

## Review

# The New Field of Neuroskeletal Biology

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**Abstract.** The fields of neuroscience and bone biology have recently converged following the discovery that bone remodeling is directly regulated by the brain. This work has defined bone remodeling as one of the cardinal physiological functions of the body, subject to homeostatic regulation and integrated with the other major physiological functions by the hypothalamus. Central to this discovery was the definition of the adipocyte-derived hormone leptin as a regulator of both arms of bone remodeling, formation and resorption, through its action on the ventromedial hypothalamus and subsequently via the sympathetic nervous system to osteoblasts. The characterization of the sympathetic nervous system as a regulator of bone remodeling has led to several large clinical studies demonstrating a substantial protective effect of  $\beta$ -blockers, particularly  $\beta_1$ -blockers, on fracture risk. Studies in model organisms have reinforced the role of the central nervous system in the regulation of bone remodeling *in vivo* by the identification of several additional genes, namely cocaine and amphetamine regulated transcript (*Cart*), melanocortin 4 receptor (*Mcr4R*), neuropeptide Y (*NPY*), Y2 receptor, cannabinoid receptor CB1 (*Cnr1*), and the genes of the circadian clock. These genes have several common features, including high levels of expression in the hypothalamus and the ability to regulate other major physiological functions in addition to bone remodeling including energy homeostasis, body weight, and reproduction. We review the major pathways that define the new field of neuroskeletal biology and identify further avenues of inquiry.

**Key words:** Skeleton — Bone — Adrenergic receptor — Peptin — Sympathetic nervous system

Beyond development, bone is a physiologically active and reactive tissue responding to dietary, hormonal, paracrine/autocrine, and mechanical signals necessary to release minerals into the bloodstream and to adapt to physiological and mechanical demands. The responsiveness of bone is accomplished by the action of os-

teocytes that modulate mineral homeostasis and respond to mechanical strain as well as by osteoclasts and osteoblasts through the process of bone remodeling, a mechanism that promotes conservation of an appropriate architecture and stable bone mass during adulthood [1, 2]. Defects in bone remodeling, generally induced by an imbalance between osteoblast and osteoclast activity, lead to pathological conditions such as osteoporosis, a disease characterized by reduced bone mineral density (BMD) and increased fracture risk [3]. This disease, the most prevalent degenerative condition in developed countries, has become of prime importance due to our ever-increasing life span and the as yet poorly understood deterioration of bone quality over time.

Clues about the existence of neuronal and central regulatory components of the bone remodeling process have been reported in the clinical literature for several decades. Those correlations between bone and the nervous system include the reflex sympathetic dystrophy syndrome (or algodystrophy) characterized by localized sympathetic hyperactivity and osteopenia; increased osteogenic activity and BMD associated with severe head injury [4]; the associations between stroke, spinal cord injury, or peripheral neuropathy and osteopenia, bone fragility, or poor fracture healing [5–10]; and observations of robust neurogenesis during fracture healing [6, 11, 12]. It also seems logical that bone physiology would be coupled to the regulation of other homeostatic functions to better integrate skeletal physiology with the daily physiological changes triggered by the environment or by endogenous rhythms. The hypothalamus in that regard would be the preferential center to achieve this function, thanks to its “sensing” properties, its ability to integrate peripheral and central signals, and its ability to send efferent hormonal and neuronal signals in response to stimulation.

The hypothalamus was implicated as a regulator of bone remodeling by melding two independent and well-established epidemiological observations: obese patients

are protected from osteoporosis and osteoporosis typically follows gonadal deficiency. These clinical observations, which were previously viewed as unconnected, were synthesized to suggest the existence of a common mechanism regulating reproduction, body weight, and bone remodeling [13]. Based on the fact that reproduction and body weight are centrally regulated, the hypothesis implied the existence of a novel central locus for the regulation of bone remodeling. A number of studies by the Karsenty group using genetic and pharmacological manipulation of mouse models confirmed the notion that bone homeostasis is under the influence of a hypothalamic control center and that the output pathway is neuronal in nature.

### Multiple Hypothalamic Pathways Regulate Bone Remodeling

#### *Leptin*

The first experimental evidence of functional regulation of bone remodeling by the hypothalamus was provided by mice that are both hypogonadal and obese. The discovery that absence of leptin (*Ob*) or its signaling receptor (*ObRb*) induces a high bone mass in mice, in spite of hypogonadism, suggested a major inhibitory role for leptin in the regulation of bone mass [13]. Leptin is an adipocyte-derived hormone that acts on the brain to reduce food intake, mostly by regulating the activity of neurons in the hypothalamic arcuate nucleus that highly express *ObRb*. This anorexigenic function of leptin occurs via stimulation of neurons that express the anorexigenic peptide alpha-melanocyte-stimulating hormone ( $\alpha$ -MSH) and inhibition of neurons that coexpress the orexigenic peptides neuropeptide Y (NPY) and agouti-related protein (AgRP) [14]. However, along with its better-known role in the regulation of food intake and energy expenditure, leptin also regulates and integrates via the hypothalamus, or by direct peripheral effects, many other physiological functions, including the regulation of lipid and glucose metabolism, cardiovascular function, angiogenesis, reproduction, hematopoiesis, wound healing, and brain development [15].

Experimental evidence that leptin uses a central relay to regulate bone remodeling was provided first in mice and later in rats and sheep by demonstrating that low doses infused intracerebroventricularly (icv) significantly decrease trabecular bone mass [13, 16, 17]. Additional studies using mouse models with high or low serum leptin levels confirmed the pronounced effect of leptin on bone mass. Markedly increasing serum leptin level by transgenic liver overexpression reduced bone mass [18]. In contrast, mice characterized by low serum leptin level, such as A-ZIP lipodystrophic or PPAR<sup>hyp/hyp</sup> mice, displayed a high bone mass phenotype [18, 19]. Follow-

up studies using chemical lesioning and icv infusion of leptin defined a population of ventromedial hypothalamic (VMH) neurons as constituting the major center responsive to leptin for its regulation of bone remodeling [20]. Indeed, destruction of VMH neurons, which highly express the leptin receptor *ObRb*, recapitulated the bone phenotype of mice lacking leptin (*ob/ob*); and leptin icv infusion could rescue the high bone mass phenotype of *ob/ob* mice but not that of VMH-lesioned *ob/ob* mice, thereby convincingly demonstrating that leptin requires VMH neurons to decrease bone mass.

Surprisingly, a recent study using *Sfl-cre* mice to delete hypothalamic *ObRb* receptors in a VMH-specific manner reported the absence of a bone density phenotype in these mutant mice [21]. Unfortunately, bone mass was not assessed in this mouse model, and the field awaits a more appropriate histomorphometric or micro-computed tomographic (microCT) analysis. If it turns out to be correct that these mice have no bone phenotype, since 20% of their VMH neurons remained responsive to leptin (i.e., the *ObRb* deletion was only 80% complete), these findings would suggest that the VMH is highly sensitive to leptin for bone mass regulation.

The strong expression of *ObRb* in hypothalamic neurons together with the previously described *in vivo* results allowed elucidation of a novel physiological pathway for the control of bone mass by the brain, a discovery that has led to several controversies. The high bone mass and decreased fracture risk associated with obesity are counterintuitive based on this pathway, where high leptin levels should reduce bone mass. One explanation for this observation invokes the notion of leptin resistance, where leptin levels above a critical threshold create a state of resistance to the hormone. This explanation has been used to explain why obesity is associated with high leptin levels and may hold true to explain the increased bone mass in obese patients who would have a state of low leptin signaling centrally.

Another controversy revolves around the central vs. peripheral effects of leptin. Unfortunately, the literature on the peripheral effects of leptin is difficult to interpret as central leptin resistance may have been caused by the use of pharmacological doses of leptin; e.g., the cumulative dose of leptin that is required in mice to decrease bone mass centrally is approximately 5  $\mu$ g, whereas the cumulative dose used to demonstrate a peripheral action of leptin on resistance to load [22] was 860  $\mu$ g, both given over similar time frames. In another example, Burguera et al. [23] showed partial protection against ovariectomy-induced bone loss in rats with 100  $\mu$ g/day of peripheral leptin administration. Food intake, one of the most important central functions regulated by leptin, was not different between leptin- and vehicle-treated rats in the latter half of the study, suggesting the development of central leptin resistance. This was consistent

with the authors' finding that cerebrospinal fluid leptin levels were virtually identical in the two groups of animals. A second consideration in interpreting this body of work is that some peripheral leptin effects on bone can only be demonstrated in *ob/ob* mice and not in wild-type (WT) mice [24]. However, the reason leptin produced *in vivo* by liver overexpression was able to decrease bone mass [18] is at present unclear but may relate to the short half-life of leptin (5 min), to leaky expression centrally, or to inappropriate (asynchronous) peaks of exogenous leptin vis-à-vis its endogenous circadian secretion rhythm.

A separate controversy is whether leptin is produced peripherally. Several groups have shown that leptin (and its signaling receptor *ObRb*) can be found in human primary trabecular osteoblast cultures [24–26], whereas others have shown the absence of *leptin*, *ObRb*, and leptin signaling in mouse primary calvarial osteoblast cultures [12]. A mouse knock-in of  $\beta$ -galactosidase into the leptin locus demonstrated X-gal staining only in white fat and no other tissue, and overexpression of leptin in mouse osteoblasts *in vivo* via the 2.3kb  $\alpha 1$  collagen promoter did not affect trabecular bone mass [18, 20]. These studies indicate there may be species-specific differences in the production and signaling of leptin. If human leptin is produced in osteoblasts and if it directly and positively regulates bone formation, then clinical studies would be expected to show a positive association between serum leptin and BMD. On the other hand, if leptin mainly signals centrally to inhibit bone formation, then clinical studies should show an inverse association between serum leptin and BMD [27]. As discussed below, a large majority of studies find an inverse association, arguing against the peripheral mode of action. It is, however, possible that in the obese state peripheral production or positive signaling of leptin in bone becomes more prominent and this, along with central leptin resistance effects, could contribute to the higher bone mass and reduced fracture risk seen with obesity. If this is true, then clinical studies limited to obese participants should show a positive association between serum leptin and BMD.

Finally, there has been controversy surrounding the phenotype of *ob/ob* mice that were reported to have high trabecular bone mass in femora and vertebrae [12]. Bone studies of male *ob/ob* mice confirmed a high vertebral bone mass but identified low femoral bone mass, both trabecular and cortical, indicating that the response of bone to the absence of leptin signaling varies between the axial and appendicular skeleton in a gender-specific manner [13, 28]. Gender-specific differences in bone mass have been seen by us in several mouse models used to study the leptin pathway, validating the observations of Hamrick et al. [28] and suggesting that this difference is a general feature of the pathway. How this occurs is a fascinating and open research question.

Clinical studies have confirmed a role for leptin in the regulation of bone biology in humans. However, the skeletal sites, age, gender, and strength of the effect(s) of leptin are still being defined. Numerous studies have suggested that serum leptin level is associated with BMD, independently of fat mass and other covariates [27, 29–36]; but the association is not seen in many, often smaller, studies [37–42] and appears stronger for women. The stronger association in women is notable for two reasons, first because women have a two- to threefold higher leptin level than men and second because virtually all of the mouse studies elucidating the role of leptin and its pathway to bone were done in female mice [13, 18, 20, 27]. Moreover, the low bone mass phenotype in the femora of male *ob/ob* mice may identify a confounding variable that reduced the power to detect an effect in some of the negative clinical studies [39–44]. When detected, the association between serum leptin and BMD is almost always inverse, as would be expected from the mouse data; however, positive associations have occasionally been reported [43, 44]. Despite their hypogonadism, leptin-deficient patients have a high BMD phenotype that is equivalent to that seen in leptin-replete, eugonadal, obese controls [45]. Leptin-deficient patients also have accelerated bone age, indicating that leptin is an inhibitor of both chondrogenesis and osteogenesis in humans, as suggested by animal studies [46]. This effect was more pronounced in younger patients, suggesting that rapidly growing bone may be more sensitive to regulation by leptin [18].

#### *NPY and NPY Receptors*

NPY is a target of leptin signaling in the hypothalamus, with the potential to act through at least five receptors (Y1, Y2, Y4, Y5, and Y6) that differ not only in their binding profiles but also in their distribution in the central nervous system (CNS) and the periphery. All of these receptors are expressed in the hypothalamus, and several respond to other ligands. One important ligand for Y receptors, NPY, is widely distributed within the CNS. Strong expression is found in the hypothalamic area, where NPY fibers project from the arcuate nucleus, which integrates emotional, vegetative, sensory, and autonomic functions with endocrine functions.

NPY functions in the CNS include the regulation of anxiety-related behavior, feeding, and cardiovascular and memory functions [47]. Its role at the periphery is still unclear, but immunoreactivity for NPY is found in nerve fibers distributed throughout bone [48, 49]. Strongly supporting a role of NPY receptor signaling in the regulation of bone formation,  $Y2^{-/-}$  mice display an increase in long bone trabecular bone mass that can be reproduced by hypothalamus-specific deletion of  $Y2$ , indicating that  $Y2$  signaling in the hypothalamus inhibits bone formation [50]. Similarly, *ob/ob* mice have

a high trabecular bone mass phenotype that can be reproduced by destruction of hypothalamic neurons expressing *ObRB* [13, 20]. This similarity between *ob/ob* and *Y2<sup>-/-</sup>* mice and the elevation of NPY levels in the hypothalamus of both mouse models [51, 52] pointed toward a mechanistic link between NPY and leptin for the regulation of bone mass and to the suggestion that NPY may be a common mediator of the high bone mass of these two mouse models [53]. However, a dichotomy between the *ObRb* and *Y2* pathways is supported by comparison of the long bones of male *Y2<sup>-/-</sup>* and *ob/ob* mice that revealed that *Y2* deficiency stimulates both cortical and trabecular bone mass, while *leptin* deficiency has the opposite effect on both bone compartments, diminishing trabecular and cortical bone mass [54]. Furthermore, absence of the *Y2* receptor stimulated cortical bone formation with or without blockade of leptin action [54]. Interestingly, inactivation of both *Y2* and *Y4* receptors led to a further increase in bone mass compared to *Y2* alone, which was accompanied by reduced serum leptin level, suggesting a *Y4*-mediated additional effect of leptin deficiency on the *Y2<sup>-/-</sup>* bone phenotype [55].

Thus, *Y2* receptor signaling clearly regulates, via a hypothalamic relay, the bone formation arm of the bone remodeling process in both femoral trabecular and cortical bone compartments. Whether NPY is the ligand for *Y2* receptor for this function on bone mass and whether the role of NPY on bone formation is restricted to long bones is still a question since a bone mass phenotype was observed in NPY-deficient femurs but not vertebrae [56, 57]. The role of other *Y* receptors in bone remodeling awaits analysis of mutant mice lacking *Y1* or *Y5*. The peripheral function of NPY also remains to be investigated based on its rich expression in the bone microenvironment and during fracture healing. Lastly, one will have to verify whether the role of *Y* receptor and NPY signaling is conserved from mouse to humans since no human data are available yet to correlate these exciting mouse results with human physiology.

#### *The Cannabinoid System*

The cannabinoid system, mostly known for its involvement in psychotropic, analgesic, and orexigenic processes, has also recently been shown to regulate bone mass *in vivo*. The cannabinoid type 1 (CB1) receptor is expressed in the CNS and sympathetic nervous system (SNS), as well as in osteoclasts. It accounts for most of the CNS actions of cannabinoid drugs and endocannabinoid molecules synthesized by the body. Its absence in mutant animals induces a bone phenotype that is dependent on strain background differences, as has been observed for their other phenotypes [58]. Indeed, *CNRI<sup>-/-</sup>* mice on the outbred CD1 background exhibit a high bone mass phenotype [59], while *CNRI<sup>-/-</sup>* mice

on the inbred C57BL6/J background display a low bone mass phenotype, decreased bone formation, and elevated osteoclast number [60]. The molecular explanation for these opposing phenotypes is not known. It is, however, interesting to note that *CNRI<sup>-/-</sup>* mice on a C57BL6/J background are hypersensitive to leptin *icv*, which could explain at least part of their phenotype [61]. Because it inhibits norepinephrine release, CB1 signaling in peripheral nerves may affect signaling of the SNS to bone (described in more detail below) [62]. However, since CB1 is expressed in both the CNS and peripheral neurons and cells, the contribution of central versus peripheral CB1 receptors in mediating the effect of cannabinoids on bone remains to be determined. Here again, conditional deletion of *Cnr1* or increased expression of CB1 ligands through transgenesis will bring meaningful information regarding the importance and site of action of this receptor for its regulatory action on bone mass.

The CB2 receptor encoded by *Cnr2* is more specific for peripheral tissues and is notably expressed in immune cells, osteoblasts, and osteoclasts. Mice deficient for the *Cnr2* gene display a low bone mass phenotype resulting from high bone turnover, and *in vitro* analyses demonstrated a direct effect of CB2 agonists on the generation of osteoclasts, indicating a peripheral mode of action [63].

The importance of the cannabinoid system in bone biology is implicated in the bone response to traumatic brain injury (TBI), which enhances osteogenesis and stimulates central endocannabinoid production. In a TBI mouse model, Tam et al. [64] reported that *Cnr1<sup>-/-</sup>* mice, but not *Cnr2<sup>-/-</sup>* mice, are resistant to TBI-induced stimulation of bone formation, suggesting that CB1 signaling mediates the pro-osteogenic state in TBI.

In human studies, a significant association of single-nucleotide polymorphisms and haplotypes encompassing the CB2 gene (*CNR2*) at the susceptibility locus for low hip BMD [65] on human chromosome 1p36 was found in patients with postmenopausal osteoporosis [66]. An association was not found for *CNRI*, but based on the mouse results, studies of TBI in humans may be required to demonstrate an effect of this locus.

#### *CART and Melanocortin 4 Receptor*

Cocaine and amphetamine-regulated transcript (CART), another neuropeptide whose expression is positively controlled by leptin signaling [67, 68], has recently been shown to regulate the bone resorption arm of bone remodeling [69]. *Cart* is broadly expressed in the CNS, including in hypothalamic neurons, as well as in peripheral organs such as the pancreas and the adrenal gland. Mice deficient for *Cart*, although eugonadal, display a low bone mass phenotype solely due to an increase in bone resorption, indicating that CART



inhibits bone resorption *in vivo* [69]. The importance of CART in regulating bone remodeling and the hypothalamic nature of this regulation are supported by animal models characterized by low or high hypothalamic *Cart* expression and significant bone phenotypes. Low *Cart* expression in *ob/ob* mice accompanies the increased bone resorption observed in these mice, while increased hypothalamic *Cart* expression in obese and hyperleptinemic melanocortin 4 receptor (*Mc4r*)-deficient mice correlates with their low bone resorption and high bone mass [69]. Furthermore, decreasing *Cart* by genetic means in *Mc4r*-deficient mice rescues their resorption phenotype, indicating that *Cart* overexpression in *Mc4r*-deficient mice is required for their high bone mass phenotype [70]. Importantly, this CART-mediated regulatory loop of bone resorption may be conserved in humans as people lacking *MC4R* have increased CART serum levels and a high bone mass phenotype associated with decreased bone resorption [69–71]. The mode of action of CART on bone resorption is not yet defined and awaits identification of the CART receptor.

#### Neuronal Nitric Oxide Synthase and Central Nitric Oxide Signaling

Nitric oxide (NO) and enzymes producing it (endothelial, inducible, and neuronal NO synthases) play important regulatory roles in multiple cell types, including osteoblasts. In particular, endothelial NO synthase (eNOS) appears to be a positive regulator of osteoblast proliferation and differentiation, based on its predominance in bone cells (compared to other isoforms) and the low bone mass phenotype of mice deficient for eNOS [72, 73]. Inducible NOS (iNOS) activity in osteoblasts is essential for mediating interleukin-1 (IL-1)-induced bone resorption and regulating the effects of proinflammatory cytokines on bone [74–76]. On the other hand, neuronal NOS (nNOS) expression is minimal in bone cells, and osteoblasts derived from nNOS-deficient mice do not display obvious proliferation or differentiation defects, whereas *in vitro* receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF)-induced osteoclastogenesis from bone marrow macrophages is increased in the absence of nNOS [77]. In contrast to these *in vitro* observations, nNOS-deficient mice display a high bone mass phenotype and low bone turnover. This bone phenotype along with the high expression of nNOS in the CNS, including the hypothalamus, suggest that nNOS might regulate bone homeostasis by a central relay, although a role in the peripheral nervous system cannot be excluded for now. eNOS and iNOS are also expressed in the CNS and could play a role in the regulation of bone mass via a central relay as well. Experimental interventions aimed

at modifying NO production or NOS activity specifically in the hypothalamus or other CNS regions are essential to determine whether a central relay involving NO signaling regulates bone homeostasis.

#### Sympathetic Signaling Downstream of Hypothalamic Leptin-Responsive Neurons Regulates Bone Formation

The identification of a central relay for leptin's antiosseogenic function led to the question of the nature of the downstream mechanism whereby hypothalamic neurons regulate the activity of distant cells like osteoblasts. One could imagine the existence of two alternate pathways: a humoral pathway involving a molecule secreted by the hypothalamus or pituitary and a neuronal pathway. Parabiosis experiments have argued against a humoral mechanism [20], while several observations support a neuronal pathway: (1) *ob/ob* mice have a low sympathetic tone, (2) electrical stimulation of the VMH [78] and leptin injection directly into the VMH nucleus result in enhanced sympathetic tone [79], and (3) innervation of bone and the presence of immunological reactivity for diverse neuropeptides within the bone microenvironment were described several decades ago [for review, see 80], although the physiological relevance of these observations remained obscure. Along with these observations, the selective detection, among all post-synaptic adrenergic receptors, of the  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR) in mouse primary osteoblasts supported the hypothesis that the SNS could relay leptin signaling from the hypothalamus to osteoblasts [69]. In agreement with this hypothesis, mice lacking dopamine  $\beta$ -hydroxylase (*Dbh*), the enzyme generating norepinephrine, were found to have an increased bone mass, as were mice treated with the  $\beta$ -blocker propranolol. In contrast, mice treated with the adrenergic agonist isoproterenol, used as a surrogate of sympathetic activation, lost bone mass [20]. In rats, a  $\beta_2$ AR agonist decreased BMD as well as mechanical strength [81], while  $\beta$ -blockade had been previously shown to have the opposite effects [82]. These data strongly suggested that  $\beta$ -adrenergic signaling, downstream of leptin signaling, inhibits bone formation by osteoblasts. However, because of the multiple endocrine abnormalities observed in *ob/ob* and *Dbh* mice, these data and concepts have been questioned. Phenotypic analysis of mice lacking *Adrb2* (the  $\beta_2$ AR gene), which are not plagued by any of the endocrine abnormalities seen in *ob/ob* or *Dbh* mice, provided a definitive answer to this question. Indeed, *Adrb2*-deficient mice, as opposed to *ob/ob*, VMH-lesioned, or *Dbh*<sup>-/-</sup> mice, have a normal body weight and normal hormonal status but show an increased trabecular bone formation rate and increased bone mass [69]. Bone marrow transplantation experiments between WT and *Adrb2*-deficient mice demonstrated that this bone phenotype was due to a bone cell-autonomous defect

and not a central defect, which was a possibility based on the widespread expression of *Adrb2* in the CNS. Importantly, *Adrb2*-deficient mice, in contrast to WT littermates, were resistant to leptin icv infusion, indicating that  $\beta_2$ AR signaling is required for leptin's anti-osteogenic function [69]. At the cellular level, *Adrb2*-deficient mice, like *ob/ob* mice, showed increased numbers of osteoblasts and an increased bone formation rate, suggesting that inhibition of osteoblast proliferation (or differentiation) and function is the final effect of sympathetic signaling. Together, this *in vivo* pharmacological and genetic body of evidence demonstrated that leptin regulates bone formation via VMH neurons, the SNS, and  $\beta_2$ AR signaling in osteoblasts.

Although mouse osteoblasts do not normally express *Adrb1* and *Adrb1*-deficient mice have a normal bone mass, mice deficient for both *Adrb1* and *Adrb2* display a low bone mass phenotype, suggesting an indirect role for  $\beta_1$ AR signaling in bone biology. The reduced femur length and low bone mass characterizing *Adrb1/2*-deficient mice are accompanied by decreased serum levels of insulin-like growth factor I (IGF-I), suggesting that this indirect role may proceed through an osteoblast-independent mechanism [83].

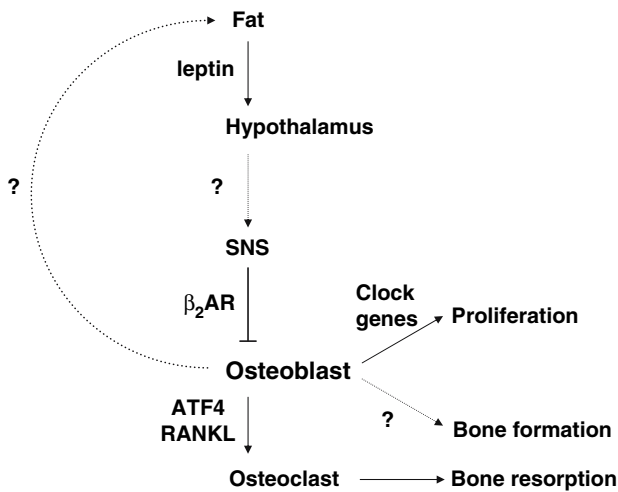
In addition to the examples cited at the beginning of this article, a role for  $\beta$ -adrenergic signaling in human bone biology is supported by several recent epidemiological studies [84, 85]. A meta-analysis of seven studies assessing fracture risk in patients using  $\beta$ -blockers found their use was associated with a 28% reduction in hip fracture risk (95% confidence interval [CI] 19–37% reduction) and a 14% reduction (95% CI 2–24% reduction) in the risk for any fracture [84]. These case-control studies yielded several important pieces of information. First, it appears that  $\beta$ -blocker effects on BMD, which is different from bone mass, account for only a small portion of the observed fracture protection [77]. Thus, the influence of sympathetic signaling on bone metabolism may be somewhat different for humans and mice, with the caveat that human bone mass has not been properly examined. Second, one of the largest studies [86] found that only  $\beta$ -blockers without intrinsic sympathomimetic activity were protective, indicating that the observed protection was not a side effect of the drugs but rather due to sympathetic blockade. Third,  $\alpha$ -adrenergic blockers have no detectable effect on fracture risk, indicating specificity for the  $\beta$ -adrenergic pathway [84]. Fourth, the protective effects of  $\beta$ -blockers appear to be mediated through effects on sympathetic blockade of  $\beta_1$ -adrenoceptors, perhaps explaining the failure of one study that excluded  $\beta_1$ -blockers to find an effect on fracture risk [87]. Taken together with the mouse data showing that *Adrb1* deficiency rescues the high bone mass of *Adrb2* deficiency, these results point to an as yet unexplored complexity in the regulation of bone metabolism by sympathetic sig-

naling. Long-term prospective randomized trials are required to demonstrate unequivocally a protective effect of  $\beta$ -blockers, but these will take some time to complete. For now, the human data remain quite promising and open to further refinement.

### Sympathetic Signaling Regulates Both Arms of Bone Remodeling

Further analysis of the bone phenotype of *Adrb2*-deficient mice revealed that  $\beta_2$ AR signaling regulates not only bone formation but also bone resorption. Indeed, *Adrb2*-deficient mice display decreased bone resorption due to a decrease in osteoclast differentiation. To understand the cellular and molecular basis of this phenotype, osteoblast-osteoclast coculture assays were performed using combinations of WT and *Adrb2*<sup>-/-</sup> cells. This strategy demonstrated that  $\beta_2$ AR stimulation in osteoblasts indirectly favors osteoclast differentiation. Further biochemical and molecular studies indicated that  $\beta_2$ AR stimulation in osteoblasts increases the cyclic adenosine monophosphate (cAMP) level and activates protein kinase A (PKA), which eventually results in the phosphorylation of ATF4, a cAMP response element binding protein (CREB) family member previously shown to regulate osteoblast differentiation and collagen synthesis [88]. Mutation/phosphorylation and promoter studies pinpointed ATF4's serine 254 as the target of PKA and as a necessary step for ATF4 to bind to and transactivate the *Rankl* promoter. In agreement with this result, isoproterenol, a surrogate of sympathetic signaling, increased *Rankl* expression in WT cells but not in *Aft4*<sup>-/-</sup> osteoblasts [69]. These studies thus characterized a new transcriptional target of  $\beta_2$ AR signaling in osteoblasts and identified a key role for  $\beta_2$ AR signaling in the regulation of bone formation and bone resorption. The *in vivo* relevance of the role of sympathetic signaling in regulating bone resorption was confirmed in mice and rats by showing that  $\beta$ -blockers could ameliorate or even prevent bone loss associated with ovariectomy [25, 81]. Moreover, a short-term randomized trial confirmed that  $\beta$ -blockers could suppress bone resorption in humans [89]. The pathway whereby leptin regulates bone formation and bone resorption is illustrated in Figure 1.

The sympathetic/ $\beta$ -adrenergic pathway is so far the only identified signaling pathway linking the hypothalamus and bone cells, downstream of leptin. Whether the autonomic nervous system, and in particular  $\beta_2$ AR signaling, also mediates hypothalamic NPY or Y2 receptor antiosteogenic function remains unknown. The lack of any changes in plasma total calcium, leptin, hypothalamo-pituitary-corticotrophic, -thyrotrophic, -somatotrophic, or -gonadotropic status in *Y2*<sup>-/-</sup> mice suggests that the Y2 receptor does not modulate bone formation by a known humoral mechanism and, there-



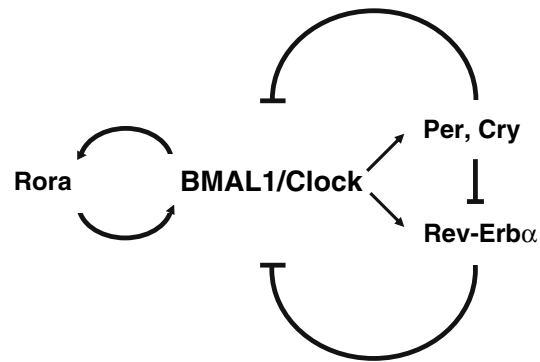
**Fig. 1.** Pathway for the regulation of bone formation and resorption by white adipose tissue. Structures are listed in the *central axis* of the figure; functions are on the *right* and molecular mediators on the *left* of the *arrows*. *Arrows* indicate enhancement of function, while the *blunted line* between SNS and *Osteoblast* indicates inhibition of osteoblast function and proliferation by the SNS. *Question marks* indicate regulators that remain to be discovered. Homeostasis requires feedback; therefore, an *arrow* representing a hypothetical pathway is shown from *Osteoblast* to *Fat*, but osteoblasts could just as well provide feedback to the hypothalamus and/or the SNS.

fore, that alteration of autonomic function through hypothalamic Y2 receptors could possibly play a role in the central regulatory circuit involved in bone formation [50]. Interestingly, viral retrograde neuronal labeling recently confirmed the neuronal nature of the link between bone and brain and, in particular, revealed targeting to the parvocellular nuclei where NPY is released and the Y1 receptor is expressed [90]. Additional studies including infusion of NPY to *Adrb2<sup>-/-</sup>* mice should address the question of the relationship between NPY signaling in the hypothalamus and  $\beta_2$ AR-mediated sympathetic signaling to bone.

### The Circadian Clock Regulates Sympathetic Signaling to Bone by Gating Osteoblast Proliferation

Daily variation in bone marrow proliferation with a peak occurring at night has long been known [91], as have daily (circadian) variations in the synthesis of collagen [92] and, more recently, in the variation in bone turnover markers [93–95]. Since the body's main clock resides in the hypothalamus and leptin-regulated bone remodeling was demonstrated to be under hypothalamic control, these observations suggested that leptin's effects on bone may be physiologically integrated by the molecular clock.

The molecular clock is evolutionarily conserved from archaeobacteria to humans and is found in most organs of the body. It is composed of a positive transcriptional



**Fig. 2.** Simplified view of the molecular clock. BMAL1 and Clock form a heterodimeric transcription factor that stimulates transcription of the major negative arm genes *Per1*, *Per2*, *Cry1*, and *Cry2*. This stimulation is self-limited because, along with *Rev-Erbα*, *Per* and *Cry* disrupt BMAL1/Clock-mediated transcription. The next phase sees declining *Per* and *Cry* levels, and this in combination with their inhibition of *Rev-Erbα* function relieves the repression of BMAL1/Clock. BMAL1 and Clock are then free to be reinduced by positive regulators like the retinoic acid-related orphan receptor  $\alpha$  (*Rora*) to begin the cycle anew.

arm that turns on a group of genes termed the *negative arm*. The negative arm then suppresses the positive arm, with the whole process lasting around 24 hours and ending with reinduction of the positive arm genes to restart the cycle (Fig. 2). The positive arm of the clock is a heterodimeric transcription factor composed of the Clock and BMAL1 (MOP3) proteins. The main negative arm genes are *Period (Per) 1* and 2 and *Cryptochrome (Cry) 1* and 2 [89].

To test the role of the molecular clock in the regulation of bone mass, female mice lacking both *Per* genes, which are molecularly arrhythmic, were examined and found to have a high bone mass phenotype in vertebrae and femora that was due to increased numbers of osteoblasts and an increased bone formation rate [96]. Mice lacking both *Cry* genes showed the same phenotype, indicating that the molecular clock is indeed an inhibitor of bone mass. Mice lacking a molecular clock were not deficient in leptin or sympathetic tone but were found to be resistant to the antiosteogenic effect of icv leptin infusion. Since increasing sympathetic signaling by icv leptin infusion did not lead to the expected bone loss in these mice, the results suggested an interruption of the leptin-SNS-bone pathway at the level of the bone. Consistent with this, bone was found to harbor all components of the molecular clock with up to 70-fold variation in the level of gene expression over a 24-hour period. Moreover, osteoblast-specific deletion of *Per* function using the *2.3 kb α1(I)-collagen* promoter *cre* transgene resulted in high bone mass [96].

In WT mice, osteoblasts showed a diurnal variation in proliferation with a peak of DNA synthesis at night, whereas mice lacking a molecular clock showed no variation but instead had a tonically elevated rate of

proliferation, as did their osteoblasts *in vitro*, which showed shortening of the G<sub>1</sub> phase of the cell cycle. These results suggested that the molecular clock may mediate the effects of sympathetic signaling on osteoblasts. Consistent with this,  $\beta_2$ AR was found to signal via CREB to directly activate the negative arm clock genes *Per1* and *Per2* in osteoblasts. These clock genes then inhibit *c-myc* transcription, leading to downregulation of its target *cyclin D1*, thus describing a complete molecular pathway for leptin's antiosteogenic function [96]. In addition to the clock genes,  $\beta_2$ AR signaling activated gene expression of AP-1, a family of transcription factors known to regulate osteoblast proliferation in part through activation of *c-myc* and *cyclin D1* [90]. Thus, the data are consistent with a model whereby the molecular clock acts downstream of  $\beta_2$ AR to gate sympathetic signaling in osteoblasts to ensure that proliferation preferentially occurs at certain times. In its absence, osteoblast proliferation is increased throughout the 24-hour day, and this eventually leads to high bone mass. Looking forward, the molecular mechanisms whereby the clock regulates the synthesis of bone extracellular matrix remain to be elucidated.

The clocks found in peripheral organs are synchronized by the master clock in the hypothalamus [97]. The regulation of bone mass by the clock is therefore likely to be influenced by factors that entrain the master clock, such as day length, and could therefore be one of the mechanisms underlying the seasonal changes observed in bone mass for most mammals, including humans [98, 99]. Intriguingly, peripheral clocks are able to function independently of the master clock under certain circumstances [100, 101]; thus, there may be ways to selectively affect the osteoblast clock to achieve a therapeutic gain in bone mass.

## Conclusions

The increasing number of articles reporting bone phenotypes in mutant animal models deficient for neuropeptides or their receptors confirms the physiological relevance of a central and neuronal regulatory arm of bone remodeling and emphasizes the importance of genetically engineered mouse models for understanding bone physiology and homeostasis. Although of major importance, these studies still require follow-up clinical investigation to confirm the conservation of function between murine and human physiology and disease and to allow the design of new therapeutic strategies for the treatment of bone diseases. The results obtained during the last 15 years using genetically engineered mouse models and *in vivo* studies also highlight by their sometimes contradictory and/or complex results the importance of using appropriate genetic controls (mouse background), technology (e.g., histomorphometry with

microCT analyses, which are far superior to BMD analysis in mice), nomenclature, and definition of the sex, bone, bone compartment, and age of the studied animals.

The demonstration that bone remodeling is regulated by hypothalamic centers suggests that this process is an integral part of the complex homeostatic control system that allows the body to respond to internal and external environmental changes, allowing the coordinated integration of bone homeostasis with diet, reproduction, lactation, and physical activity via hypothalamic neuronal networks responsive to mediators generated by each of these physiological processes. One can hypothesize that the evolutionary selection of common mediators for functions of a disparate nature may have occurred as a mechanism to jointly coordinate these functions, via the hypothalamus. This is best exemplified by the complex central effect of the hormone leptin on reproduction, bone remodeling, food intake, energy expenditure, and immunity.

The knowledge brought by these studies is fascinating and allows a deeper understanding of mammalian homeostasis. However, the complexity of hypothalamic signaling networks will make it difficult to intervene therapeutically at the hypothalamic level to modulate any aspect of bone remodeling without affecting other homeostatic processes, such as the regulation of body weight homeostasis or reproduction. An alternative, more feasible strategy may be to intervene downstream of the hypothalamus with drugs that block signals from the hypothalamus to bone cells, the reception of these signals by bone cells, or intracellular transduction pathways in bone cells.  $\beta$ -Blockers may be one way to achieve such a goal based on their dual ability, demonstrated in mice and rats, to increase bone formation and decrease bone resorption. The existence of  $\beta$ -blockers that do not cross the brain barrier will also be of interest, to limit their action to peripheral tissues. Although there are no NPY receptor antagonists clinically available, the recent results obtained with mice for the regulation of both body weight and bone mass may trigger more interest in developing such agents.

Since feedback loops are required for homeostasis to occur, these new findings imply the existence of a pathway whereby bone cells can signal back to the hypothalamus (or other brain centers). The nature and existence of this putative signal are still unknown but could involve factors of either a humoral or neuronal nature. The emerging description of neuroskeletal biology is a major conceptual advance in the field of bone biology that is allowing old questions to be asked in new ways, and it has already proven to be promising for identifying new targets for the amelioration of bone disease. What makes it particularly stimulating is not only the chance to identify new therapeutics but also the opportunity to use knowledge, concepts, and tools from other fields and apply them to the field of bone biology.



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