

The β -Adrenergic System and Bone Mineral Remodeling

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Abstract Known bone functions include maintaining the homeostasis of the calcium–phosphate metabolism, repairing damage produced by daily exercise and maintaining the bone architecture according to mechanical requirements, meaning that bone remodeling is a true homeostatic function. Bone is a dynamic tissue that is constantly changing through bone remodeling, which requires a lot of energy. The sympathetic nervous system contributes to bone remodeling and is one form of interaction between the skeleton and the brain, through leptin, an adipocyte-derived hormone, which uses this route to induce expression of the gene for the receptor activator of nuclear factor kappa- β ligand, an osteoclast differentiation factor. This review summarizes basic research findings on the role of the sympathetic nervous system in bone metabolism.

Keywords β -adrenergic receptors · Leptin · Catecholamines · Central nervous system

Introduction

Known bone functions include maintaining the homeostasis of the calcium–phosphate metabolism, repairing damage produced by daily exercise and maintaining the bone

architecture according to mechanical requirements, meaning that bone remodeling is a true homeostatic function [1]. In the last two decades, by inactivating different genes in animal models, studies have identified new growth factors, hormones and circulating proteins that have identified bone as an important regulator of energy metabolism. These recent discoveries suggest that bone may play a role as an endocrine organ [2]. Bone is a dynamic tissue that is constantly changing through bone remodeling, which requires a lot of energy. Many homeostatic functions, such as appetite or reproduction, are controlled by the hypothalamus. It is not surprising that bone remodeling may be, at least partially, regulated by the central nervous system (CNS) [2–6].

The sympathetic nervous system (SNS) contributes to bone remodeling and is one form of interaction between the skeleton and the brain, through leptin, an adipocyte-derived hormone, which uses this route to induce expression of the receptor activator nuclear factor kB ligand (RANKL) gene, an osteoclast differentiation factor [5].

Skeletal Innervation

The first studies to determine the innervation of bone date from 1846 [7]. Since then, histological studies have shown the presence of a dense and intimate bone tissue innervated by displaying different neuropeptides containing sympathetic and sensory fibers [8].

Immunofluorescence studies in long mouse bones have shown the existence of NF 200, a marker of neurofilaments present in tissues and cells of neural origin [8], and of tyrosine hydroxylase, calcitonin gene-related peptide, vasoactive intestinal peptide, synaptophysin, neuropeptide Y (NPY), substance P and MAP2, through antibodies directed

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against other neuronal markers or noradrenergic fibers [9–12]. Most nerves are located in irrigated zones such as the periosteum, Volkmann's canals, bone marrow, the osteochondral junction, the growth plate and the vicinity of the synovial membrane.

This extensive network of neural markers is distributed through the metaphyses and is in contact with the bone or bone marrow cells. This indicates that the neuropeptides used by the postganglionic or sensory sympathetic fibers may play a role in regulating bone cell activity, such as growth, repair or remodeling, as suggested by clinical observations in patients with neurological disease who present bone abnormalities and in experimental studies showing the influence of the nervous system on fracture healing, bone growth, heterotopic ossification and the development of arthropathies or the alterations produced by denervation or sympathectomy on bone remodeling [13].

Studies have also shown various anatomical neural connections between the skeleton and specific CNS centers, such as the brain stem, motor cortex, paraventricular nucleus and prelimbic cortex, providing anatomical evidence that the CNS exercises control of the bone marrow through different areas of the brain [14].

Receptor Signaling in β 2-Adrenergic Osteoblasts

Expression

Bone regulation by the SNS requires adrenergic receptors in the osteoblasts. Sympathetic nerve fibers are involved in the synthesis, storage and release of norepinephrine, which subsequently binds to these receptors [15]. β -adrenergic receptors are a subtype of adrenergic receptors or adrenoceptors. There are three subtypes: β 1-, β 2- and β 3-adrenoceptors. β 1-adrenergic receptors (Adrb1) have different effects on cardiovascular and renal regulation. β 2 (Adrb2) receptors act on uterine muscle contraction and muscle of the airway, bladder, eye ciliary, gastrointestinal tract and liver vascular system. β 3 (Adrb3) is involved in increased lipolysis in adipose tissue and thermogenesis in skeletal muscle.

Pharmacological and genetic studies have shown that bone cells express adrenoceptors. The effects of catecholamines on bone growth were first described in 1950 [16] and, in the 1970s, the first observations on the role of cyclic adenosine monophosphate (cAMP) and its increase in bone marrow cells emerged after treatment with epinephrine or isoproterenol, suggesting that these cells had β -adrenergic receptors [17]. The expression of β 2-adrenergic receptors in various osteoblast-like cell lines (ROS 17/2.8 and SaOS-2) has been confirmed specifically by PCR amplification of cDNA copied from the mRNA. In

these lines, β receptors were activated by increased cAMP production and stimulated bone resorption in intact mouse calvaria after stimulation by an adrenergic agonist, norepinephrine [18].

The expression of different levels of Adrb receptors in human osteoblast-like cell lines (TE 85, SaOS-2, MG63 and OHS-4) was analyzed using Northern blot. Adrb1 was found in SaOS-2, OHS-4 and TE 85 but not in MG 63. Adrb2 was expressed in MG-63 at greater levels than in TE-85 and SaOS-2 but not in OHS-4, whereas no lines expressed Adrb3. Specific (formoterol) and nonspecific (isoproterenol) β -adrenergic agonists induced the expression of c-fos, and this was inhibited by a specific Adrb2 receptor antagonist but not by Adrb1, indicating that induction of the c-fos gene was mediated by the β 2 receptor through an increase in cAMP that activated a protein kinase A (PKA)-dependent pathway [19].

In osteoblastic cells derived from human periosteum (SaM-1), human osteoclast cells and cells derived from human osteosarcoma (Saos-2, HOS and MG-63), mRNA expression of all β -adrenergic receptors (Adrb1, β 2 and β 3) and α 1 and α 2 receptors, which may participate in the regulation of bone remodeling was identified by RT-PCR. However, Adrb2 seems to be the principal postsynaptic adrenergic receptor involved in remodeling [19–24].

Signaling

β 2-adrenergic receptors belong to the G protein-coupled receptor (GPCR) family and are membrane proteins capable of receiving information from external stimuli and transmitting it to the cell interior. They act as signal transducers, i.e., they bind specifically and selectively with one or more ligands and respond to this binding by a conformational change that involves their activation or inactivation. They are activated by various endogenous or exogenous ligands and modulate different signaling pathways, principally the cAMP and phosphatidylinositol pathways, thus participating in many biological processes.

The G protein couples to other receptors bound to the same protein that are important for bone homeostasis, such as the parathyroid hormone receptor, calcitonin, prostaglandin E2 (PGE2) and 5-hydroxytryptamine 2 (5HT2). There are several types of G protein: Gs (stimulatory), which activates adenylate cyclase; Gi (inhibitory), which inhibits adenylate cyclase; and Gq, which stimulates β phospholipase C and increases intracellular calcium concentrations. G protein has a heterotrimeric structure, i.e., it consists of three different subunits, α , β and γ . In its inactive state, the α subunit contains a guanosine diphosphate (GDP) group. Binding of an extracellular agonist to the Adrb2 receptor results in a conformational change of the cytoplasmic ends of the

transmembrane segments that allows the heterotrimer Gs (α , β and γ) to bind to the receptor. GDP is released from the α subunit upon formation of the β 2AR-Gs complex. Guanosine triphosphate (GTP) binds to the free nucleotide of the α subunit, producing dissociation of the α and $\beta\gamma$ subunits from the receptor. This causes activation of adenylyl cyclase and conversion of adenosine triphosphate (ATP) to cAMP, modulated by cAMP-specific phosphodiesterases (PDEs). This contributes to the activation of protein kinase A and the phosphorylation of a series of proteins [25, 26]. These subunits regulate multiple effector proteins including: adenylyl cyclase, phospholipases, phosphodiesterases, ion channels and phosphatidylinositol 3 kinase. In this way, these receptors regulate numerous biological functions, such as sensory perception, neurotransmission, chemotaxis, and cell proliferation and differentiation [27].

This activation ceases when the regulators of G protein signaling (RGS) induce GTPase activity. After hydrolysis (GTP to GDP) of the nucleotide, the subunit α bound to GDP is reassociated with the $\alpha\gamma$ dimer and the heterotrimeric G protein remains inactive and therefore ready for a new activation cycle. Thus, GTPase activity acts as biological switches for this activity.

To ensure that the extracellular stimulus is translated into intracellular signals of adequate magnitude, most cascades are closely regulated. The β 2-adrenergic receptor is subjected to three modes of regulation: desensitization, in which the receptor becomes refractory to a continuous stimulus, internalization, where the receptors move from the cell surface via endocytosis, and down-regulation, where cell receptor levels descend [27].

Desensitization is primarily mediated by a second messenger-dependent kinases: PKA, protein kinase C and GPCR kinase (GRK). Once G α is activated, the first response is phosphorylation of one or more members of the class of serine–threonine kinases, known as GRK.

GRKs are a subfamily of protein kinases with six components. GRK2 and GRK5 are the main members involved in this process, although others can contribute. In vitro studies show that GRK2 phosphorylates Thr 384, Ser 396, Ser 401 and Ser 407 in the carboxy terminal region of the B227 receptor. GRK phosphorylation plays an important role in mediating the binding of GPCRs with arrestins. That is, GRKs reduce receptor signaling by rapid phosphorylation of specific GPCR residues to facilitate the recruitment and binding of β arrestin to the phosphorylated receptor, decreasing the affinity of the agonist and the binding of G protein by steric mechanisms [28]. Furthermore, arrestins activate the internalization of the β 2 receptor and targets it at specific endosomes, where it is dephosphorylated and then recycled to the cell surface or degraded. The binding of the β 2-arrestin complex may be a

signaling mechanism per se, as it acts as an adapter to trigger binding to other pathways, such as terminal c-Jun-N-kinase and extracellular signals regulated by kinases [27].

Activation of PKA catalytic subunits phosphorylates cytosolic proteins to increase intracellular calcium or translocate it to the nucleus, where they activate the cAMP response element binding (CREB) protein or activating transcription factor 4 (ATF4), which controls the terminal differentiation of osteoblasts and whose phosphorylation stimulates RANKL expression [29]. Subsequently, there is a second rapid phosphorylation of Adr β 2, via a cAMP-mediated PKA [26]. This phosphorylation seems to improve the interaction of the Adr β 2 receptor with Gi, acting as a switch. The subsequent activation of Gi and the release of the Gi and $\beta\gamma$ subunits contribute to the activation of mitogen-activated protein kinases (MAPKs) [30].

A-kinase anchoring proteins (AKAPs) participate in PKA phosphorylation. They coordinate various signaling cascades and are involved in desensitization, internalization and resensitization. The Adr β 2 receptor contains a C-terminal PDZ domain that interacts with a Na⁺/H⁺-exchanger regulatory factor (NHERF). This binding regulates Na⁺/H⁺ exchange and also appears to regulate internalization of the receptor. Although part of the biological action of the receptors can be attributed to their ability to stimulate cAMP, many critical components that play a role in the signaling and localization of the receptors are used by the GPCRs. Basically, these are three classes of proteins: G protein (Gs and Gi), kinase proteins such as PKA, PKC, tyrosine kinases and GRKs and scaffolding proteins such as arrestins, AKAPs and NHERF. However, in vivo studies of the importance of these cellular events are still required.

Adrenergic Effect in Osteoclastogenesis

Osteoclasts originate in the hematopoietic cells and their formation requires RANKL, which is produced by osteoblasts, stromal cells and macrophage colony stimulating factor (G-CMFS). RANKL is essential for the activation and differentiation of osteoclasts, and its effect is antagonized by osteoprotegerin (OPG). The expression of both proteins is regulated by osteotropic factors such as 1,25(OH)2D3, interleukin (IL)-1 α , IL-11, PGE2, transforming growth factor (TGF)- β 1 and parathormone (PTH) [31]. Eleftheriou et al. [24] showed that adrenergic agonists indirectly stimulated osteoclast differentiation by increasing the expression of RANKL on osteoblasts.

The participation of RANKL and/or OPG in bone resorption induced by epinephrine has been demonstrated by the finding that messenger RNA (mRNA) of both

molecules is expressed in MC3T3 and by the formation of tartrate-resistant acid phosphatase (TRAP)-positive in mesenchymal cells. Activation of Adr β 2 receptors stimulates other osteoclastogenic factors such as IL-6, IL-11 and PGE2 [32]. RT-PCR techniques have shown RANKL and OPG mRNA expression in osteoblasts after adrenergic stimulation mediated by α and β receptors, without the involvement of IL-11 or PGE2 [32].

Studies have shown that, in mouse calvaria osteoblasts, the RANKL/OPG mRNA ratio was significantly increased after stimulation with isoproterenol, compared with osteoblasts from double deficient mice (Adr1, 2 $-/-$). Conversely, the response of RANKL/OPG to PTH was similar in wild-type mice and double-deficit mice, indicating that isoproterenol induces RANKL expression and represents the lack of adrenergic signaling and not only a reflection of poor osteoblast differentiation [33]. It has also been demonstrated that catecholamines stimulate maturation and induce osteoclast activity in human osteoclast precursors [34]. Therefore, adrenergic stimulation increases the number and activity of osteoclasts and, concomitantly, inhibits osteoblast function, resulting in an imbalance in remodeling that favors resorption.

Adrenergic Effect in Osteoblasts and Bone Formation

Little is known about the role of β -adrenergic receptors in the osteogenic differentiation of mesenchymal stem cells (MSC), although studies have shown the presence of β 2 receptors in osteoblasts, osteoclasts and MSCs. Dennis et al. [35] was the first to demonstrate that MSCs had the potential to differentiate into four phenotypes: cartilage, adipose tissue, osteoblasts and osteoclasts, showing these cells can potentially adapt to the needs required within bone at any moment and can differentiate into bone progenitors during repair or into osteoclasts during bone turnover. Li et al. [36] showed the expression of the three β receptors in MSCs after the induction of osteogenesis. Levels of proteins and the mRNA of Adr β 2 and Adr β 3 were elevated after osteogenesis of MSCs, with the effect of B2 being greater. The agonists negatively regulated B2, and the antagonists positively regulated osteogenesis. This effect is carried out, in part, through the cAMP/PKA pathway, suggesting that MSCs are targets of the β -adrenergic receptor and play an important role in osteogenesis.

Adr β 2 receptor activation mediated by cAMP/PKA involves the expression of the c-fos protein, which forms a heterodimer with the Jun proteins and regulates the transcription of AP1, which is responsible for genes such as osteocalcin, ALP and type 1 collagen in human and mouse

osteosarcoma cells, and thus induces osteoblast differentiation [37]. However, these observations are contradicted by *in vivo* findings using isoproterenol.

The Central Nervous System and Bone Remodeling

The main studies of the effects of activation of adrenergic receptors in bone remodeling began following the discovery of leptin. Since then, numerous pharmacological and genetic studies have identified the involvement of the SNS in controlling the function of osteoblasts and bone mass. Bone remodeling is a biphasic process that occurs in a sequential, balanced fashion and consists of the destruction or resorption of preexisting bone by osteoclasts, followed by the formation of a new bone matrix by osteoblasts [3, 38].

Conceptually, bone resorption and formation are part of the same physiological function, implying that the differentiation and function of osteoblasts and osteoclasts should be regulated by common molecules forming a complex hormonal control, whose participants are cytokines and growth factors that act locally and hormones that act systemically [5, 6].

Hormonal regulation by the parathormone and sex steroids is one of the most important regulators of bone remodeling mechanisms. The search for the identification of hormones that may regulate bone formation shows two fundamental clinical facts. First, osteoporosis is caused by gonadal failure [39] and, secondly, obesity seems to protect against osteoporosis [40–42]. Tremolieres et al. [43] found that obesity in postmenopausal women is associated with a reduction in bone loss. This suggests there is a common regulating mechanism between appetite, reproduction and bone mass. Attempts to determine which known hormones regulate appetite and reproduction show that only leptin significantly influences both functions.

Leptin is a hormone-like 16 kilodalton (kDa) cytokine that occurs predominantly in adipose tissue and circulates freely and bound to proteins [44]. Leptin inhibits the appetite and promotes reproduction and energy consumption through binding to a receptor (ObRb) located in the brain, which makes it an excellent candidate to investigate the existence of a common regulatory mechanism between bone remodeling and energy metabolism. Among the most important studies are those using genetically modified leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice [45]. These mice have a metabolic phenotype with increased appetite, obesity, hypogonadism and a small increase in resorption, with high bone mass and elevated bone formation parameters. This shows that leptin uses a

central pathway, probably hypothalamic, to act on bone and is a potent inhibitor of increases in bone mass [24, 46].

This phenotype is remarkable, as it is the only animal model in which hypogonadism and elevated bone mass coexist [46]. The fact that the absence of leptin entails high bone mass in spite of the hypogonadism suggests that the regulation of bone remodeling is a critical function of leptin. This conjecture was proven by testing mice who were thin and fat free due to a deficit of leptin and the virtual absence of adipocytes, and showing that this phenotype developed regardless of the presence of adipose tissue.

In ob/ob mice, intracerebroventricular infusion of leptin corrected the bone phenotype caused by the absence of leptin without producing a detectable peak in circulation, causing bone loss in ob/ob mice and wild mice and confirming the existence of a central mechanism [46–48].

Takeda et al. [23] showed a phenotype similar to the ob/ob and db/db mouse was obtained by wounding the glucose-sensitive neurons in the ventromedial hypothalamus (VMH), where leptin signaling occurs, with gold thioglucose. Ob/ob mice with a sectioned arcuate nucleus corrected bone mass but did not suffer weight change, suggesting there are different regulatory sites in bone remodeling and appetite.

Other neuropeptides are involved in bone homeostasis and metabolic control. Different efferent signals go from the hypothalamus to the osteoblasts, including the SNS, melanocortin, NPY, and cocaine and amphetamine-regulated transcript (CART) peptides.

Leptin uses two pathways, the sympathetic pathway, which acts on osteoblasts and promotes osteoclast differentiation by inducing the expression of RANKL, an osteoclast differentiating factor [5], and CART, which, in the VMH, inhibits RANKL expression in osteoblasts via an unknown mechanism [24].

CART is a peptide of 116 amino acids encoded by the CARTPT gene whose expression in the hypothalamus is positively regulated by leptin. It is also present in the systemic circulation due to secretion by the pituitary glands and pancreatic islets [49]. CART-deficient mice (CART^{-/-}) show no abnormalities in weight or reproduction, but have delayed onset of the low bone mass phenotype, caused by increased resorption mediated by RANKL [23, 49]. In ob/ob mice, bone formation is increased and CART expression is absent in the hypothalamus. However, CART expression in macrophages and stromal cells is normally differentiated and there are no receptors nor evidence of the expression of CART in bone, suggesting the presence of another central mediator of remodeling [24, 46].

Melanocortin belongs to a family of peptides produced by posttranslational processing of pro-opiomelanocortin and had originally been considered as the major signaling

molecule in bone remodeling. Two melanocortin receptors are located in the arcuate nucleus, and the deletion of one in animal models results in high bone mass and suppressed resorption [24].

Mice with deletion of the melanocortin 4 receptor (MC4R^{-/-}) show high levels of CART, which suppresses the expression of RANKL, which is produced in osteoblasts and is essential for osteoclast differentiation. This results in a decrease in bone resorption parameters, high bone mass and increased serum and/or hypothalamic levels of CART [50]. Moreover, reduced bone resorption in MC4R^{-/-} mice is genetically normalized by reductions in CART levels [51]. Evidence in humans is less well documented. MC4R-deficient patients have a metabolic phenotype and increased bone mineral density [50].

Neuromedin U (NMU) is a neuropeptide produced in the dorsomedial nucleus, pituitary gland and myenteric plexus of the small intestine. It binds to the NMUR2 receptor and, like other neuropeptides, regulates appetite and sympathetic activation [52]. The ob/ob mouse is NMU deficient (NMU^{-/-}), but this is corrected by leptin administration. Sato et al. found that NMU^{-/-} mice had high bone mass and increased bone formation compared with wild-type mice, even though the proliferation of osteoblasts and osteoclasts was normal [52, 53]. This meant that NMU must act centrally in the control of remodeling. However, intracerebroventricular infusion of leptin or the administration of β 2-adrenergic agonists in NMU^{-/-} mice did not reduce bone mass [54].

Sato et al. also found that central administration of leptin, paradoxically, increased the number of osteoblasts in NMU^{-/-} mice. This, together with the finding that the mice had high levels of urinary catecholamines and metanephrine but a normal increase in bone resorption, means that the lack of NMU leads the skeleton to resist sympathetic activation by leptin or that compensatory changes occur in other neuropeptides and thereby balance any increase in sympathetic tone [52]. The explanation for increased bone formation in these mice, if osteoblasts and osteoclasts in vitro proliferate and differentiate normally, is that the circadian locomotor output cycles Kaput (Clock) genes, Per1 and Per 2, were significantly lowered. This is like the osteoblasts of ob/ob mice osteoblasts in which the absence of an Adr β 2 signal due to the absence of leptin, reduces the Clock genes, with a subsequent increase in bone formation [52].

Most biological processes in mammals are subjected to circadian rhythms driven by endogenous clocks, which are comprised of a center located in the suprachiasmatic nucleus and subordinate nuclei in peripheral tissues. Both central and peripheral clocks are controlled by positive and negative feedback from circadian genes. The positive feedback is due to Bmal 1 and Clock, which stimulate the

expression of circadian genes such as Per 1 and 2, Cry 1 and 2 and Rev Erb. The negative feedback is due to the Per and Cry proteins and casein kinase, which suppress the transcription of Per, Cry and Rev Erb directly suppressing the activity of Bmal 1/Clock.

The similarity between the Per1 $-/-$, Per2 $-/-$, ob/ob and B2-ADR $-/-$ bone phenotypes, with an increase in formation parameters, osteoblasts and bone mass, suggests that they may regulate bone inhibition through the sympathetic-leptin pathway by inhibiting cyclin G1 and osteoblast proliferation. The fact that Per-deficient mice had normal levels of free leptin and a slight increase in sympathetic tone, rules out the possibility that the high bone mass was due to a mere decrease in both leptin and sympathetic tone, and may be attributed to the molecular clock. In fact, the parameters of bone mass and formation are reduced in wild mice after the infusion of leptin, which increased bone formation in Per-deficient mice [51].

Studies show that in the clock genes, induced by sympathetic signaling, the expression of AP-1, which is overexpressed in Per-deficient mice. This leads to the expression of cyclin D1, which is significantly elevated in Per-deficient mice, and is regulated through an indirect mechanism that inhibits the expression of c-myc, a critical regulator of cyclin and a gene that is highly expressed in osteoblasts. The expression of c-myc and cyclin D1 was increased in bones of Per-deficient mice and this explains the increased number of osteoblasts after intracerebroventricular leptin infusion [51].

Sympathetic regulation of the clock genes and G1 in osteoblasts, the similarity in the cell cycle of the B2-ADR and Per-deficient models, and the failure of bone inhibition after intracerebroventricular infusion of leptin all indicate that the clock genes act by reducing sympathetic signaling to inhibit bone formation [51]. Through the CNS, NMU alters the molecular clock in bone through unknown mechanisms and acts as a central leptin-dependent mediator of bone regulation. Furthermore, in NMU $-/-$ mice, CART expression is increased, and this involves a fine balance between the suppressive effects of CART in osteoclastogenesis and of SNS-induced bone resorption [52].

NPY belongs to the class of peptides that includes the pancreatic polypeptide (PP) and the peptide YY (PYY) and which are expressed in the CNS. They signal through the Y1, Y2, Y4, Y5 and Y6 receptors expressed in tissues. Like CART, they are synthesized in the arcuate nucleus and increase appetite when overexpressed. They have also been identified in bone marrow, the peripheral nervous system, intestines, periosteum and the vessels that surround the marrow cells. NPY mRNA expression in the arcuate nucleus increases in response to fasting and increases markedly in ob/ob and db/db mice. Leptin administration in these mice suppresses the overexpression of NPY in the arcuate nucleus.

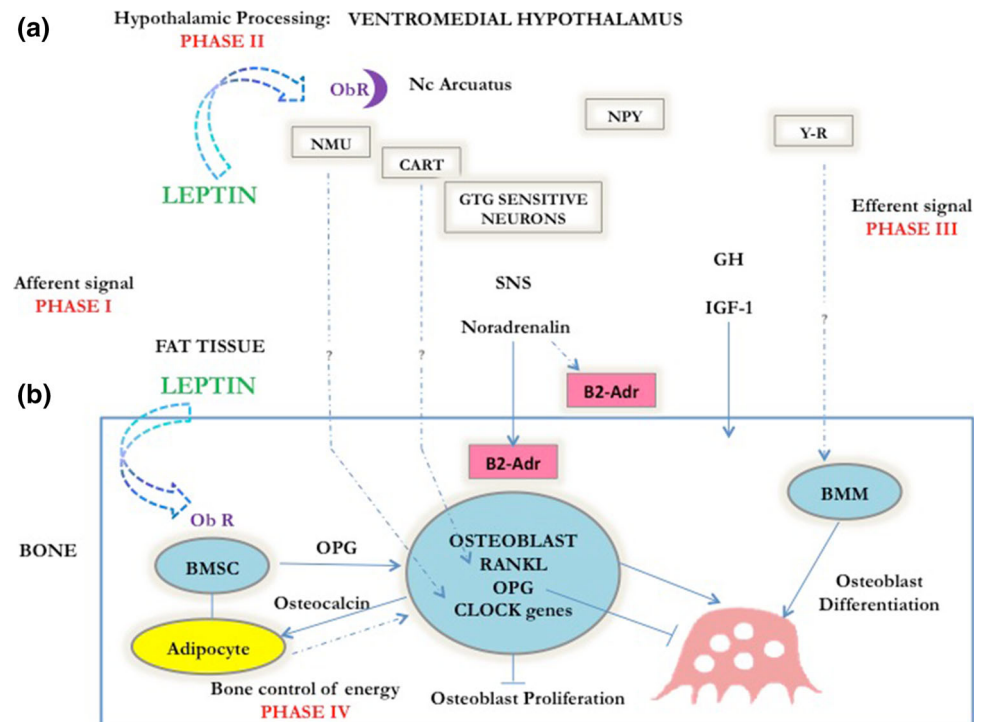
Ob/ob mice without the NPY gene have a phenotype marked by decreased food intake and reduced weight compared to ob/ob mice, indicating that NPY is an important target of leptin. Studies in mice with a germline hypothalamic deletion of the Y2 receptor ($-/-$ Y2) showed that cortical bone and bone formation increased, but resorption remained unchanged [56]. Mice with a deletion of the Y1 receptor also showed high bone mass. However, mice with hypothalamic deletion of Y1 genes showed no alterations in bone density.

The proliferation of bone marrow stromal cells was increased in Y2 $-/-$ mice and was accompanied by in vitro evidence of increased mineralization and easier adipocyte differentiation in appropriate conditions compared with wild-type cells, indicating that the cells of these mice are more pluripotent than those of wild mice. Surprisingly, these mice had no Y1 receptors in osteoblasts [52]. This suggests that the NPY signal can modulate bone remodeling, but that the anabolic effect of Y2 gene deletion is mediated by peripheral regulation of the Y1 receptor in osteoblasts.

To integrate the significance of these findings, four phases in the interaction of bone and the brain as a regulatory unit can be defined (Fig. 1). In the first phase, the skeleton detects the energy status and afferent signals are sent from storage deposits to the hypothalamus. Fat cells control the metabolic function by storing and releasing energy in response to nutrient intake. Adipocytes synthesize cytokines, hormones and growth factors, which modulate the activity of other cells. Leptin is the prototype of the adipokine that exhibits classical endocrinal activity by crossing the blood–brain barrier, improving the reproductive function, reducing appetite through the arcuate nucleus and regulating bone remodeling through the SNS via the VMH [23, 46, 52, 55]. In the second phase, a complex neural process occurs in the VMH in conjunction with other neural circuits such as the glutamatergic, serotonergic, dopaminergic, cannabinoid and NPY-ergic systems, which may also be activated by these afferent signals.

In the third phase, efferent signals are sent from the SNS to the Adr β 2 in osteoblasts. The molecular pathways include transcriptional and posttranslational regulation of the Clock genes, which indirectly regulate the proliferation of osteoblasts by inhibiting c-myc [52]. In the fourth and final phase, the osteoblasts modulate the expression of adiponectin in adipocytes, completing the reciprocal regulation between bone and brain [52]. The SNS appears to be the link between the leptin-mediated action of the hypothalamus and the osteoblasts. This hypothesis is supported by evidence: First, ob/ob mice have a high bone mass phenotype and a low sympathetic tone, which contributes to this. Second, intraventricular injection of leptin in the VMH increases the release of epinephrine and

Fig. 1 Interaction bone and brain. Adapted from Cirmanova (2008)



norepinephrine in plasma, without secretion of catecholamines in other nuclei such as Arc, the paraventricular nucleus and the dorsomedial hypothalamus [57]. The discovery of different neuropeptides in contact with bone cells and the expression of $\text{Ard}\beta 2$ in osteoblasts confirm this linkage. The development of genetic models with autonomic dysfunction and pharmacological studies has demonstrated the existence in vivo of a neural pathway between the hypothalamus and osteoblasts.

Genetic Studies

Genetic studies have provided strong evidence of $\text{Adr}\beta 2$ receptor signaling in bone remodeling (Table 1). Models with decreased SNS activity, such as *ob/ob* mice and dopamine β -hydroxylase-deficient (*Dbh -/-*) mice first demonstrated a phenotype with high bone mass. *Ob/ob* mice had increased vertebral cancellous bone mass but reduced distal femur cancellous bone volume, cortical area and cortical thickness [46]. Restoration of sympathetic activity resulted in a 45 % reduction in bone mass without the appetite or reproduction being affected [58].

Reid suggested that leptin might have a dual action: a direct anabolic effect on cortical bone and an indirect catabolic effect in SNS-mediated cancellous bone [59]. The absence of dopamine β -hydroxylase, an enzyme required for the synthesis of catecholamines (epinephrine and norepinephrine), caused a high bone mass phenotype in

6-month-old mice. These findings were significant despite increases in cortisol and dopamine, which promote bone loss. This increase in bone mass was due to an increase in the rate of formation and the number of osteoblasts, whereas markers of bone remodeling were normal [23].

As catecholamines are released by the SNS and the adrenal gland, another experiment was carried out in which the adrenal gland was removed in mice. This did not affect bone mass, confirming the existence of neural regulation of the skeleton [23, 60]. Subsequently, *Dbh -/-* mice were infused with intracerebroventricular leptin without reduction in bone mass, showing that the antiosteogenic effect of leptin requires the integrity of the SNS.

Mouse models without β receptors are very interesting because they permit the exploration of central and peripheral regulatory pathways of bone mass. $\text{Adr}\beta 2$ -deficient mice (*Adr}\beta 2 -/-*) developed high bone mass in the vertebrae and long bones at 24 weeks, but no other metabolic alterations, unlike other models with decreased sympathetic tone such as *ob/ob* mice, which were obese and hypogonadal [24]. These mice with normal weight and no hormonal changes had increases in cancellous bone volume, trabecular volume fraction and the rate of bone formation, increased osteoblasts and a reduced number of osteoclasts, which did not involve a decrease in resorption, measured by the telopeptide, compared to wild-type mice of the same age. Ovariectomy during growth, which reduced estrogen, did not reduce bone mass, showing the importance of SNS integrity [24]. This indicated that $\text{Adr}\beta$

Table 1 Genetic ablation studies in animals

Genetic ablation studies	Specific gene knock-out	Bone phenotype alteration
Ducy et al. [46]	Ob/ob Db/db	High bone mass
Takeda et al. [23]	Dopamine β -hydroxylase (Dbh)	High bone mass
Elefteriou et al. [24]	β 2 adrenergic receptor (Adr β 2)	High bone mass
Pierroz et al. [63]	β -1,2 adrenergic receptors (Adr β 1, β 2)	Low bone mass
Yan et al. [65]	Adenylyl cyclase 5 (AC5)	Bone quality preserved in old mice
Bouxsein et al. [64]	β 1,2,3 adrenergic receptors	High bone mass

signaling directly inhibits bone formation and promotes osteoclastogenesis by augmenting RANKL [61]. The fact that this mouse does not have metabolic changes that may contribute to high bone mass indicates that, at least partially, this is due to low sympathetic tone, especially when not corrected by intracerebroventricular infusion of leptin in β 2-deficient mice.

When the other adrenergic receptors were inactivated, significant alterations were found in bone. Adr β 1 $-/-$ mice had a lower trabecular volume fraction, whereas femoral and cortical mineral densities were similar to those of wild mice [62]. Analysis of β 1- and β 2-deficient mice (Adr1-2 $-/-$) showed slightly increased weight and insulin-like growth factor and a significant reduction in total bone mass in the femur and spine and, unlike Adr β 1 $-/-$ mice, there was no protection of the cortical or trabecular microstructure against oophorectomy [63]. In contrast, mice with deletion of β 1, β 2 and β 3 showed higher bone mass accompanied by weight gain and had no protection against the bone loss which occurs with age and oophorectomy, suggesting that loss of β 1 and β 3 seems to counteract the protective effect of β 2 deficit [64]. The marked inhibition of bone formation in Adr1-2 $-/-$ mice suggests that Adr β 2 $-/-$ requires functional β 1 receptors, and therefore, these may indirectly stimulate bone formation, contrary to the direct inhibitory effect of β 2 receptor signaling. Mice deficient in adenylyl cyclase 5, a β 2 receptor mediator, also developed high bone mass. AC5 loss improved osteoblast function through activation of extracellular-signal-regulated kinases (ERKs) [65].

Pharmacological Analyses in Animals

Models with autonomic alterations induced by drugs allow better exploration of the link between neural connections and the control of remodeling (Table 2). Findings suggest that the pharmacological antagonism of β 2 receptors, like genetic ablation studies, cause anabolic effects on bone,

which are much more pronounced in situations that favor bone loss, such as gonadectomy, low doses of propranolol or unloading [62, 66]. According to the results obtained in β -hydroxylase mouse models, when mice are treated with a non-selective beta blocker, propranolol, they show an increase in bone mass [23, 67–69] and improved fracture healing [70]. Baek et al. [71] found that using this antagonist attenuates the decline in trabecular mass in mice fed on high-calorie diets.

Beta blockers improve trabecular bone architecture in ovariectomized mice, preventing the inhibition of bone formation and the stimulation of resorption induced by the SNS. The primary effect of low doses of beta blockers may be blockade of the Adr β 2 receptors in osteoblasts, while high doses may antagonize other β receptors in bone cells or other tissues [72].

Pierroz et al. [33] studied whether the combination of propranolol and PTH, whose intermittent administration induced remodeling, had a synergistic effect on the increase in bone mass. The study found that, in 15-week-old ovariectomized mice, the combination improved the balance of remodeling. Pataki et al. [73] found that salbutamol had beneficial effects on bone. Arai et al. [74] studied the effect of butoxamine, a selective β 2-adrenoreceptor antagonist, on bone metabolism and found that low doses reduced osteoclast numbers and activity and increased osteoblast activity. When mice were treated with the non-selective β agonist, isoproterenol, there was massive bone loss in the vertebrae and long bones, due to a reduction in the rate of bone formation and the number of osteoblasts in the bone perimeter [63, 66]. Isoproterenol also increased the percentage of lean mass and reduced the percentage of fat, with no major change in body weight but with a decrease in leptin levels [23]. Bonnet et al. [75] demonstrated the negative effects of other selective agonists, such as salbutamol and clenbuterol, in trabecular bone microarchitecture. The clenbuterol-treated group had a higher risk of fracture despite an increase in bone mass. Most studies show the positive effect of beta blockers on bone mass,

Table 2 Models with autonomic alterations induced by drugs and bone remodeling

Genetic ablation studies	Bone phenotype alteration
Propranolol: non-selective adrenergic antagonist	
Minkowitz et al. [70]	High bone mass
Takeda et al. [23]	High bone mass
Levasseur et al. [68]	High bone mass
Bonnet et al. [62]	High bone mass
Pierroz et al. [33]	High bone mass
Zhang et al. [67]	High bone mass
Bonnet et al. [62]	Reduces bone resorption
Rodrigues et al. [69]	Reduces the decrease in trabecular mass
Baek et al. [71]	Attenuates the decrease in trabecular bone mass, bone volume and trabecular number
de Souza et al. [76]	No difference
Marenzana et al. [77]	No difference
Butoxamine: β2-AR selective antagonist	
Arai [74]	
Isoproterenol: non-selective adrenergic agonist	
Takeda et al. [23]	Low bone mass
Kondo et al. [66]	Low bone mass
Pierroz et al. [63]	Low bone mass
Clenbuterol or salbutamol: selective β2-AR agonists	
Bonnet et al. [75]	Low bone mass

although some studies [76, 77] have found no increase in cortical and trabecular bone. This may be due to methodological differences and the use of different models (stress, unloading, fracture and ovariectomy), different mice ages, different routes and parameters of drug administration and, above all, different doses.

Alpha Adrenergic System

The α -adrenergic receptors, like the Adr β receptors, are members of the GPCR superfamily. There are two types: α 1 (a Gq-coupled receptor) and α 2 (a Gi-coupled receptor). Alpha 1 is responsible for smooth muscle contraction and the α 2 receptors (2a, 2b and 2c) are found in the pancreas and are responsible for the release of neurotransmitters in the CNS and SNS. Alpha receptors have been detected in human osteoblasts [22], although their functional significance remains to be defined. Fonseca et al. [78] deleted the α (2a) R $-/(2c)$ R $-$) presynaptic autoreceptors, which inhibit catecholamine release, in mice and found that, surprisingly, the mice had a high bone mass. This suggests that activation of the α 2a receptors promotes osteoclast differentiation. Alpha 2a blockade with doxazosin increased osteogenic differentiation of mesenchymal cells. These findings show that Adr β 2 is not the only adrenoceptor

involved in the regulation of bone remodeling, where alpha receptors appear to play a role yet to be elucidated [79].

Disclosures

Conflict of interest The authors (Marta Gonzalez-Rozas, Antonio Dueñas-Laita and José-Luis Pérez-Castrillón) do not have a potential conflict of interest directly or indirectly related to the research.

Animal/Human Studies This article does not include any studies with human or animal subjects performed by the author.

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