Baldock et al.: Hypothalamic Control of Bone Formation: Distinct Actions of Leptin and Y2
Receptor Pathways
Journal of Bone and Mineral Research, 20(10) 1851-1857, 2005

Hypothalamic Control of Bone Formation: Distinct Actions of Leptin and Y2 Receptor Pathways

Paul A Baldock,[1] Amanda Sainsbury,[2] Susan Allison,[1] En-JuDLin,[3] Michelle Couzens,[2] Dana Boey,[2] Ronaldo Enriquez,[1] Matthew During,[3] Herbert Herzog,[2] and Edith M Gardiner[2,4]

[1]*Bone and Mineral Program, Garvan Institute of Medical Research, St Vincent's Hospital, Darlinghurst, Sydney, Australia;* [2]*Neuro- biology Program, Garvan Institute of Medical Research, St Vincent's Hospital, Darlinghurst, Sydney, Australia;* [3]*Department of Molecular Medicine and Pathology, University of Auckland, Auckland, New Zealand;* [4]*Present address: University of Queensland School of Medicine, Princess Alexandra Hospital, Brisbane, Queensland, Australia.*

ABSTRACT: **Leptin and Y2 receptors on hypothalamic NPY neurons mediate leptin effects on energy homeostasis; however, their interaction in modulating osteoblast activity is not established. Here, direct testing of this possibility indicates distinct mechanisms of action for leptin anti-osteogenic and Y2−/− anabolic pathways in modulating bone formation.**

Introduction: Central enhancement of bone formation by hypothalamic neurons is observed in leptin-deficient *ob/ob* and Y2 receptor null mice. Similar elevation in central neuropeptide Y (NPY) expression and effects on osteoblast activity in these two models suggest a shared pathway between leptin and Y2 receptors in the central control of bone physiology. The aim of this study was to test whether the leptin and Y2 receptor pathways regulate bone by the same or distinct mechanisms.
Materials and Methods: The interaction of concomitant leptin and Y2 receptor deficiency in controlling bone was examined in Y2−/−*ob/ob* double mutant mice, to determine whether leptin and Y2 receptor deficiency have additive effects. Interaction between leptin excess and Y2 receptor deletion was examined using recombinant adeno-associated viral vector overproduction of NPY (AAV-NPY) to produce weight gain and thus leptin excess in adult Y2−/− mice. Cancellous bone volume and bone cell function were assessed.
Results: Osteoblast activity was comparably elevated in *ob/ob*, Y2−/−, and Y2−/−*ob/ob* mice. However, greater bone resorption in *ob/ob* and Y2−/−*ob/ob* mice reduced cancellous bone volume compared with Y2−/−. Both wildtype and Y2−/− AAV-NPY mice exhibited marked elevation of white adipose tissue accumulation and hence leptin expression, thereby reducing osteoblast activity. Despite this anti-osteogenic leptin effect in the obese AAV-NPY model, osteoblast activity in Y2−/− AAV-NPY mice remained significantly greater than in wildtype AAV-NPY mice.
Conclusions: This study suggests that NPY is not a key regulator of the leptin-dependent osteoblast activity, because both the leptin-deficient stimulation of bone formation and the excess leptin inhibition of bone formation can occur in the presence of high hypothalamic NPY. The Y2−/− pathway acts consistently to stimulate bone formation; in contrast, leptin continues to suppress bone formation as circulating levels increase. As a result, they act increasingly in opposition as obesity becomes more marked. Thus, in the absence of leptin, the cancellous bone response to loss of Y2 receptor and leptin activity can not be distinguished. However, as leptin levels increase to physiological levels, distinct signaling pathways are revealed.

INTRODUCTION

Skeletal tissue is constantly being remodeled in a process that maintains both mechanical integrity and mineral homeostasis. Control of this process occurs primarily through interactions between endocrine hormones, local growth factors, and cytokines that orchestrate the activities

of both osteoclastic and osteoblastic cells. Recently, however, two potent bone anabolic models have been described that involve osteoblast regulation by neurons of the hypothalamus. Both leptin and Y2 receptors have been shown to modulate osteoblast activity, with inactivation of the gene encoding either protein leading to a high cancellous bone mass phenotype associated with elevated osteoblastic activity in mice. (**1**, **2**) Importantly, the involvement of hypothalamic activity in the phenotypes of leptin-deficient *ob/ob* and Y2−/− mice has been experimentally shown. The high bone mass of leptin-deficient *ob/ob* mice was corrected after central leptin supplementation using microdoses that had no effect on bone when injected peripherally. (**1**) Conditional deletion of hypothalamic Y2 receptors in adult mice recapitulates the high BMD of germline Y2−/− mice. (**2**)

From these studies, it is clear that alterations of central leptin or Y2 signaling can influence osteoblast activity. However, the extent to which the leptin and Y2 receptor pathways interact in regulating bone has not been resolved. Leptin and Y2 receptors have been shown to interact in the regulation of adipose tissue, with Y2−/−*ob/ob* double knockout mice displaying a significant reduction of the obesity phenotype characteristic of *ob/ob* mice. (**3**, **4**) Furthermore, *ob/ob* and Y2−/− mice share key characteristics, with similar increases in osteoblast activity, suggesting a commonality of mechanism. In addition, neuropeptide Y (NPY) levels are significantly elevated in the hypothalamus of both *ob/ob*(**3**, **5**) and Y2−/− mice. (**6**) These observations support a mechanistic link between leptin and Y2 in the regulation of bone physiology. The role of NPY in the central control of bone formation has been questioned, however, because no skeletal phenotype was observed in NPY knockout mice. (**7**) Notably, these mice also lacked an obvious phenotype with respect to food intake and body weight, (**8**) despite a well-documented association of obesity with elevated NPY activity. (**9**) In contrast, when the NPY−/− mouse was crossed onto the leptin-deficient *ob/ob* mutant, the obese phenotype of the *ob/ob* was dramatically reduced. (**10**) This suggests that elevated central NPYergic activity contributes to the obesity phenotypes but that loss of NPY does not cause lean phenotypes. Similarly, in bone, it is possible that high, but not low, central NPYergic tonus is involved in regulation of bone physiology. We therefore hypothesize that elevated hypothalamic NPY expression may be a common mediator of the high BMD phenotype of both *ob/ob* and Y2−/− mice. In keeping with the hypothesis that central NPY and not leptin deficiency is the common stimulator of osteoblastic activity, circulating leptin levels vary between normal in Y2−/− mice(**6**) and absent in *ob/ob*,(**11**) despite both exhibiting elevated osteoblast activity. In this manner the leptin- and Y2-mediated pathways may interact through a shared pathway, possibly through the increase in central NPY expression.

In contrast, however, there is evidence from Y2−/−Y4−/− receptor double knockout mice that leptin-related pathways may mediate osteoblast function by a pathway distinct from Y2 receptor signaling. Male Y2−/−Y4−/− double knockout mice have a lean phenotype and a significantly reduced serum leptin compared with wildtype or Y2−/− mice. (**6**) These male mice also display a significant increase in cancellous bone volume compared with Y2−/− mice. Importantly, female Y2−/−Y4−/− mice that did not display a reduction in serum leptin also lacked the additional increase in cancellous bone volume over Y2−/− mice (P Baldock, A Sainsbury, H Hertzog, and E Gardiner, unpublished observations, 2001), suggesting that at reduced but still physiological levels of leptin, there may be a stimulation of bone formation induced by reduced leptin levels, in addition to that associated with lack of Y2 signaling. This study therefore investigated the hypothesis that the control of osteoblast activity by leptin and Y2 receptor deficiency occur by distinct pathways. To test this hypothesis, osteoblast activity was examined in Y2 knockout, leptin-deficient *ob/ob*, and Y2−/−*ob/ob* double mutant mice. This comparison was used to determine the extent to which a lack of Y2 receptors would alter the leptin-deficient bone anabolic response and to determine whether leptin and Y2 receptor deficiency have additive affects (suggesting distinct pathways) or not (suggesting common mechanisms). To further study the interrelationship among leptin, Y2, and NPY, a second contrasting model was used to increase circulating leptin while still maintaining elevated hypothalamic NPY. This high leptin-high NPY model was generated by constitutive viral overexpression of NPY in the hypothalamus, resulting in increased deposition of adipose tissue and consequently leptin production. Thus, the high leptin-high NPY model contrasted with the normal leptin-high NPY model of Y2−/− and the no leptin-high NPY model of *ob/ob*. Together, these comparisons studied the effect of consistently elevated hypothalamic NPY expression in osteoblast function over a range of circulating leptin concentrations and the role of Y2 receptors

in the leptin-NPY-bone interaction.

MATERIALS AND METHODS

Animals

Y2−/−, *ob/ob*, and Y2−/−*ob/ob* mice were generated as previously described. (**1**, **3**) Mice were maintained on a mixed C57 BL/6-129/SvJ background, which was maintained as wildtype. Mice received chow diet and water ad libitum. Skeletal phenotypes were assessed in 16-week-old male animals for comparison of germline knockout models and 18-week-old male mice for viral overproduction studies.

Viral overproduction of NPY

Recombinant adeno-associated viral vector expressing NPY under the control of a neuron specific enolase promoter (AAV-NPY) was bilaterally injected into the hypothalamic area (coordinates relative to Bregma: posterior, 2.1 mm; lateral, ±0.4 mm; ventral, 5.3 mm), as previously described. (**12**) Elevated hypothalamic NPY action stimulates feeding, energy homeostasis, and adipose tissue deposition, thereby leading to increased leptin expression. (**13**, **14**) One microliter of vector with a genomic titer of 1×10^{12} genome copies/ml was injected into each side over 5 minutes using a 30-gauge cannula connected to an automated syringe pump. Control groups were injected with vehicle alone (AAV-empty) in the hypothalamus, and an additional control group was injected with AAV-NPY or AAV-empty vector in the hippocampus, a brain region functionally removed from the neural pathways of the hypothalamus (coordinates relative to Bregma: posterior, 1.7 mm; lateral, ±0.8 mm; ventral, 2.2 mm}. The efficacy of vector injection and expression was confirmed by detection of NPY transcripts using in situ hybridization on brain slices. (**15**) The efficacy of viral NPY expression to induce a functional response was confirmed by the resultant weight gain, a characteristic response to elevated hypothalamic NPY production. (**13**)

Tissue collection and analysis

Germline knockout mice were collected at 16 weeks of age in groups of 6–10. AAV-injected animals were collected at 18 weeks of age, 3 weeks after viral injection, which was at 15 weeks of age. Groups of 10–12 mice per genotype and virus were killed by cervical dislocation and decapitation. Trunk blood was collected, and plasma was frozen at −20 C for later analysis, with leptin levels measured by radioimmunoassay (Linco Research, St Louis, MO, USA). The brain was removed and immediately frozen on dry ice. White adipose tissue (WAT) deposits (right inguinal, right epididymal, right retroperitoneal, and mesenteric) were collected and weighed. Both femora were excised and fixed in 4% paraformaldehyde at 4°C for 16 h.

The right femora were bisected transversely at the midpoint of the shaft. Distal halves were embedded undecalcified in methyl-methacrylate (APS Chemicals, Sydney, Australia) and 5-μm sagittal sections were cut using a Jung RM2055 semiautomatic microtome (Leica Microsystems, Sydney, Australia) stained for mineralized bone using a modified von Kossa technique and analyzed for cancellous bone volume, trabecular thickness, and number (Bioquant, R&M Biometrics, Nashville, TN, USA) as described previously. (**2**) Osteoblast parameters (osteoblast surface and osteoblast number) were measured using Masson's trichrome-stained sections at x400 magnification, with osteoid surface and osteoid thickness measured on the same sections at x1000 magnification. Osteoid volume was calculated as the product of mean osteoid surface and mean osteoid thickness for each section. Unlike the germline knockout comparison, in which calcein (20 mg/kg) and demeclocycline (30 mg/kg) were injected 10 and 3 days before tissue collection, respectively, tetracycline labels were not included in the AAV study to eliminate any possible adverse interaction of the tetracycline with AAV activity. Osteoclast surface and osteoclast number were measured at x400 from sections stained for TRACP activity as described previously. (**2**) All cancellous measurements were conducted in a sample area bordering the epiphyseal growth plate, beginning 0.25 mm distal to the mineralization zone to exclude primary spongiosa and extending proximally 4.2 mm, encompassing all the cancellous bone within the cortices, as described previously. (**2**)

Statistical analyses

Differences between genotypes in the germline knockout comparisons were made by one-way ANOVA for genotype, with subsequent Fisher's posthoc tests for those variables that returned significant results. The NPY overexpression studies were assessed by two-way ANOVA for genotype and injection, again with Fisher's posthoc tests where appropriate. Results for change in body weight were compared among viral-injected groups by repeated-measures ANOVA followed by Fisher's posthoc tests. StatView version 4.5 (Abacus Concepts, Berkeley, CA, USA) was used for all statistical analyses, and $p < 0.05$ was accepted as being statistically significant for both ANOVA and posthoc analyses.

RESULTS

Y2–/– ob/ob double knockout mice

Cancellous bone volume was significantly elevated in Y2–/–, *ob/ob*, and Y2–/–*ob/ob* mice compared with wildtype mice (Fig. 1A), with greater trabecular thickness in all three models (Fig. 1B). Trabecular number, elevated compared with wildtype in Y2–/–, was not elevated in either *ob/ob* or Y2–/–*ob/ob* mice (Fig. 1C), consistent with the significantly greater cancellous bone volume in Y2–/– mice compared with *ob/ob* or Y2–/–*ob/ob*. Importantly, the enhanced trabecular number and bone volume in Y2–/– compared with wildtype, *ob/ob*, and Y2–/–*ob/ob* mice was not associated with a greater osteoblast activity, because mineral apposition rate was similarly elevated in all three mutant models compared with wildtype mice (Fig. 1D). Rather, the greater cancellous bone volume in Y2–/– was consistent with greater bone resorption in the *ob/ob* and Y2–/–*ob/ob* models compared with Y2–/– or wildtype, evident in the increased osteoclast surface (Fig. 1E). The wildtype level of bone resorption in the Y2–/– mice is consistent with the eugonadal status of these animals(**2**, **15**) compared with the hypogonadal status of *ob/ob* and Y2–/–*ob/ob* models. (**3**)
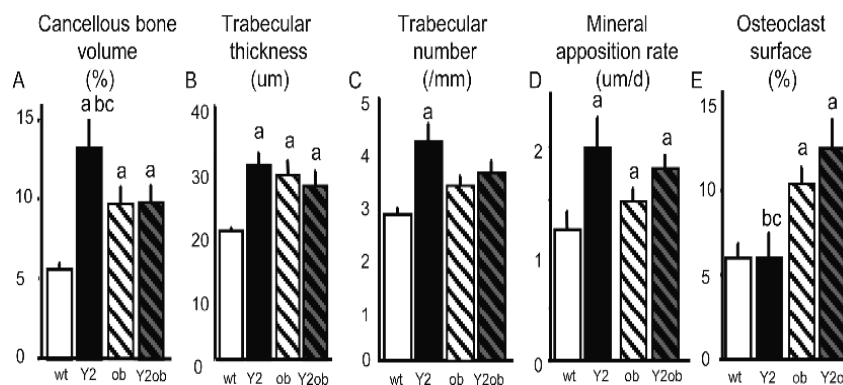

FIG. 1. Cancellous bone phenotype in Y2ob mice. (A) Cancellous bone volume. (B) Trabecular thickness. (C) Trabecular number. (D) Mineral apposition rate. (E) Osteoclast surface. Both *ob/ob* and Y2$^{-/-}$ mice have greater cancellous bone volume and trabecular thickness associated with increased mineral apposition rate; only Y2$^{-/-}$ have greater trabecular number. The combined Y2$^{-/-}$ *ob/ob* mice resemble the *ob/ob* with respect to cancellous bone volume and architecture and intermediate with respect to mineral apposition rate, and consistent with the hypogonadal state, increased osteoclast surface. Data are mean ± SE, $n = 6$–14; [a]$p < 0.05$ vs. wildtype, [b]$p < 0.05$ vs. *ob/ob*, [c]$p < 0.05$ vs. *Y2/ob*.

Hypothalamic NPY overexpression mediated by viral vector

Bilateral injection of AAV-NPY resulted in a marked overproduction of NPY transcripts in the hypothalamus of these mice. This expression was localized to the injection site (Fig. 2), with no obvious signs of axonal transport. Injection of AAV-NPY into the hippocampus caused no changes in bone or adipose tissue measurements, confirming the restricted localization of the virally expressed product and the specificity of the hypothalamic circuit in the NPY-regulated bone and adipose tissue pathways. Body weight, serum leptin (Figs. 3A and 3B), cancellous bone volume (Fig. 3C), and osteoblast and osteoclast indices (Figs. 3D-3H) were unchanged between AAV-NPY and AAV-empty injected wildtype mice in these hippocampal injected control groups.
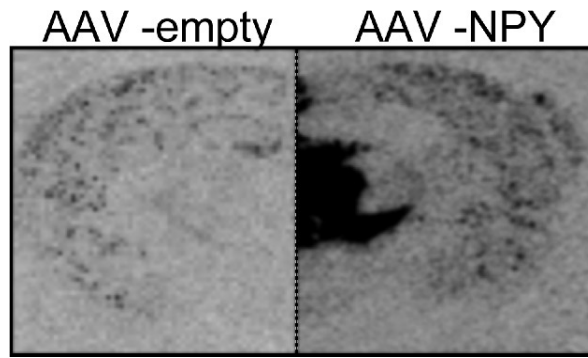
Baldock et al.: Hypothalamic Control of Bone Formation: Distinct Actions of Leptin and Y2
Receptor Pathways
Journal of Bone and Mineral Research, 20(10) 1851-1857, 2005

FIG. 2. Viral overexpression of NPY in the hypothalamus. Coronal brain sections showing in situ PCR for NPY mRNA expression after injection of empty or NPY-expressing AAV.
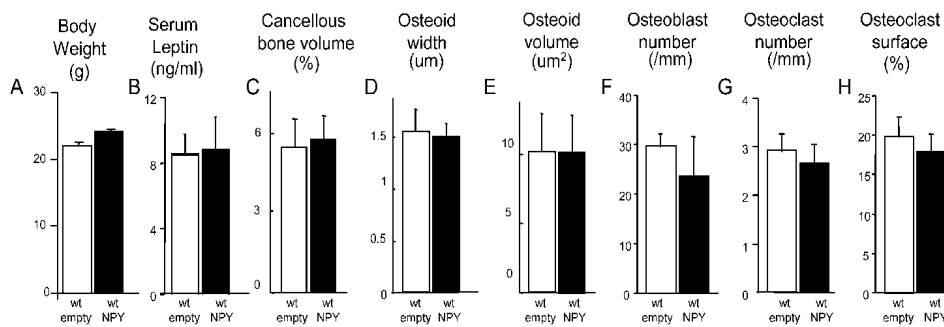

FIG. 3. Femoral cancellous bone 3 weeks after injection into the hippocampus of either empty or NPY-expressing AAV (empty or NPY) in wildtype mice. (A) Body weight. (B) Serum leptin. (C) Cancellous bone volume. (D) Osteoid width. (E) Osteoid volume. (F) Osteoblast number. (G) Osteoclast number. (H) Osteoclast surface. NPY overexpression does not alter energy homeostasis or cancellous bone volume or cell activity. Data are mean ± SE, $n = 6$–7; there were no significant differences between empty and NPY virus-injected groups.

Consistent with the known role of NPY in regulation of energy homeostasis, AAV-mediated overexpression of NPY in the hypothalamus resulted in significantly increased body weight in wildtype and Y2−/− mice. This increase was detected within the first week postinjection and rose steadily thereafter and was similar in AAV-NPY-injected wildtype and Y2−/− mice (Fig. 4A). The body weights of AAV-empty-injected wildtype and Y2−/− mice did not change significantly over the course of the experiment. The weight gain in AAV-NPY-injected groups was consistent with greater WAT deposition; WAT mass increased significantly after vector injection in wildtype and Y2−/− mice, respectively (Fig. 4B). This increase in fat mass was associated with greater serum leptin in NPY-overexpressed groups (Fig. 4C).
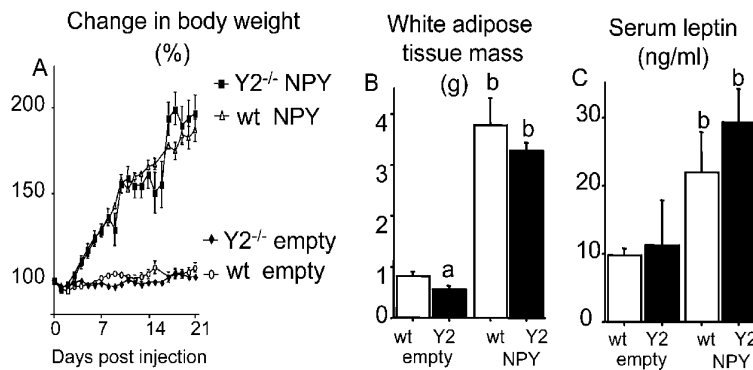

FIG. 4. Body weight and WAT mass and leptin after injection of either empty or NPY-expressing AAV (empty or NPY) in the hypothalamus of wildtype and Y2−/− mice. (A) Change in body weight. (B) WAT mass. NPY overexpression leads to a marked increase in body weight and WAT mass, overcoming the decrease in WAT by Y2 receptor deletion. Data are mean ± SE, $n = 4$–6; [a]$p < 0.05$ vs. wildtype, [b]$p < 0.05$ vs. AAV-empty.

In keeping with our previous observations of uninjected mice, cancellous bone volume was significantly greater in AAV-empty Y2−/− mice compared with AAV-empty wildtype; however, this difference was abolished by AAV-NPY injection (Fig. 5A). This decrease in cancellous bone volume in AAV-NPY-injected Y2−/− mice was accompanied by significantly reduced trabecular thickness, whereas AAV-NPY administration to wildtype mice was associated with significantly increased trabecular number (Figs. 5B and 5C) that did not induce a significant rise in cancellous bone volume.

Osteoblast activity was estimated using osteoid parameters. Consistent with the greater cancellous bone volume, osteoid width in AAV-empty injected mice was 2-fold greater in Y2−/− than wildtype mice (Fig. 5D), AAV-NPY significantly reduced osteoid width in both wildtype and Y2−/− groups. Importantly, however, osteoid width and thus osteoblast activity[16] remained greater in Y2−/− compared with wildtype animals in both AAV-NPY-injected and AAV-empty-injected groups. Similarly, osteoid volume in Y2−/− was significantly greater than wildtype in both AAV-empty and AAV-NPY groups, respectively (Fig. 5E). There were no significant effects of genetic or viral treatment on osteoblast or osteoclast number (Figs. 5F and 5G). Osteoclast surface, however, was significantly elevated in AAV-NPY Y2−/− mice compared with AAV-empty Y2−/− mice, with no difference observed in wildtype groups (Fig. 5H).

DISCUSSION

Leptin and Y2 receptors are coexpressed on neurons of the hypothalamus, and there is clear evidence that both receptors mediate regulatory circuits that inhibit bone formation. (**1**, **2**) This study examined the relationship between these leptin-sensitive and Y2 receptor-mediated bone responses in mice. In the presence of normal levels of leptin, as in Y2−/− mice, osteoblastic function was regulated through a Y2-dependent circuit, with lack of Y2 receptor activity consistently associated with elevated bone formation. At the high leptin levels induced by viral overexpression of NPY, the distinction between the leptin- and Y2-regulated pathways was enhanced, with osteoblast activity suppressed by NPY overexpression and concomitant leptin elevation but stimulated by lack of Y2 activity. Conversely, in the absence of leptin, as in *ob/ob* mice, the leptin- and Y2-mediated anabolic pathways could not be distinguished (i.e., bone formation was enhanced in the *ob/ob* mice and concurrent deletion of Y2 receptor did not further elevate osteoblast function). Elevated hypothalamic NPY levels were a common feature of all anabolic models. Reduction of osteoblast activity by leptin in the NPY overexpression model occurred despite the constitutively high NPY, suggesting that the leptin anti-osteogenic pathway is not mediated by increased hypothalamic expression of NPY. Importantly, the consistent stimulation of osteoblast activity after loss of Y2 signaling, despite suppression of osteoblast activity by increasing leptin levels, indicates that the pathway by which the Y2 receptor regulates bone formation is functionally distinct from the leptin anti-osteogenic pathway.

The effect of leptin deficiency on Y2−/−-mediated control of osteoblast function was evaluated by crossing Y2−/− and leptin-deficient *ob/ob* mice. Interestingly, the cancellous bone volumes of *ob/ob* and Y2−/−*ob/ob* models were indistinguishable from one another but reduced compared with Y2−/− mice. This reduction, however, was associated with greater bone resorption in the *ob/ob* and Y2−/−*ob/ob* models, consistent with their hypogonadal status relative to wildtype or Y2−/− mice. (**3**, **15**) More importantly, parameters of osteoblast function were not different between all three anabolic models, indicating that deletion of Y2 receptor signaling did not convey additional osteoblastic stimulation in the absence of leptin. The comparable elevation of osteoblast activity in Y2−/−, *ob/ob*, and Y2−/−*ob/ob* mice is associated with similar neuropeptide changes in the hypothalamic regions of these mice, most notably a significant increase in NPY expression. (**3**, **5**, **6**) Central leptin signaling inhibits NPY production in leptin-responsive NPYergic hypothalamic neurons. (**17**) Furthermore, Y2 receptors, abundant on these NPY neurons, also inhibit production of NPY. (**18**) Therefore, a lack of either leptin or Y2 signaling would result in increased hypothalamic NPY, as observed in both *ob/ob* and Y2−/−*ob/ob* leptin-deficient models(**3**, **5**) and in both germline(**6**) and hypothalamus-specific Y2−/− models. (**2**) The comparable level of osteoblast activity in these models suggests that the hypothalamic elevation of NPY and not the absence of leptin or Y2 signaling, per se, may be a critical determinant in the observed increases in osteoblast function. Alternately, considering the possibility that distinct osteoblast stimulatory pathways may be activated by leptin deficiency and Y2 deletion, the lack of additive effects in the Y2−/−*ob/ob* compound mutant model may indicate the involvement of a shared osteoblast regulatory system.

To address these hypotheses, experimental elevation of hypothalamic NPY by injection of NPY-expressing recombinant virus into wildtype and Y2−/− mice achieved concurrent elevation of leptin and hypothalamic NPY signaling. Although removed from normal physiological regulation using this constitutive expression system, NPY effects on energy homeostasis were in keeping with characteristic responses, notably increased body weight, adiposity[8, 9] and, in turn, elevated circulating leptin levels. [19] The speed and magnitude of the change in body weight indicated the potency of these hypothalamic pathways and made possible the relatively brief study design, thereby reducing the potential for development of central leptin resistance. [18] The surprising result of stable bone volume in the wildtype AAV-NPY mice despite such significant decreases in parameters of osteoblast function may relate to this short experimental duration compared with a previous study in which loss of bone followed more prolonged NPY infusion. [1]

The reduction in osteoblast activity with increased leptin and NPY levels in AAV-NPY-injected mice is consistent with a central anti-osteogenic action of leptin in cancellous bone. [20] This is evident in the decrease in osteoblast activity from the leptin-deficient *ob/ob* mice to the normal leptin wildtypes and the further decrease in the high leptin wildtype AAV-NPY mice. Notably, in contrast to this consistent relationship between leptin and osteoblast function, hypothalamic NPY levels are not consistently changed across these models, with high hypothalamic NPY in both *ob/ob* and AAV-NPY models, suggesting that NPY does not regulate the leptin anti-osteogenic pathway. NPY activity can not be definitively ruled out of the Y2−/− pathway, however, with high NPY levels, a consistent feature in all Y2-deficient models studied to date. It is interesting to note that NPY−/− mice lack a bone phenotype, [7] which has been taken as evidence for a lack of a role in bone. This lack of response induced by a reduction in NPY in both bone and adipose[8] is reminiscent of the actions of agouti-related protein, another neuropeptide expressed in the hypothalamus, which induces obesity in transgenic mice, with no effect in knockout models. [21] Thus, it may be that bone does not respond to NPY reduction but rather to NPY increase alone.

Despite the decrease in osteoblast activity in both AAV-NPY-injected groups, osteoid width and volume remained significantly greater in Y2−/− animals compared with wildtype. Thus, a lack of Y2 signaling was associated with a stimulation of osteoblast activity, even in the presence of increased serum leptin levels and high central NPYergic tonus. Similarly, in the Y2−/− model, bone formation was stimulated in association with high central NPYergic tonus but normal serum leptin levels. Taken together, these findings reveal a pathway modulated by Y2 receptors acting to stimulate osteoblast activity distinct from leptin. Whereas circulating leptin levels clearly influence the baseline ambient osteoblast activity on which the Y2 pathway acts, once stimulated, the Y2-mediated pathway is independent of leptin. Furthermore, the absence of leptin seems to influence the Y2 pathway in a different manner. The lack of additive effects in Y2−/−*ob/ob* mice, despite the stimulation seen in other Y2−/− models, suggests several possibilities. A permissive level of leptin may be necessary for activation of the Y2-dependent anabolic pathway to be detected, or both pathways may share a similar feedback mechanism that acts to control the levels of cancellous bone formation. However, the synergistic increase in bone volume in the previously published Y2−/−Y4−/− model that has significantly greater cancellous bone volume compared with the Y2−/− model shows that greater increases in bone accretion are possible and indicates that the former may be the case. In terms of the role central NPYergic tonus has on the activity of this Y2-dependent pathway, the reduction of osteoblast activity in the high NPY AAV-NPY-injected groups indicates that elevation in NPY in the hypothalamus is not expressly associated with enhanced osteoblast activity. Again, the greater osteoblast activity in Y2−/− AAV-NPY mice compared with wildtype AAV-NPY mice occurred under elevated NPY conditions; thus, as with previous models, NPY activity can not be excluded from the Y2−/− pathway.

This study indicates the presence of and distinct actions resulting from the leptin- and Y2-dependent circuits in the hypothalamus. In terms of the ambient level of osteoblast activity in these models, bone formation seems to be inhibited by leptin as serum levels of this hormone increase. In contrast, the pathway stimulated by loss of Y2 signaling seems to be purely stimulatory, elevating osteoblast activity by ~2-fold. Thus, at increased leptin levels, these two pathways act in opposition. Whereas the Y2−/− pathway involved in this regulation is likely to involve the sympathetic nervous system, [22] an indirect pathway, although unlikely based on

current experimental data, cannot be absolutely ruled out.

In summary, both Y2- and leptin-regulated pathways modulate osteoblast activity in the cancellous bone of the distal femur. The leptin pathway acts both to stimulate bone formation when serum leptin levels are absent and to suppress bone formation when present in excess, independent of central NPY tonus. In contrast, the pathway activated by Y2 deletion functions only to stimulate osteoblasts, and to date, has been observed only when hypothalamic NPY is elevated. Under normal physiological conditions, with leptin present, a lack of Y2 receptor signaling acts to increase osteoblast activity 2-fold. The Y2-mediated increase is evident independent of the serum leptin concentration. However, in the absence of leptin, these two pathways are not additive, suggesting that a permissive level of leptin may be necessary for activation of the Y2-dependent anabolic pathway. In conclusion, the pathways whereby serum leptin and Y2 receptors influence osteoblast activity seem to be both common and distinct components and likely represent alternate but related mechanisms. These findings support Y2-regulated pathway as a novel target for anabolic bone therapy.

ACKNOWLEDGMENTS

REFERENCES

1 Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, Shen J, Vinson C, Rueger JM, Karsenty G 2000 Leptin inhibits bone formation through a hypothalamic relay: A central control of bone mass. *Cell* **100**:197–207.

2 Baldock PA, Sainsbury A, Couzens M, Enriquez RF, Thomas GP, Gardiner EM, Herzog H 2002 Hypothalamic Y2 receptors regulate bone formation. *J Clin Invest* **109**:915–921.

3 Sainsbury A, Schwarzer C, Couzens M, Herzog H 2002 Y2 receptor deletion attenuates the type 2 diabetic syndrome of *ob/ob* mice. *Diabetes* **51**:3420–3427.

4 Naveilhan P, Svensson L, Nystrom S, Ekstrand AJ, Ernfors P 2002 Attenuation of hypercholesterolemia and hyperglycemia in *ob/ob* mice by NPY Y2 receptor ablation. *Peptides* **23**:1087–1091.

5 Wilding JP, Gilbey SG, Bailey CJ, Batt RA, Williams G, Ghatei MA, Bloom SR 1993 Increased neuropeptide-Y messenger ribonucleic acid (mRNA) and decreased neurotensin mRNA in the hypothalamus of the obese (*ob/ob*) mouse. *Endocrinology* **132**:1939–1944.

6 Sainsbury A, Baldock PA, Schwarzer C, Ueno N, Enriquez RF, Couzens M, Inui A, Herzog H, Gardiner EM 2003 Synergistic reduction in adiposity and increase in bone mass in Y2/Y4 receptor double knockout mice. *Mol Cell Biol* **23**:5225–5233.

7 Elefteriou F, Takeda S, Liu X, Armstrong D, Karsenty G 2003 Monosodium glutamate-sensitive hypothalamic neurons contribute to the control of bone mass. *Endocrinology* **144**:3842–3847.

8 Erickson JC, Clegg KE, Palmiter RD 1996 Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y. *Nature* **381**:415–421.

9 Stanley BG, Kyrkouli SE, Lampert S, Leibowitz SF 1986 Neuropeptide Y chronically injected into the hypothalamus: A powerful neurochemical inducer of hyperphagia and obesity. *Peptides* **7**:1189–1192.

10 Erickson JC, Hollopeter G, Palmiter RD 1996 Attenuation of the obesity syndrome of *ob/ob* mice by the loss of neuropeptide Y. *Science* **274**:1704–1707.

11 Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM 1995 Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* **269**:543–546.

12 Lin EJ, Richichi C, Young D, Baer K, Vezzani A, During MJ 2003 Recombinant AAV-mediated expression of galanin in rat hippocampus suppresses seizure development. *Eur J Neurosci* **18**:2087–2092.

13 Stanley BG, Leibowitz SF 1984 Neuropeptide Y: Stimulation of feeding and drinking by Injection into the paraventricular nucleus. *Life Sci* **35**:2635–2642.

14 Stanley BG, Kyrkouli SE, Lampert S, Leibowitz SF 1986 Neuropeptide Y chronically injected into the hypothalamus: A powerful neurochemical inducer of hyperphagia and obesity.

Peptides **7**:1189–1192.

15 Sainsbury A, Schwarzer C, Couzens M, Fetissov S, Furtinger S, Jenkins A, Cox HM, Sperk G, Hokfelt T, Herzog H 2002 Important role of hypothalamic Y2 receptors in body weight regulation revealed in conditional knockout mice. *Proc Natl Acad Sci USA* **99**:8938–8943.

16 Melsen F, Mosekilde L 1980 Trabecular bone mineralization lag time determined by tetracycline double-labeling in normal and certain pathological conditions. *Acta Pathol Microbiol Scand* **88**:83–88. [A].

17 Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM, Kriauciunas A 1995 The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature* **377**:530–532.

18 King PJ, Williams G, Doods H, Widdowson PS 2000 Effect of a selective neuropeptide Y Y(2) receptor antagonist, BIIE0246 on neuropeptide Y release. *Eur J Pharmacol* **396**:R1–R3.

19 Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS 1995 Leptin levels reflect body lipid content in mice: Evidence for diet-induced resistance to leptin action. *Nat Med* **1**:1311–1314.

20 Elefteriou F, Takeda S, Ebihara K, Magre J, Patano N, Kim CA, Ogawa Y, Liu X, Ware SM, Craigen WJ, Robert JJ, Vinson C, Nakao K, Capeau J, Karsenty G 2004 Serum leptin level is a regulator of bone mass. *Proc Natl Acad Sci USA* **101**:3258–3263.

21 Qian S, Chen H, Weingarth D, Trumbauer ME, Novi DE, Guan X, Yu H, Shen Z, Feng Y, Frazier E, Chen A, Camacho RE, Shearman LP, Gopal-Truter S, MacNeil DJ, Van Der Ploeg LH, Marsh DJ 2002 Neither agouti-related protein nor neuropeptide Y is critically required for the regulation of energy homeostasis in mice. *Mol Cell Biol* **22**:5027–5035.

22 Herzog H 2002 Hypothalamic Y2 receptors: Central coordination of energy homeostasis and bone mass regulation. *Drug News Perspect* **15**:506–510.