIP receptors were cloned. The receptors for VIP belong to the type II family of G protein-coupled receptors denoted the VIP/secretin/PTH receptor family, which also includes receptors for the related proteins secretin, GHRH, GIP, and glucagon, as well as PTH/PTHrP, calcitonin, and CGRP[80]. All receptor family members have a serpentine, seven transmembrane structure with a long N-terminal extracellular domain with conserved cysteine residues and a cytoplasmic tail that activates G proteins as a result of ligand binding.

VIP and the related peptide PACAP share many biological functions, which can be explained by the fact that they largely use the same receptors. The three receptors are designated $VPAC_1$, $VPAC_2$, binding both VIP and PACAP, and the PACAP-preferring PAC_1 receptor[81]. All three receptors are present both in the central and peripheral nervous systems and can be pharmacologically characterized by their affinity for VIP, PACAP, and secretin. PAC_1 preferentially binds PACAP but also VIP, although with a 100-1,000 times lower affinity. Both $VPAC_1$ and $VPAC_2$ bind VIP and PACAP with equal affinity, which is a reason for the comparable effects of VIP and PACAP in many tissues and cell types. However, the $VPAC_1$ receptor also binds secretin, a fact that can be used to distinguish between the involvement of the different receptor subtypes in signaling[81,82].

In 1983, three years before the discovery of VIP-containing nerves in bone, Hohmann et al. provided the first *in vitro* evidence for the presence of functional neuropeptide receptors on bone cells, demonstrating that VIP stimulates calcium release in neonatal mouse calvariae[83]. In osteoblasts, the presence of functional receptors for VIP, linked to enhanced formation of cyclic AMP, has been demonstrated in cells from the human osteosarcoma cell line Saos-2 and the rat osteosarcoma cell line UMR 106-01[37,84,85]. In addition, VIP has been found to stimulate cyclic AMP formation in isolated mouse calvarial osteoblasts and in the cloned, non-transformed osteoblastic cell line MC3T3-E1[37,85]. Our group has characterized the VIP-binding receptors subtypes in mouse calvarial osteoblasts. By comparing the rank order of response of peptides in the VIP/secretin/glucagon family on cyclic AMP formation, we found that PACAP-38 was 10-fold more potent than VIP[52]. A similar 10-fold difference in potency between PACAP and VIP has also been detected in the rat osteoblast-like tumor cell line UMR 106-01[86] and in the murine osteoblastic cell line MC3T3-E1[87]. By using atomic force microscopy (AFM), we have demonstrated specific binding of VIP, but not secretin, on neonatal mouse calvarial osteoblasts[88]. Furthermore, mRNA for $VPAC_2$, but not for $VPAC_1$ or PAC_1, was also detected in these undifferentiated osteoblasts. Interestingly, the expression of $VPAC_1$ mRNA was shown to be differentiation-induced after 12 days of culture[52]. In contrast to our results, Togari and coworkers demonstrated that human periosteum-derived osteoblastic cells and cells from several human osteosarcoma cell lines express $VPAC_1$, but not $VPAC_2$ or PAC_1[49]. The observed differences in VIP receptor expression in mouse and human osteoblasts may be a matter of species variations or related to phenotypic differences between tumour cell lines and primary osteoblasts.

In addition to studies on osteoblasts, the presence of receptors for VIP and related neuropeptides on osteoclasts has also been reported. By using AFM and measurements of intracellular calcium, specific VIP-binding sites on osteoclasts were found[52]. Further evidence for the presence of VIP receptors in osteoclasts is our finding of mRNA for $VPAC_1$ and PAC_1, but not $VPAC_2$, in mouse bone marrow osteoclasts isolated by micromanipulation[89].

The presence of functional receptors for VIP on both osteoblasts and osteoclasts suggests that this neuropeptide, localized in nerves in the bone microenvironment, may play an important role as a local mediator regulating bone cell

function. In osteoblasts, VIP was found to stimulate both the activity and mRNA expression of the bone mineralization-associated enzyme alkaline phosphatase (ALP)[90]. This effect was seen in osteoblasts after 6 days of culture, and this observation, together with the absent effect by secretin, indicated that the increased differentiation of committed osteoblasts and the anabolic effects by VIP in bone are mediated by the $VPAC_2$ receptor, which is in line with our finding that mouse calvarial osteoblasts, at an early stage of differentiation, express $VPAC_2$ mRNA[52].

The first documented effect by VIP on bone was that of Tashjian and co-workers demonstrating a catabolic effect by VIP on bone metabolism in calvarial bones *in vitro*[83]. This stimulatory effect by VIP on calvarial bone resorption may be due either to enhanced activity of osteoclasts or to stimulation of osteoclast formation. Our group has, using morphological studies of isolated rat bone marrow osteoclasts, revealed that VIP treatment causes a rapid cytoplasmic contraction of osteoclasts along with an associated decrease in motility[88]. Functional studies using an *in vitro* resorption assay showed that VIP caused a transient inhibition of osteoclastic bone resorption in devitalized bone slices. When the osteoclast incubations were extended over time and performed in the presence of marrow-derived stromal cells/osteoblasts, the osteoclasts escaped from the initial inhibition and VIP caused a delayed stimulation of osteoclastic pit formation in bone slices. At variance, CT also causes a transient inhibition of osteoclastic bone resorption, which, however, is not followed by a delayed stimulation. The late stimulatory effect of VIP is probably the basis of the first finding that VIP stimulates resorption in calvarial organ culture[83]. When AFM was used to analyze the presence of VIP receptors in stromal cells/osteoblasts, we found that stromal cells/osteoblasts expressed specific binding sites for VIP[88]. These receptors might mediate the indirect bone-resorbing effect caused by VIP, both in the resorption pit assay and in the calvariae. When osteoclastogenesis was studied in mouse bone marrow cultures, VIP did not enhance the number of osteoclasts[91]. In contrast, VIP caused an inhibition of osteoclast formation induced either by $1,25(OH)_2$ vitamin D_3 or by PTH. The anti-osteoclastogenic effect of VIP is associated with inhibitory effects of these peptides on the $1,25(OH)_2$ vitamin D_3-induced upregulation of receptor activator of NF-κB (RANK) and RANK ligand (RANKL). In addition, VIP counteracts the decrease of osteoprotegerin (OPG) caused by $1,25(OH)_2$ vitamin D_3[91]. VIP is not only able to inhibit osteoclastogenesis through VIP receptor expression on stromal cells/osteoblasts, but also through a direct effect mediated by VIP receptors on osteoclast progenitor cells. In ongoing studies, we have found that VIP can inhibit osteoclast formation in M-CSF and RANKL-stimulated purified bone marrow macrophage (BMM) cultures (Mukohyama et al. in preparation). Similarly, CT inhibits osteoclastogenesis in M-CSF and RANKL-stimulated BMM cultures[31]. This effect is not associated with any regulation of the mRNA expression of a wide variety of M-CSF/RANKL-induced osteoclastic genes. If the inhibitory effect by VIP on osteoclast formation is similarly associated, without any effects on gene expression, is currently under investigation.

In addition to bone marrow cultures, we have also studied the effects by VIP on the expression of osteoclastogenic factors in isolated calvarial osteoblasts. In previous reports, VIP has been shown to stimulate IL-6 production in stromal cells[92] and in cells from the rat osteosarcoma cell line UMR 106-01[93]. We have confirmed these observations using mouse calvarial osteoblast. VIP was shown to stimulate IL-6 mRNA and protein expression in both a time- and concentration-dependent manner[94]. Furthermore, VIP caused enhanced IL-6 promoter activity in cells from the osteoblastic cell line MC3T3-E1 transfected with an IL-6 promoter construct coupled to a luciferase reporter gene. By using several techniques, including electrophoretic mobility shift assay (EMSA), we also showed that treatment of osteoblasts with VIP resulted in activation of the cAMP/PKA pathway and increased DNA binding of the transcription factor C/EBP, whereas the DNA binding of the AP-1 complex was decreased by VIP. In contrast, the regulation by VIP of IL-6 synthesis did not require activation of the NF-κB pathway[94]. In addition to the effects by VIP, PACAP-38 also increased IL-6 synthesis whereas secretin did not have any effect, indicating that the effects by VIP and PACAP-38 are mediated by the $VPAC_2$ receptor[94].

VIP is a well-known regulator of inflammatory cytokine production in several cell types[95]. After our first study showing stimulatory effects by VIP on IL-6 production in murine osteoblasts, we addressed the question whether VIP may interact with known osteotropic cytokines to regulate the IL-6 production in these cells. Indeed, VIP was found to increase the stimulatory effect by IL-1β, IL-11, IL-17, LIF, OSM, and TGF-β on IL-6 production in a synergistic manner[96]. Furthermore, VIP also potentiated the IL-1β-induced stimulation of IL-6 promoter activity in osteoblastic MC3T3-E1 cells transfected with the IL-6 promoter/luciferase construct[96].

In addition to the effects by VIP on bone cell function, recent studies have demonstrated that VIP also has a protective effect on bone destruction in experimentally-induced arthritis. Treatment of mice exhibiting collagen-induced arthritis (CIA) with VIP resulted in delayed onset, reduced incidence and decreased severity of the arthritis, as well as a dramatic decrease of cartilage damage and bone erosion in arthritic joints[97]. Furthermore, VIP decreased levels of IL-1β, IL-6, IL-11, IL-17, and TNF-α, whereas the levels of IL-4 and IL-10 were elevated. VIP also decreased the RANKL/OPG ratio, mainly because of increased levels of circulating OPG[98]. In contrast to the effects of VIP on bone cell function and production of osteotropic cytokines by these cells, the effects seen in the arthritis models are believed to be a result of a neuroimmunomodulatory interaction, where VIP regulates

the proliferation and differentiation of T lymphocytes, as well as pro-inflammatory cytokine production by T lymphocytes, macrophages, and fibroblasts in the synovial tissue. These findings suggest that VIP, in addition to the effects on bone cell function, may also have a possible role as a therapeutic target for treatment of arthritis.

In summary, the findings by our group and others demonstrate that VIP affects both osteoblasts and osteoclasts through regulation of cell activity and expression of osteotropic factors, including IL-6 and the RANK/RANKL/OPG system, suggesting that VIP may have a unique role in bone metabolic processes by acting as a fine tuner of bone cell function through a neuro-osteological interplay.

Acknowledgements

Studies performed in the authors laboratory have been supported by The Swedish Research Council, The Swedish Rheumatism Association, The Royal 80 Year Fund of King Gustav V, The County Council of Västerbotten, The Centre for Musculoskeletal Research, The Medical Faculty of Umeå University and SalusAnsvar.

References

1. Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, Shen J, Vinson C, Rueger JM, Karsenty G. Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. Cell 2000;100:197-207.
2. Takeda S, Elefteriou F, Lavasseur R, Liu X, Zhao L, Parker KL, Armstrong D, Ducy P, Karsenty G. Leptin regulates bone formation via the sympathetic nervous system. Cell 2002;111:305-17.
3. Elefteriou F, Ahn JD, Takeda S, Starbuck M, Yang X, Liu X, Kondo H, Richards WG, Bannon TW, Noda M, Clement K, Vaisse C, Karsenty G. Leptin regulation of bone resorption by the sympathetic nervous system and CART. Nature 2005;434:514-20.
4. Lee NK, Sowa H, Hinoi E, Ferron M, Ahn JD, Confavreuz C, Dacquin R, Mee PJ, McKee MD, Jung DJ, Zhang Z, Kim JK, Mauvais-Jarvis F, Ducy P, Karsenty G. Endocrine regulation of energy metabolism by the skeleton. Cell 2007;130:456-69.
5. Baldock PA, Sainsbury A, Couzens M, Enriquez RF, Thomas GP, Gardiner EM, Herzog H. Hypothalamic Y2 receptors regulate bone formation. J Clin Invest 2002;109:915-21.
6. Lerner UH, Persson E, Lundberg P. Kinins and neuro-osteogenic factors. In: Bilezikian JP, Raisz LG, Rodan GA, editors. Principles of Bone Biology, 3rd edition. San Diego: Academic Press; 2007 (in press).
7. Bjurholm A, Kreicbergs A, Brodin E, Schultzberg M. Substance P- and CGRP-immunoreactive nerves in bone. Peptides 1988;9:165-71.
8. Hill EL, Elde R. Distribution of CGRP-, VIP-, D beta H-, SP-, and NPY-immunoreactive nerves in the periosteum of the rat. Cell Tissue Res 1991;264:469-80.
9. Hukkanen M, Konttinen YT, Rees G, Santavirta S, Terenghi G, Polak JM. Distribution of nerve endings and sensory neuropeptides in rat synovium, meniscus and bone. Int J Tissue React 1992;14:1-10.
10. Dénes A, Boldogkoi Z, Hornyák A, Palkovits M, Kovács KJ. Attenuated pseudorabies virus-evoked rapid innate immune response in the rat brain. J Neuroimmunol 2005;180:88-103.
11. Hukkanen M, Konttinen YT, Santavirta S, Paavolainen P, Gu XH, Terenghi G, Polak JM. Rapid proliferation of calcitonin gene-related peptide-immunoreactive nerves during healing of rat tibial fracture suggests neuronal involvement in bone growth and remodelling. Neuroscience 1993;54:969-79.
12. Li J, Kreicbergs A, Bergström J, Stark A, Ahmed M. Site-specific CGRP innervation coincides with bone formation during fracture healing and modeling: a study in rat angulated tibia. J Orthop Res 2007;25:1204-12.
13. Lerner UH. Deletions of the genes encoding calcitonin/a-CGRP, amylin and calcitonin receptor have given new and unexpected insights into the function of calcitonin receptors and calcitonin receptor-like receptors in bone. J Musculoskelet Neuronal Interact 2006;6:87-95.
14. Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W, Muff R, Fischer JA, Foord SM. International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin and calcitonin receptors. Pharmacol Rev 2002;54:233-46.
15. Granholm S, Lundberg P, Lerner UH. Expression of the calcitonin receptor, calcitonin receptor-like receptor and receptor activity modifying proteins during osteoclast differentiation. J Cell Biochem 2008;104:920-33.
16. Nakamura M, Morimoto S, Yang Q, Hisamatsu T, Hanai N, Nakamura Y, Mori I, Kakudo K. Osteoclast-like cells express receptor activity modifying protein 2: application of laser capture microdissection. J Mol Endocrinol 2005;34:257-61.
17. Tippins JR, Morris HR, Panico M, Etienne T, Bevis P, Girgis S, MacIntyre I, Azria M, Attinger M. The myotropic and plasma calcium-modulating effects of calcitonin gene-related peptide (CGRP). Neuropeptides 1984;4:425-34.
18. Roos BA, Fischer JA, Pignaat W, Alander CB, Raisz LG. Evaluation of the *in vivo* and *in vitro* calcium-regulating actions of noncalcitonin peptides produced via calcitonin gene expression. Endocrinology 1986;118:45-51.
19. D'Souza SM, MacIntyre I, Girgis SI, Mundy GR. Human synthetic calcitonin gene-related peptide

inhibits bone resorption *in vitro*. Endocrinology 1986;119:58-66.
20. Tamura T, Miyaura C, Owan I, Suda T. Mechanism of action of amylin on bone. J Cell Physiol 1992;153:6-14.
21. Yamamoto I, Kitamura N, Aoki J, Shigeno C, Hino M, Asonuma K, Torizaka K, Fujii N, Otaka A, Yajima H. Human calcitonin gene-related peptide possesses weak inhibitory potency of bone resorption *in vitro*. Calcif Tissue Int 1986;38:339-41.
22. Granholm S, Lerner UH. The members of the calcitonin gene family of peptides, including recently discovered intermedin and CRSP, inhibit both osteoclast activity and formation. J Bone Miner Res 2006; 21(Suppl.1):S364.
23. Alam ASMT, Moonga BS, Bevis PJR, Huang CLH, Zaidi M. Selective antagonism of calcitonin-induced osteoclastic quiescence (Q effect) by human calcitonin gene-related peptide-(Val^8Phe^{37}). Biochem Biophys Res Commun 1991;179:134-9.
24. Alam AS, Moonga BS, Bevis PJ, Huang CL, Zaidi M. Amylin inhibits bone resorption by a direct effect on the motility of rat osteoclasts. Exp Physiol 1992;78:183-96.
25. Alam ASMT, Bax CMR, Shankar VS, Bax BE, Bevis PJR, Huang CLH, Moonga BS, Pazianas M, Zaidi M. Further studies on the mode of action of calcitonin on isolated rat osteoclasts: pharmacological evidence for a second site mediating intracellular Ca^{2+} mobilization and cell retraction. J Endocrinol 1992;136:7-15.
26. 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.
27. Akopian A, Demulder A, Ouriaghli F, Corazza F, Fondu P, Bergmann P. Effects of CGRP on human osteoclast-like cell formation: a possible connection with the bone loss in neurological disorders? Peptides 2000;21:559-64.
28. Cornish J, Callon KE, Bava U, Kamona SA, Cooper GJS, Reid IR. Effects of calcitonin, amylin, and calcitonin gene-related peptide on osteoclast development. Bone 2001;29:162-8.
29. Ibbotson KJ, Roodman GD, McManus LM, Mundy GR. Identification and characterization of osteoclast-like cells and their progenitors in cultures of feline mononuclear cells. J Cell Biol 1984;99:471-80.
30. Roodman GD, Ibbotson KJ, MacDonald BR, Kuehl TJ, Mundy GR. 1,25-dihydroxyvitamin D3 causes formation of multinucleated cells with several osteoclast characteristics in cultures of primate marrow. Proc Natl Acad Sci USA 1985;82:8213-7.
31. Granholm S, Lundberg P, Lerner UH. Calcitonin inhibits osteoclast formation independently on transcriptional regulation by RANK and c-Fms. J Endocrinol 2007;195:415-27.
32. Hoff AO, Catala-Lehnen P, Thomas PM, Priemel M, Rueger JM, Nasonkin I, Bradley A, Hughes MR, Ordonez N, Cote GJ, Amling M, Gagel RF. Increased bone mass is an unexpected phenotype associated with deletion of the calcitonin gene. J Clin Invest 2002;110:1849-57.
33. Schinke T, Liese S, Priemel M, Haberland M, Schilling AF, Catala-Lehnen P, Blicharski D, Rueger JM, Gagel RF, Emeson RB, Amling M. Decreased bone formation and osteopenia in mice lacking α-calcitonin gene-related peptide. J Bone Miner Res 2004;19:2049-56.
34. Dacquin R, Davey RA, Laplace C, Levasseur R, Morris HA, Goldring SR, Gebre-Medhin S, Galson DL, Zajac JD, Karsenty G. Amylin inhibits bone resorption while the calcitonin receptor controls bone formation *in vivo*. J Cell Biol 2004;164:509-14.
35. Woodrow JP, Sharpe CJ, Fudge NJ, Hoff AO, Gagel RF, Kovacs CS. Calcitonin plays a critical role in regulating skeletal mineral metabolism during lactation. Endocrinology 2006;147:4010-21.
36. Michelangeli VP, Findlay DM, Fletcher A, Martin TJ. Calcitonin gene-related peptide (CGRP) acts independently of calcitonin on cyclic AMP formation in clonal osteogenic sarcoma cells (UMR 106-01). Calcif Tissue Int 1986;39:44-8.
37. Michelangeli VP, Fletcher AE, Allan EH, Nicholson GC, Martin TJ. Effects of calcitonin gene-related peptide on cyclic AMP formation in chicken, rat, and mouse bone cells. J Bone Miner Res 1989;4:269-72.
38. Thiebaud D, Akatsu T, Yamashita T, Suda T, Noda T, Martin RE, Fletscher AE, Martin TJ. Stucture-activity relationships in calcitonin gene-related peptide: cyclic AMP response in a preosteoblast cell line (KS-4). J Bone Miner Res 1991;6:1137-42.
39. Bjurholm A, Kreicbergs A, Schultzberg M, Lerner UH. Neuroendocrine regulation of cyclic AMP formation in osteoblastic cell lines (UMR 106–01, ROS 17/2.8, MC3T3-E1, and Saos-2) and primary bone cells. J Bone Miner Res 1992;7:1011-9.
40. Pondel M. Calcitonin and calcitonin receptors: bone and beyond. Int J Exp Pathol 2000;81:405-22.
41. Uzan B, de Vernejoul MC, Cressent M. RAMPs and CRLR expressions in osteoblastic cells after dexamethasone treatment. Biochem Biophys Res Commun 2004;321:802-8.
42. Cornish J, Callon KE, Lin CQ, Xiao CL, Gamble GD, Cooper GJ, Reid IR. Comparison of the effects of calcitonin gene-related peptide and amylin on osteoblasts. J Bone Miner Res 1999;14:1302-9.
43. Ballica R, Valentijn K, Khachatryan A, Guerder S, Kapadia S, Gundberg C, Gilligan J, Flavell RA, Vignery A. Targeted expression of calcitonin gene-related peptide to osteoblasts increases bone density in mice. J

Bone Miner Res 1999;14:1067-74.
44. Offley SC, Guo TZ, Wei T, Clark JD, Vogel H, Lindsey DP, Jacobs CR, Yao W, Lane NE, Kingery WS. Capsaicin-sensitive sensory neurons contribute to the maintenance of trabecular bone integrity. J Bone Miner Res 2005;20:257-67.
45. Imai S, Matsusue Y. Neuronal regulation of bone metabolism and anabolism: calcitonin gene-related peptide-, substance P-, and tyrosine hydroxylase-containing nerves and the bone. Microsc Res Tech 2002;58:61-9.
46. Goto T, Tanaka T. Tachykinins and tachykinin receptors in bone. Microsc Res Tech 2002;58:91-7.
47. Liu D, Jiang LS, Dai LY. Substance P and its receptors in bone metabolism. Neuropeptides 2007;41:271-83.
48. Goto T, Yamaza T, Kido MA, Tanaka T. Light- and electron-microscopic study of the distribution of axons containing substance P and the localization of the neurokinin-1 receptor in bone. Cell Tissue Res 1998;293:87-93.
49. Togari A, Arai M, Mizutani S, Mizutani S, Koshihara Y, Nagatsu T. Expression of mRNAs for neuropeptide receptors and β-adrenergic receptors in human osteoblasts and human osteogenic sarcoma cells. Neurosci Lett 1997;233:125-8.
50. Shih C, Bernard GW. Neurogenic substance P stimulates osteogenesis *in vitro*. Peptides 1997;18:323-6.
51. Goto T, Nakao K, Gunjigake KK, Kido MA, Kobayashi S, Tanaka T. Substance P stimulates late-stage rat osteoblastic bone formation through neurokinin-1 receptors. Neuropeptides 2007;41:25-31.
52. Lundberg P, Lundgren I, Mukohyama H, Lehenkari PP, Horton MA, Lerner UH. Vasoactive intestinal peptide(VIP)/pituitary adenylate cyclase-activating peptide receptor subtype in mouse calvarial steoblasts: presence of VIP-2 receptors and differentiation-induced expression of VIP-1 receptors. Endocrinology 2001;142:339-47.
53. Azuma H, Kido J, Ikedo D, Kataoka M, Nagata T. Substance P enhances the inhibition of osteoblastic cell differentiation induced by lipopolysaccharide from Porphyromonas gingivalis. J Periodontol 2004;75:974-81.
54. Adamus MA, Dabrowski ZJ. Effect of the neuropeptide substance P on the rat bone marrow-derived osteogenic cells *in vitro*. J Cell Biochem 2001;81:499-506.
55. Mori T, Ogata T, Okumura H, Shibata T, Nakamura Y, Kataoka K. Substance P regulates the function of rabbit cultured osteoclasts; increase of intracellular free calcium concentration and enhancement of bone resorption. Biochem Biophys Res Commun 1999;262:418-22.
56. Sherman BE, Cole RA. A mechanism for sympathectomy-induced bone resorption in the middle ear. Otolaryngol Head Neck Surg 1995;113:569-81.
57. Sohn SJ. Substance P upregulates osteoclastogenesis by activating nuclear factor kappa B in osteoclast precursors. Acta Otolaryngol 2005;125:130-3.
58. Matayoshi T, Goto T, Fukuhara E, Takano H, Kobayashi S, Takahashi T. Neuropeptide substance P stimulates the formation of osteoclasts via synovial fibroblastic cells. Biochem Biophys Res Commun 2005;327:756-64.
59. Liu D, Li H, Zhao CQ, Jiang LS, Dai LY. Changes of substance P-immunoreactive nerve fiber innervation density in the sublesional bones in young growing rats at an early stage after spinal cord injury. Osteoporos Int 2007;19:559-69.
60. Wedemeyer C, Neuerburg C, Pfeiffer A, Heckelei A, von Knoch F, Hilken G, Brankamp J, Hensche F, von Koch M, Löer F, Saxler G. Polyethylene particle-induced bone resorption in substance P-deficient mice. Calcif Tissue Int 2007;80:268-74.
61. Ahmed M, Bergström J, Lundblad H, Gillespie WJ, Kreicbergs A. Sensory nerves in the interface membrane of aseptic loose hip prosthesis. J Bone Joint Surg Br 1998;80:151-5.
62. Said SI, Mutt V. Long acting vasodilator peptide from lung tissue. Nature 1969;224:699-700.
63. Said SI, Mutt V. Polypeptide with broad biological activity: isolation from small intestine. Science 1970;169:1217-8.
64. Said SI, Mutt V. Isolation from porcine-intestinal wall of a vasoactive octacosapeptide related to secretin and to glucagon. Eur J Biochem 1972;28:199-204.
65. Bodanszky M, Klausner YS, Said SI. Biological activities of synthetic peptides corresponding to fragments of and to the entire sequence of the vasoactive intestinal peptide. Proc Natl Acad Sci USA 1973;70:382-4.
66. Mutt V, Said SI. Structure of the porcine vasoactive intestinal octacosapeptide. The amino-acid sequence. Use of kallikrein in its determination. Eur J Biochem 1974;42:581-9.
67. Said SI, Rosenberg RN. Vasoactive intestinal polypeptide: abundant immunoreactivity in neural cell lines and normal nervous tissue. Science 1976;192:907-8.
68. Brenneman DE, Hauser J, Philips TM, Davidson A, Bassan M, Gozes I. Vasoactive intestinal peptide. Link between electrical activity and glial-mediated neurotrophism. Ann NY Acad Sci 1999;897:17-26.
69. Blondel O, Collin C, McCarran WJ, Zhu S, Zamostiano R, Gozes I, Brenneman DE, McKay RD. A glia-derived signal regulating neuronal differentiation. J Neurosci 2000;20:8012-20.
70. Groneberg DA, Rabe KF, Fischer A. Novel concepts of neuropeptide-based drug therapy: vasoactive intestinal peptide and its receptors. Eur J Pharmacol 2006;533:182-94.
71. Henning RJ, Sawmiller DR. Vasoactive intestinal peptide:

cardiovascular effects. Cardiovasc Res 2001; 49:27-37.
72. Cooke HJ. Neurotransmitters in neuronal reflexes regulating intestinal secretion. Ann NY Acad Sci 2000;915:77-80.
73. van Geldre LA, Lefebvre RA. Interaction of NO and VIP in gastrointestinal smooth muscle relaxation. Curr Pharm Des 2004;10:2483-97.
74. Fahrenkrug J. Gut/brain peptides in the genital tract: VIP and PACAP. Scand J Clin Lab Invest Suppl 2001;234:35-9.
75. Voice JK, Dorsam G, Chan RC, Grinninger C, Kong Y, Goetzl EJ. Immunoeffector and immunoregulatory activities of vasoactive intestinal peptide. Regul Pept 2002;109:199-208.
76. Delgado M, Pozo D, Ganea D. The significance of vasoactive intestinal peptide in immunomodulation. Pharmacol Rev 2004;56:249-90.
77. Gozes I, Furman S. Clinical endocrinology and metabolism. Potential clinical applications of vasoactive intestinal peptide: a selected update. Best Pract Res Clin Endocrinol Metab 2004;18:623-40.
78. Hohmann EL, Elde RP, Rysavy JA, Einzig S, Gebhard RL. Innervation of periosteum and bone by sympathetic vasoactive intestinal peptide-containing fibers. Science 1986;232:868-71.
79. Bjurholm A, Kreicbergs A, Terenius L, Goldstein M, Schultzberg M. Neuropeptide Y-, tyrosine hydroxylase, and vasoactive intestinal peptide-immunoreactive nerves in bone and surrounding tissues. J Autonom Nerv Syst 1988;25:119-25.
80. Segre GV, Goldring SR. Receptors for secretin, calcitonin, parathyroid hormone (PTH)/PTH-related peptide, vasoactive intestinal peptide, glucagonlike peptide 1, growth hormone-releasing hormone, and glucagon belong to a newly discovered G-protein-linked receptor family. Trends Endocrinol Metab 1993;4:309-14.
81. Laburthe M, Couvineau A. Molecular pharmacology and structure of VPAC receptors for VIP and PACAP. Regul Pept 2002;108:165-73.
82. Usdin TB, Bonner TI, Mezey E. Two receptors for vasoactive intestinal polypeptide with similar specificity and complementary distributions. Endocrinology 1994;135:2662-79.
83. Hohmann EL, Levine L, Tashjian AH Jr. Vasoactive intestinal peptide stimulates bone resorption via a cyclic adenosine 3'5'-monophosphate-dependent mechanism. Endocrinology 1983;112:1233-9.
84. Hohmann EL, Tashjian AH Jr. Functional receptors for vasoactive intestinal peptide on human osteosarcoma cells. Endocrinology 1984;114:1321-7.
85. Lerner UH, Lundberg P, Ransjö M, Persson P, Håkanson R. Helodermin, helospectin, and PACAP stimulate cyclic AMP formation in intact bone, isolated osteoblasts, and osteoblastic cell lines. Calcif Tissue Int 1994;54:284-9.
86. Kovacs CS, Chik CL, Li B, Karpinski E, Ho AK. Pituitary adenylate cyclase-activating peptide stimulates cyclic AMP accumulation in UMR 106 osteoblast-like cells. J Endocrinol 1996;149:287-95.
87. Susuki A, Kontoyori J, Oiso Y, Kozawa O. Pituitary adenylate cyclase-activating peptide induces cAMP production independently from vasoactive intestinal polypeptide in osteoblast-like cells. Cell Signal 1994;6:11-6.
88. Lundberg P, Lie A, Bjurholm A, Lehenkari PP, Horton MA, Lerner UH, Ransjö M. Vasoactive intestinal peptide regulates osteoclast activity via specific binding sites on both osteoclasts and osteoblasts. Bone 2000;27:803-10.
89. Ransjö M, Lie A, Mukohyama H, Lundberg P, Lerner UH. Microisolated mouse osteoclasts express VIP-1 and PACAP receptors. Biochem Biophys Res Commun 2000;274:400-4.
90. Lundberg P, Boström I, Mukohyama H, Bjurholm A, Smans K, Lerner UH. Neuro-hormonal control of bone metabolism: vasoactive intestinal peptide stimulates alkaline phosphatase activity and mRNA expression in mouse calvarial osteoblasts as well as calcium accumulation in mineralised bone nodules. Regul Pept 1999;85:47-58.
91. Mukohyama H, Ransjö M, Taniguchi H, Ohyama T, Lerner UH. The inhibitory effects of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide on osteoclast formation are associated with upregulation of osteoprotegerin and downregulation of RANKL and RANK. Biochem Biophys Res Commun 2000;271:158-63.
92. Cai Y, Xin X, Shim GJ, Mokuno Y, Uehara H, Yamada T, Agui T, Matsumo K. Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) stimulate interleukin-6 production through the third subtype of VIP/PACAP receptor in rat bone marrow-derived cells. Endocrinology 1997;138:2515-20.
93. Greenfield EM, Horowitz MC, Lavish SA. Stimulation by parathyroid hormone of interleukin-6 and leukemia inhibitory factor expression in osteoblasts is an immediate-early gene response induced by cAMP signal transduction. J Biol Chem 1996;271:10984-9.
94. Persson E, Voznesensky OS, Huang YF, Lerner UH. Increased expression of interleukin-6 by vasoactive intestinal peptide is associated with regulation of CREB, AP-1, and C/EBP, but not of NF-κB, in mouse calvarial osteoblasts. Bone 2005;37:513-29.
95. Delgado M, Pozo D, Ganea D. The significance of vasoactive intestinal peptide in immunomodulation. Pharmacol Rev 2004;56:249-90.
96. Persson E, Lerner UH. The neuropeptide VIP potenti-

ates IL-6 production induced by pro-inflammatory osteotropic cytokines in calvarial osteoblasts and the osteoblastic cell line MC3T3-E1. Biochem Biophys Res Commun 2005;335:705-11.
97. Delgado M, Abad C, Martinez C, Leceta J, Gomariz RP. Vasoactive intestinal peptide prevents experimental arthritis by down-regulating both autoimmune and inflammatory components of the disease. Nature Med 2001;7:563-8.
98. Juarranz Y, Abad C, Martinez C, Arranz A, Gutierrez-Cañas I, Rosignoli F, Gomariz RP, Leceta J. Protective effect of vasoactive intestinal peptide on bone destruction in the collagen-induced arthritis model of rheumatoid arthritis. Arthritis Res Ther 2005;7:R1034-45.