Crosstalk between the brain and bone

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Summary

Bone alters its metabolic and anabolic activities in response to the variety of systemic and local factors such as hormones and growth factors. The responsiveness of bone is accomplished by the action of osteoblasts, osteoclasts and osteocytes through the process of bone remodeling. The importance of the nervous system on body homeostasis systems has been described (1) and has been suggested that organogenesis and tissue repair are under neuronal control.
The first documentation of an anatomic relationship between nerves and bone was made via woodcut, by Charles Estienne in Paris in 1545, which demonstrated nerves entering and leaving the bones of a skeleton (2). Later, several authors showed that cortical bone is densely innervated (3) and differentiated myelinated and non-myelinated fibers are associated with the arterial vessels and venous sinusoids in bone (4). This began a steady flow of studies of various nerve types in bone by a number of different groups (2). The field has recently been reinvigorated by the observation of an important role of neural control of many aspects of bone metabolism (2).

KEY WORDS: bone nerves; neuropeptides; beta-adrenergic signaling; serotonin; leptin.

Anatomy and physiology

The distribution of nerves in bone, specifically those with neuropeptide-containing fibers, has been extensively studied. A number of histological studies revealed the existence of neuropeptides in bones including enzymes, neuropeptides of sensory, symphatic and glutaminergic types (5). The distribution of nerves in bone is most frequently found in metabolically active area and the majority of nerves are localized along blood vessels. To date, no classical synapses have been found to involve osteoblasts, osteoclast, or osteocytes (2). However, nerve fibers with active expression of various neural transmission ligands have been demonstrated to be in close spatial association with bone cells and receptors for these neural ligands have been found expressed by bone cells. In addition administration of these neural transmission molecules has potent effects on bone cells (2). 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 (6, 7). Essentially, bone nerves have been implicated in two different roles: a) as regulators of bony mechanical forces and b) as a source of trophic factors essential for structure and bone function. 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 (7). 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 (6-8).
Chemical nature of bone innervation appears to change along developmental stages, suggesting the existence of a bidirectional signaling system between nerves and bone cells and a possible influence of bone cells on nerve behavior, survival and signaling. For instance, rat thoracic sympathetic axons mostly display catecholaminergic properties [thyrosine hydrossilase (TH) and norepinephrine (NE)-positive] when they reach the periosteal region of the sternum, but switch their properties to cholinergic/peptidergic traits (acetylcholine transporter and VIP-positive) after contact with the sternum tissue during the first two post-natal weeks suggesting that the targeted bony tissue plays a role in determining neurotransmitter type in innervating neurons (9). Bone is richly innervated by sensory, symphatic and glutaminergic fibers. Substance P (SP), calcitonin gene-related peptide (CGRP), vasointestinal peptide (VIP), neuropeptide Y (NPY), serotonin, glutamate, TH and NE are among the neuronal products that have been detected in bone. Finally, together with neuronal products act on bone there are evidence for crosstalk between the brain and bone through two distinct routes. The first pathway comprises well-defined hormonal signals arising from neuroendocrine neurons of the hypothalamus and subsequently processed within the pituitary. The second pathway consists of efferent neuronal discharges originating from the hypothalamus and processed through the brainstem (10).

Neuropeptides involved in bone metabolism

Glutamate
Glutamatergic synaptic transmission dominates internervous signaling in the central and peripheral nervous systems. Glutamate has been identified in bone both in association with other nerve markers in proximity to bone cells and blood vessels, and as a product released by osteoblasts themselves (2, 11, 12). Osteoblasts and osteoblast like cells actively release glutamate at concentrations that are sufficient to activate receptors expressed on bone cell surfaces, providing convincing evidence for an intrinsic osteo-glutamatergic signaling mechanism (13). In addition, glutamate signaling in osteoblasts is regulated by the cytokines TNF-α and IFN-γ, which are known to have potent effects on bone remodeling. These cytokines have been shown to induce apoptosis in osteoblasts, and this may be mediated at least in part through decreased glutamate release (13).
Osteoblasts, osteoclasts, and osteocytes express the NMDA (inotropic glutamate receptors: NMDAR1) and other glutamate recep-

tors and induce patch-clamp-demonstrable currents (the standard measure of ion channel controlled currents in nerves) in response to glutamate signaling. Mice under express NMDAR1 are smaller in comparison with wild type, which may reflect a disruption in skeletal development (5). Glutamate has also a paracrine function on bone cells and it is suspected to be at work when expression of transporters of glutamate is down regulating in response to mechanical loading of osteocytes (14).

Calcitonin Gene-Related Protein (CGRP)

CGRP is a 37 amino acid neuropeptide generated by alternative splicing of the calcitonin gene. In bone, nerve fibers immunostaining for CGRP are found in the periostum, bone marrow, and preferentially in the epiphyseal trabecular bone (2, 15, 16). CGRP-immunoareactive fibers have been shown to serve three functions. Acting on the vasculature, CGRP is the most potent vasodilator among sensory neuropeptides. CGRP acts also in skeletal muscle where the primary effects are the inhibition of glycogen synthesis and stimulation of glycogenolysis with a reduction of glucose uptake and increased glycolysis. In the liver they stimulate the glucose output as a result of gluconeogenesis. By these ways CGRP act as noncompetitive antagonist of insulin and produce insulin resistance (17). On bone cells, CGRP has been reported to stimulate ontogenesis, either by activating stem cell mitosis or osteoprogenitor cell differentiation, or both. In addition, CGRP increased the number and size of bone colonies *in vitro* (6, 18). Cultured osteoblasts from multiple species demonstrate that CGRP exposure increases insulin-like growth factor expression dramatically and interleukin-6 expression weakly. In addition it decreases TNF-α expression. These findings suggest that CGRP should increase bone formation and decrease resorption (2). These effects appeared to be dose-dependent. A similar finding was obtained by intravenous injection of CGRP 2 hours before bone marrow cells were harvested (6). The effect of CGRP on osteogenesis is mediated via CGRP1 and CGRP2 receptors, which are distinct from calcitonin C receptors. It has been shown that CGRP receptors activate the cAMP or the protein kinase C (PKC) pathways (6, 7). In contrast to CT, however, CGRP does not affect osteoclast retraction (19). These observations confirm 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 (19).

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 (7). VIP-immunoreactive nerves are distributed in bones in a similar pattern to CGRP-related fibers. 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. This peptide elicits a broad spectrum of biological functions, including anti-inflammatory and immunoregulatory properties, that lead to the amelioration or prevention of several inflammatory and autoimmune disorders in animal models and in human Rheumatoid Arthritis (RA) (20). The biological effects of VIP are mediated by G protein-coupled receptors ($VPAC_1$ and $VPAC_2$) that bind VIP and pituitary adenylate cyclase-activating polypeptide (PACAP) with equal affinity, and a PACAP selective receptor (PAC_1) (20). Osteoclasts and osteoblasts have been shown to express different subtypes of VIP receptors (20, 21). The prevalent function of VIP-immunoreactive nerves in bone seems to be the stimulation of resorption (6). However, when osteoclastogenesis was studied in mouse bone marrow cultures, VIP did not enhance the number of osteoclasts (6). In contrast, VIP caused an inhibition of osteoclast formation induced either by 1,25(OH)2 vitamin D3 or by PTH. The anti-osteoclastogenic effect of VIP is associated with inhibitory effects of these peptides on the 1,25(OH)2 vitamin D3-induced up regulation of receptor activator of NF-kB (RANK) and RANK ligand (RANKL) (6). 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 (6, 22). VIP reduces the expression of RANK and RANKL in the joints of arthritic mice, and may account for the bone protective properties of VIP in RA. On the other hand, its effects on the expression of OPG further support the postulated bone protective property of VIP. In addition OPG circulating levels rise in collagen-induced arthritis (CIA) and these levels were even higher in VIP treated mice. In this way, the ratio of RANKLRANK to OPG that determines the erosive nature of RA is greatly reduced by VIP, accounting for the bone protection (20).

Substance P (SP)

Nerve fibers staining for substance P, a well-known nociceptive signaling molecule typically associated with sensory nerves, enter the bone marrow in association with vessels (23). Substance P has been shown to increase osteoblast differentiation, bone colony formation, and osteoblast cyclic AMP production (2).

Table 1 shows the neuropeptide receptors on bone.

Beta-adrenergic signaling

Among all post-synaptic beta-adrenergic receptors (β1AR, β2AR and β3AR), β2AR is the main, if not the only adrenergic receptor expressed in osteoblasts (5). Activated βARs couple to Gsα proteins to activate adenylyl cyclase, which increases cAMP intracellular levels. Increased cAMP levels then activate protein kinase A (PKA), which can phosphorylate various protein targets, including transcription factors, kinases and cell surface receptors, including β2AR. This signaling system is fully functional in osteoblasts, as demonstrated by the increase in intracellular cAMP following osteoblast stimulation with norepinephrine or with βAR pharmacological agonists such as isoproterenol (5).

The involvement of the sympathetic nervous system (SNS) in the regulation of bone mass has been demonstrated both pharmacologically and genetically by an increase in osteoblast number and activity and a subsequent increase in bone mass in mice charac-

Table 1 - Receptors for neuropeptides on bone cells.

Neuropeptide	Osteoblast receptors	Osteoclast receptors	Bone formation	Bone resorption	Osteoclast formation
CGRP	+	+	⇑	⇓	⇓
SP	+	+	⇑⇓	⇑	⇑
VIP	+	+	⇑	⇓	⇓
NMDA	+	+	⇑	⇓	⇓

CGRP: calcitonin gene-related peptide; SP: substane P; VIP: vasoactive intestinal peptide; NMDA: glutamate receptors.

terized by low sympathetic tone, such as mice treated with the β-blocker propranolol, mice deficient for dopamine β-hydroxylase (DBH), the step-limiting enzyme responsible of catecholamine synthesis, and leptin-deficient ob/ob mice (5). Using osteoblast/osteoclast co-culture experiments and combinations of wild-type and Adrb2–/– cells, Elefteriou F. et al. demonstrated that adrenergic signaling stimulates osteoclast differentiation indirectly by increasing Rankl expression in osteoblasts, via β2AR (24). Both parathyroid hormone (PTH) and β-adrenergic agonists can therefore up regulate Rankl expression in osteoblasts and subsequently bone resorption by osteoclasts. However, although both signals use G-coupled-protein receptors, increase cAMP intracellular levels and activate PKA, they employ different signaling distal pathways: PTH activates cAMP response element binding protein (CREB), while β2-adrenergic stimulation leads to the phosphorylation of ATF4, a CREB family member previously shown to control osteoblast terminal differentiation and collagen synthesis (25). Finally, β2AR and Atf4 are expressed in immature osteoblasts, while PTH receptor (PTHR) expression peaks in more differentiated osteoblasts. The distinct signaling pathways leading to the stimulation of Rankl expression and the expression of β2AR and PTHR at different stages of osteoblast differentiation therefore suggest that β-adrenergic and PTH signaling regulate Rankl expression and bone resorption by acting on different populations of osteoblasts (24).

Hypothalamic regulation of bone

The hypothalamus, with its semipermeable blood-brain barrier, is thus one of the most powerful regulatory regions within the body, integrating signals not only from peripheral tissues but also from within the brain itself. These direct, neural pathways represent an emergent area of study that is identifying novel regulatory axes between the brain and the cells of bone (10). The first model to define a central, neural pathway to bone involved the action of an endocrine signal not from bone, but from fat cells. Leptin is a peptide secreted by adipocytes acting on hypothalamus to control body weight (26). Leptin-deficient ob/ob mice exhibit a phenotype with varied skeletal abnormalities. Cancellous bone mass is increased despite hypogonadism and hypercortisolism, while, in contrast, bone length and mass are reduced despite markedly greater fat mass compared to wild-type mice (27). Importantly, centrally administered leptin is able to correct the ob/ob changes in cancellous bone at inactive doses when administered peripherally, indicating a pathway confined to the brain (27). Recent studies have elucidated an important role for serotonin production in the brainstem. Leptin inhibition of bone mass growth requires the integrity of specific hypothalamic neurons (10, 28). However, loss of the leptin receptors from these neurons did not affect leptin action, suggesting that direct leptin signaling acts elsewhere in the brain to achieve these functions (10). Yadav et al. (29) have shown that ***5-hydroxytryptamine (5-HT)*** or serotonin, is able to modulate leptin's effects on bone mass. Recent studies have showed the role of leptin pathway to bone, elucidating an important role for serotonin production in the brainstem. Serotonin acts via a multiple receptors (HTR) to modulate numerous processes. These actions have now been expanded to include the regulation of bone mass (29). 5-HT receptors have been identified in all the major bone cell types (osteoblasts, osteocytes and osteoclasts), and stimulation of these receptors influences bone cell activities (30, 31). A number of *in vitro* studies have confirmed the functionality of 5-HT signaling in bone cells, but offer contrasting evidence as to its effects. Some suggest a direct stimulatory effect on bone formation pathways with 5-HT increasing prostaglandin E_2 release from osteocyte-like (MLO-Y4) cells and enhancing proliferation of MC3T3-E1 cells and primary human osteoblasts (32). In contrast, other investigations suggest an inhibitory effect of 5-HT on bone formation with 5-HT reducing proliferation of primary murine osteoblasts (33) and inhibiting nitric oxide release from mouse-derived osteoblasts (34). Interesting, gut-derived 5-HT acted as a downstream mediator for the entire skeletal effects of LRP5. To establish a more compelling link between LRP5, 5-HT and bone, transgenic mice with either gut- or osteoblast-specific loss- or gain-of-function of *Lrp5* were generated. This was achieved by crossing mice harboring either a floxed loss- or gain-of-function allele of *Lrp5* with either *Villin* (gut-specific) or *collagen type 1* (osteoblast-specific) promoter-driven *Cre* transgenic mice. Gut-specific deletion of *Lrp5* recapitulated the high circulating 5-HT and skeletal phenotype of *Lrp5*$^{-/-}$ mice, whereas osteoblast-specific deletion did not (33). In addition, the marked effects of leptin deficiency on the bone and energy homeostasis were corrected by specific inactivation of serotonin production in the brainstem, while loss of the leptin receptor in serotonergic neurons in the brainstem recapitulates them. Specifically, central serotonin stimulates bone mass accrual through binding to HTR2C receptors on ventromedial hypothalamic neurons and appetite via HTR1A and 2B receptors on arcuate neurons. One of the molecule involved in the liptin-bone pathway is neuromedin U (NMU). NMU is a neuropeptide expressed in hypothalamic neurons and in the small intestine, and is regulated by sympathetic activation (10, 35). However, it has also been shown to regulate bone mass. NMU2 receptor is expressed in the paraventricular nucleus, and central infusion of NMU rescued the high bone mass of NMU null mice. Moreover, NMU and its receptors are not detectable in bone, and osteoblast activity is not altered by *in vitro* (10). In hypothalamus is also broadly expressed cocaine- and amphetamine-regulated transcript (CART), a neuropeptide precursor protein involved in the regulation of food intake and energy expenditure (24). The ob/ob mice can be distinguished from the β2AR null mice by their decrease in hypothalamic CART expression and their increased resorption, thereby implicating CART as a potential regulator of bone resorption. Furthermore, the decreased CART expression in ob/ob mice can be restored by i.p. treatment with leptin (10). An increased of hypothalamic CART expression in melanocortin 4 receptor null mice has been shown to be necessary for their reduced bone resorption (5). Another major contributor to the neural output from the hypothalamus to bone is the neuropeptide Y (NPY) system. The NPY system involves three ligands, NPY and two ligands expressed in the periphery, peptide YY, and pancreatic polypeptide. These ligands signal through five receptor subtypes, Y1, Y2, Y4, Y5, and Y6 in mice, expressed widely in central and peripheral tissues (10). Y2 receptor null mice displayed a generalized increase in osteoblast activity in cortical and cancellous bone, with no indication of changes in bone resorption (36). Interestingly, central NPY overexpression, a model of forced central starvation, similar to that evident in ob/ob mice resulted in a reduction in bone mass despite marked increases in body weight, also evident in ob/ob (10). In this manner, as calorie restriction reduces body weight, central NPY levels rise (37) and bone formation is inhibited, conserving limited energy resources. In addition to the central actions in the hypothalamus, NPY appears to provide a local circuit in the osteoblast. a direct effect of NPY on osteoblastic cells acting via locally expressed Y1 receptors is more likely. This is supported by *in situ* hybridization revealing the presence of Y1 receptor expression in osteoblasts on endocortical and cancellous surfaces within the femoral bone tissue (38) indicating a diversity of central pathways to bone, yet another system, involving endocannabinoid signaling has been elucidated. The endocannabinoid system mediates its actions via two cannabinoid receptors, CB1 and CB2 (39). CB1 is primarily found within the central nervous system (CNS) and accounts for most of the CNS actions of cannabinoid drugs and endocannabinoids (40), while CB2 is predominantly expressed in peripheral tissues (10). Inactivation of CB1 receptors increased bone mineral density and additionally provided protection against ovariectomy-induced bone loss (41). In addition, CB1 receptor antagonism inhibited osteoclast formation and bone resorption, while CB1 knockout mice were resistant to these effects (41). Osteoblasts, osteocytes, and osteoclasts expressed CB2 receptors and null mice have accelerated age- related can-

cellous bone loss and cortical expansion due to increased bone turnover (42). *In vitro* studies indicate that CB2 signaling contributes to the maintenance of bone mass by two mechanisms: i) stimulating stromal cells/osteoblasts directly; and ii) inhibiting monocytes/osteoclasts RANKL expression both directly and indirectly (10).

Conclusions

In conclusion, the simultaneous maintenance of bone mass, mechanical integrity, and mineral homeostasis by the process of bone remodeling requires a complex regulatory milieu. These neural signals convey rapid and often marked effects on osteoblast and osteoclast activity, and thus present tempting therapeutic potential. 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. Experimental manipulation of gene gain- an loss- of function, specifically in the CNS versus bone cells, will be essential to further demonstrate the *in vivo* relevance of these nerve-derived factors on bone remodeling.

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