

## Hypothalamic Regulation of Cortical Bone Mass: Opposing Activity of Y2 Receptor and Leptin Pathways

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**ABSTRACT:** NeuropeptideY-, Y2 receptor (Y2)-, and leptin-deficient mice show similar anabolic action in cancellous bone but have not been assessed in cortical bone. Cortical bone mass is elevated in Y2<sup>-/-</sup> mice through greater osteoblast activity. In contrast, leptin deficiency results in reduced bone mass. We show opposing central regulation of cortical bone.

**Introduction:** Treatment of osteoporosis is confounded by a lack of agents capable of stimulating the formation of bone by osteoblasts. Recently, the brain has been identified as a potent anabolic regulator of bone formation. Hypothalamic leptin or Y2 receptor signaling are known to regulate osteoblast activity in cancellous bone. However, assessment of these pathways in the structural cortical bone is critical to understanding their role in skeletal health and their potential clinical relevance to osteoporosis and its treatment.

**Materials and Methods:** Long bones of 16-week male *ob/ob* and germline and hypothalamic Y2<sup>-/-</sup> mice were assessed by QCT. Cortical osteoblast activity was assessed histologically.

**Results:** The femora of skeletally mature Y2<sup>-/-</sup> mice and of leptin-deficient *ob/ob* and Y2<sup>-/-</sup>*ob/ob* mice were assessed for changes in cortical osteoblast activity and bone mass. Ablation of Y2 receptors increased osteoblast activity on both endosteal and periosteal surfaces, independent of leptin, resulting in increased cortical bone mass and density in Y2<sup>-/-</sup> mice along the entire femur. Importantly, these changes were evident after deletion of hypothalamic Y2 receptors in adult mice, with a 5-fold elevation in periosteal bone formation. This is in marked contrast to leptin-deficient models that displayed reduced cortical mass and density. These changes were associated with substantial differences in calculated strength between the Y2<sup>-/-</sup> and leptin-deficient mice.

**Conclusions:** These results indicate that the Y2-mediated anabolic pathway stimulates cortical and cancellous bone formation, whereas the leptin-mediated pathway has opposing effects in cortical and cancellous bone, diminishing the production of cortical bone. The findings from conditional hypothalamic Y2 knockout show a novel, inducible control mechanism for cortical bone formation and a potential new pathway for anabolic treatment of osteoporosis.

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**Key words:** hypothalamus, cortical bone, Y2 receptor, leptin, bone formation

### INTRODUCTION

THROUGHOUT LIFE, SKELETAL health is maintained by a continual process of bone remodeling, with minute quanta of bone tissue resorbed by osteoclasts and replaced with new bone by osteoblasts. Normally a balanced process, bone remodeling is altered with aging and sex hormone deficiency, resulting in continued decline in bone mass and

strength. This diminished bone strength is a significant and growing medical issue with osteoporotic fracture being one of the most common musculoskeletal events, suffered over life by one in two women and one in three men.<sup>(1)</sup> So serious are the consequences of skeletal fragility that osteoporotic fracture of any type is associated with a 2-fold increase in mortality in both men and women.<sup>(2)</sup> Antiresorptive treatments, which represent the majority of therapeutic agents, act to halt the continued decline in bone mass by attenuating osteoclastic activity. Anabolic agents, in contrast, are capable of far greater gains in bone mass than antiresorptive treatments<sup>(3)</sup>; however, only one is currently available, and there is a need for additional anabolic treatment options.

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In recent years, evidence of novel bone anabolic pathways originating from the central nervous system has revealed the hypothalamus as a potent regulator of osteoblastic function. Two major pathways identified involve altered leptin<sup>(4)</sup> and neuropeptide Y Y2 receptor signaling.<sup>(5)</sup> Genetic modulation of these pathways has powerful actions in cancellous bone, with a 2-fold increase in the volume of cancellous bone reported in the leptin-deficient (*ob/ob*) and Y2 receptor knockout (*Y2<sup>-/-</sup>*) mutant mouse models.<sup>(6)</sup> These effects are known to be mediated, at least in part, within the hypothalamus, because both leptin injection<sup>(4)</sup> and conditional Y2 deletion<sup>(5)</sup> in this region have effects on bone. Importantly, these changes result from greater osteoblastic bone formation, highlighting the marked anabolic potential of these pathways. While the cancellous bone response to these central pathways has been well characterized, their effects in cortical bone are less well defined.

Cortical bone is critical to skeletal strength. In the shaft region of long bones, where cancellous bone is absent, load bearing is dependent entirely on cortical bone. Moreover, even in regions of extensive cancellous bone, the cortices provide major contributions to load carrying capacity,<sup>(7)</sup> with estimates up to 75% in the vertebrae<sup>(7,8)</sup> and 96% at the femoral neck and 80% at the intertrochanteric region.<sup>(9)</sup> Thus, the contributions of these hypothalamic pathways to cancellous bone mass, while of mechanistic interest, are of lessened consequence to skeletal health in the absence of information on their effects on cortical bone. Moreover, clarification of the effect of these hypothalamic signals on cortical bone mass and consequently bone strength is vital to any assessment of their potential impact on osteoporosis therapy.

Cortical bone mass has been examined in leptin-deficient models previously, with conflicting reports. Initial studies reported that cortical bone was unaffected in leptin-deficient *ob/ob* mice, as was bone strength.<sup>(4)</sup> In contrast, several studies have reported cortical bone mass, size, and longitudinal growth to be reduced in *ob/ob*.<sup>(10,11)</sup> This raises important questions regarding this pathway, initially described as inducing a high bone mass phenotype because of the changes in cancellous bone, with the possibility that this cancellous effect is overridden by a reduction in cortical bone mass in these mice. Recently, it has been established that leptin and Y2 receptor pathways independently modulate cancellous bone homeostasis,<sup>(6)</sup> with preliminary analysis of the *Y2<sup>-/-</sup>* bones showing an increase in midfemoral cortical area.<sup>(5)</sup> These initial reports indicate that the two pathways to bone may have conflicting actions in the crucial area of cortical bone regulation.

This study addresses this issue by examining the cortical bone phenotypes of *Y2<sup>-/-</sup>* and *ob/ob* mouse models. The potential for independent signaling by these two pathways has been addressed by comparing *Y2<sup>-/-</sup>*, *ob/ob*, and *Y2<sup>-/-</sup>ob/ob* double mutant mice. Furthermore, the central nature of the Y2-mediated response in cortical bone and the importance of developmental changes have been studied using conditional, hypothalamic Y2 receptor knockout in adult mice.

TABLE 1. BODY WEIGHT AND FEMUR LENGTH OF Y2 RECEPTOR- AND LEPTIN-DEFICIENT MICE

Genotype	Wildtype	Y2 <sup>-/-</sup>	ob/ob	Y2 <sup>-/-</sup> ob/ob
Body weight (g)	30.2 ± 1	29.0 ± 1	52.4 ± 4*†	47.3 ± 5*†
Femur length (mm)	15.6 ± 0.1	15.7 ± 0.1	15.4 ± 0.3	15.6 ± 0.01

Values are mean ± SE, *n* = 5–7.

\* Significantly different from wildtype.

† Significantly different from *Y2<sup>-/-</sup>*.

## MATERIALS AND METHODS

### Animals

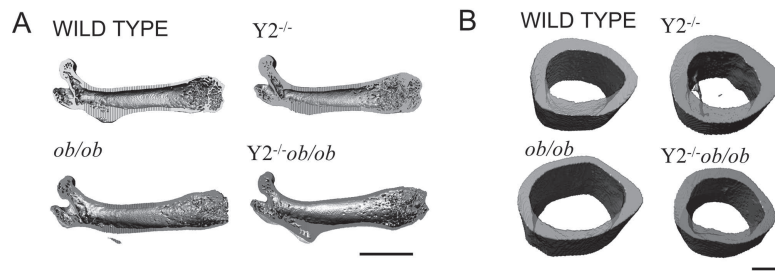
*Y2<sup>lox/lox</sup>*, *Y2<sup>-/-</sup>*, *ob/ob*, and *Y2<sup>-/-</sup>ob/ob* mice were generated as previously described.<sup>(12,13)</sup> Hypothalamic-specific deletion of Y2 receptors was induced in 10-week-old mice, and skeletal phenotypes were assessed in 16-week-old male animals for comparison with germline knockout models. Body weight was markedly increased in both leptin-deficient groups compared with leptin-sufficient groups (Table 1), necessitating body weight correction for some cortical parameters (see *Statistical analysis*).

### Tissue collection and analysis

Mice were kept under standard light circle and fed ad libitum with standard chow (Gordon's Specialty Stockfeeds, Sydney, Australia) containing 16% protein, 4% fat (11 MJ/kg). Tissues were collected from 16-week-old mice as previously described.<sup>(5)</sup> Briefly, both femora were excised and fixed in 4% paraformaldehyde at 4°C for 16 h. The right femurs were measured using a digital micrometer (Mitotoyo, Tokyo, Japan) and bisected transversely, and distal halves were embedded undecalcified in methylmethacrylate (APS Chemicals, Sydney, Australia). Five-micrometer sagittal sections were cut, which enabled examination of the tension/compression axis within the femur using sequential calcein labels. Osteoblast activity was determined by measurement of mineral apposition rate (MAR) as described previously.<sup>(5)</sup>

### QCT

QCT was used to isolate cortical bone for analysis, using a Stratec XCT Research SA (Stratec Medizintechnik, Pforzheim, Germany). Scans were conducted using a voxel size of 70 µm, scan speed of 5 mm/s, and slice width of 0.2 mm every 0.5 mm on excised left femurs. Bones were scanned in 32 slices from distal to proximal, allowing longitudinal assessment of mineral distribution along the length of the femur. The consistency in femur length between genotypes (Table 1) enabled direct comparison of scans between groups.<sup>(8,9)</sup> Because of the marked structural differences along the femur affecting volumetric BMD (vBMD), and particularly, strength index, QCT scans were grouped into distal, shaft, and proximal thirds. For comparison of *Y2<sup>lox/lox</sup>* mice, a single scan was made 6 mm proximal from the distal margin of the tibiae. Bone strength index, a measure of bending strength, was calculated.<sup>(14)</sup>



**FIG. 1.** Femoral morphology of Y2 receptor- and leptin-deficient mice. (A)  $\mu$ CT images of a coronal aspect of femora from 16-week-old  $Y2^{-/-}$ ,  $ob/ob$ , and  $Y2^{-/-}ob/ob$  mice display the lack of gross morphological deformities and a general consistency of structure between mutant groups. Bar represents 5 mm. (B) Femoral cross-sections from Y2 receptor- and leptin-deficient mice.  $\mu$ CT images of femurs from 16-week-old wildtype,  $Y2^{-/-}$ ,  $ob/ob$ , and  $Y2^{-/-}ob/ob$  mice display differences in cortical bone accrual and structure between the genotypes. Cortical thickness is greater in  $Y2^{-/-}$  mice, whereas leptin-deficient mice display reduced cortical thickness. Bar represents 0.5 mm.

### $\mu$ CT

Representative femurs were imaged using  $\mu$ CT Skyscan 1172 (Skyscan, Aartselaar, Belgium) at 80 kV with a voxel size of 17  $\mu$ m.

### Statistical analyses

Comparisons between genotypes were made by one-way ANOVA for genotype, with subsequent Bonferroni/Dunn posthoc tests for those variables, which returned significant results.

Correction for body weight was carried out on QCT cortical parameters. After determination of a significant linear relationship between a parameter and body weight in leptin-sufficient animals, a predicted value for all animals was calculated. This predicted value was calculated using the slope and intercept of the regression equation and the body weight of the individual animal. This produced a predicted value for all animals, based on the original leptin sufficient relationship. The corrected value was expressed as the measured value divided by the proportion that the predicted value was altered from the measured value i.e.,  $\text{Corrected} = \text{Measured} \times (\text{Measured}/\text{Predicted})$ .

StatView version 4.5 (Abacus Concepts) was used for all statistical analyses, and  $p < 0.05$  was accepted as being statistically significant for both ANOVA and posthoc analyses.

## RESULTS

Gross changes in femoral morphology between the four genotypes were not apparent (Fig. 1A), with no difference in femur length (Table 1).

### Cortical osteoblast activity

Deletion of Y2 receptors stimulated cortical osteoblast activity, as has been described in cancellous bone. MAR in  $Y2^{-/-}$  mice was greater than wildtype in both distal endocortical and shaft periosteal surfaces, with a 6-fold increase in the latter (Fig. 2).

Periosteal MAR in  $ob/ob$  was greater than wildtype, but was not altered at either endocortical site. Ablation of Y2 receptors in combination with leptin deficiency still resulted

in greater osteoblast activity; MAR in  $Y2^{-/-}ob/ob$  double knockout mice was greater than wildtype in the distal and shaft endocortical and shaft periosteal surfaces. Moreover, osteoblast activity in  $Y2^{-/-}ob/ob$  was greater than  $ob/ob$  in both distal and shaft endocortical surfaces; however, an 80% increase between  $ob/ob$  and  $Y2^{-/-}ob/ob$  on the periosteum was not significantly different ( $p = 0.1$ ).

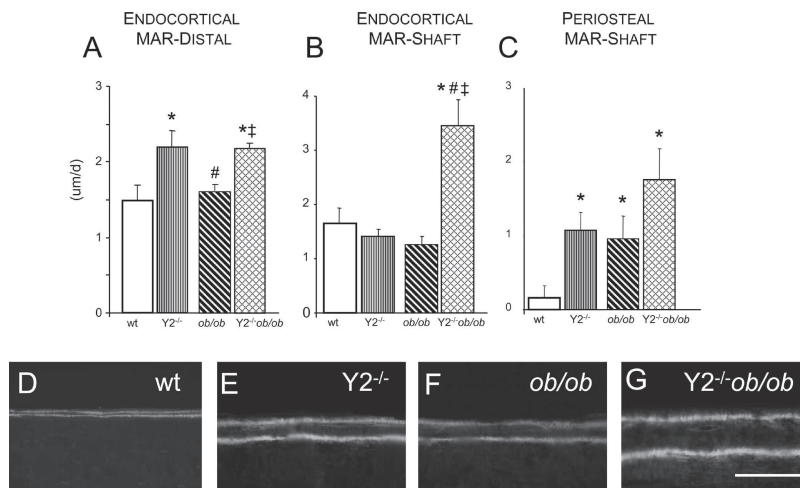
### Femoral QCT

Femurs were examined along their entire length using sequential QCT, excluding cancellous and subcortical bone. In  $Y2^{-/-}$  mice, femoral cortical BMC and vBMD were consistently increased compared with wildtype along the length of the femur (Fig. 3). Femoral cortical BMC and vBMD in  $ob/ob$  were not altered compared with wildtype but were less than  $Y2^{-/-}$ . There were no differences between  $ob/ob$  and  $Y2^{-/-}ob/ob$  in cortical BMC or vBMD.

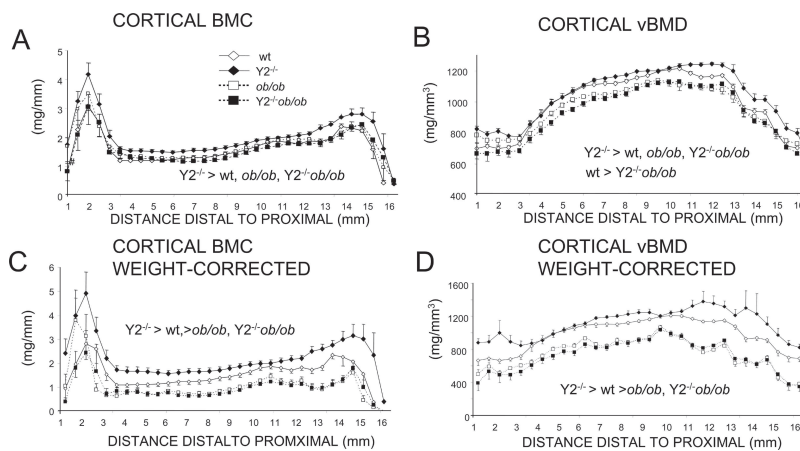
Because body weight has an effect on cortical bone accrual, particularly in the long bones,<sup>(15)</sup> it is usual to correct for weight in comparing animals. Corrected BMC and corrected vBMD in  $Y2^{-/-}$  was greater than wildtype,  $ob/ob$ , and  $Y2^{-/-}ob/ob$  mice. Conversely, both  $ob/ob$  and  $Y2^{-/-}ob/ob$  had significantly reduced corrected femoral BMC and vBMD than wildtype.

Grouping the cortical data into regions showed that  $Y2^{-/-}$  mice had consistently greater weight-corrected cortical BMC, with an average increase of 45% compared with wildtype (Fig. 4). vBMD was more modestly increased, most prominently in the proximal region, averaging 17% greater than wildtype. In contrast, leptin-deficient mice had an average reduction in BMC of 32%, which was evident in the shaft and proximal regions, and a reduction of 25% in vBMD (25%) compared with wildtype, which was evident throughout. The production of cortical bone was clearly different between these two centrally active models, such that corrected BMC was 120% and vBMD was 60% greater in  $Y2^{-/-}$  compared with  $ob/ob$  mice. Despite greater osteoblast activity in  $Y2^{-/-}ob/ob$  than  $ob/ob$ , bone mass and density in double knockout mice were similarly reduced compared with  $Y2^{-/-}$ .

These changes in bone mass were accompanied by changes in femoral structure. In the shaft region, periosteal circumference was greater in  $Y2^{-/-}$  mice compared with



**FIG. 2.** Cortical osteoblast activity in Y2 receptor- and leptin-deficient mice. (A) Endocortical MAR in the distal femur was greater in the absence of Y2 receptors independent of leptin status. (B) Endocortical MAR in the shaft was greater in  $Y2^{-/-}ob/ob$  mice compared with other genotypes. (C) Periosteal MAR in the shaft was greater in all models compared with wildtype. (D–G) Periosteal bone apposition representing 7 days of bone accrual between fluorescent bands; bar represents 15  $\mu$ m. There was no osteoblast activity on the distal periosteum for any genotype. Data are mean  $\pm$  SE,  $n = 6–14$ , \* $p < 0.05$  vs. wildtype, # $p < 0.05$  vs.  $Y2^{-/-}$ ,  $^{\dagger}p < 0.05$  vs.  $ob/ob$ .



**FIG. 3.** Serial QCT analysis of femoral cortical bone in Y2 receptor- and leptin-deficient mice. (A) Femoral cortical BMC was consistently greater in  $Y2^{-/-}$  compared with wildtype with no change in  $ob/ob$  or  $Y2^{-/-}ob/ob$  compared with wildtype. (B) Femoral cortical vBMD was again greater in  $Y2^{-/-}$  compared with wildtype but was reduced in  $Y2^{-/-}ob/ob$  compared with wildtype. (C) Body weight-corrected cortical BMC was markedly greater in  $Y2^{-/-}$  compared with wildtype and reduced in both  $ob/ob$  and  $Y2^{-/-}ob/ob$  mice. (D) Similarly, corrected cortical vBMD was greater in  $Y2^{-/-}$  and reduced in  $ob/ob$  and  $Y2^{-/-}ob/ob$  compared with wildtype. Data are mean  $\pm$  SE,  $n = 5–6$ , \* $p < 0.05$  vs. wildtype,  $^{\dagger}p < 0.05$  vs.  $Y2^{-/-}$ ,  $^{\ddagger}p < 0.05$  vs.  $ob/ob$ .

wildtype, whereas in  $ob/ob$  and  $Y2^{-/-}ob/ob$ , it was less than wildtype (Table 2). Endosteal circumference in this region was greater in both  $ob/ob$  and  $Y2^{-/-}ob/ob$  and not different between  $Y2^{-/-}$  and wildtype. These changes resulted in marked differences in the thickness of the cortical bone and can be seen in the cross-sectional  $\mu$ CT images (Fig. 1B). Cortical thickness in  $Y2^{-/-}$  was greater than wildtype in shaft and proximal regions, whereas  $ob/ob$  and  $Y2^{-/-}ob/ob$  were less than wildtype in the shaft alone. However, cortical thickness was greater in  $Y2^{-/-}$  than  $ob/ob$  and  $Y2^{-/-}ob/ob$  in all regions. Consistent with altered cortical structure, calculated strength index, a measure of bending strength, was also altered. In the shaft, where bending strength is most important to overall bone strength, strength index was 35% greater in  $Y2^{-/-}$  and 25% less in  $ob/ob$ , respectively, compared with wildtype (Fig. 4), with a similar pattern in the proximal region.

#### Conditional hypothalamic Y2 knockout

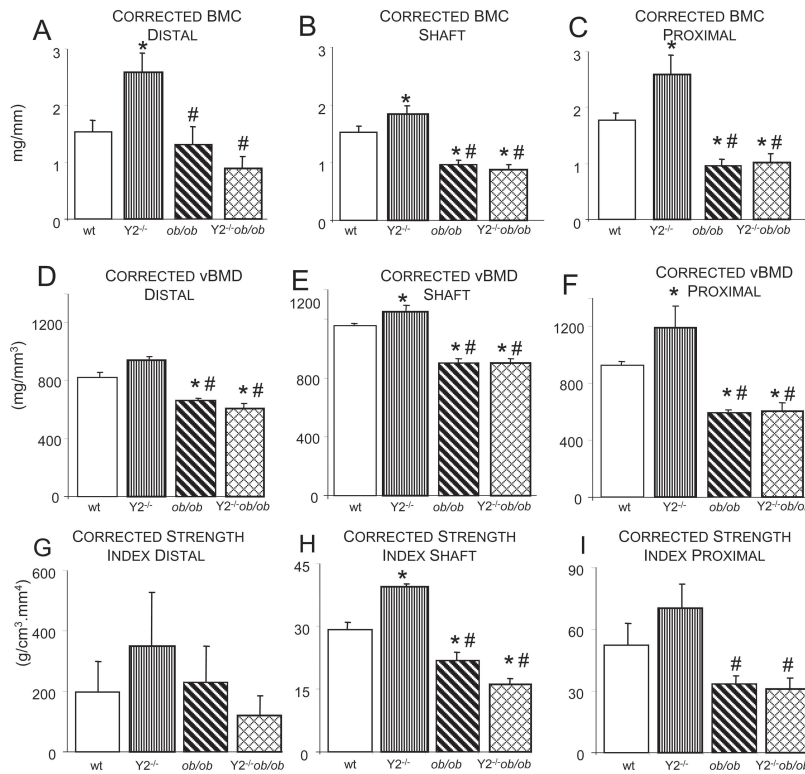
The Y2-associated stimulation of cortical bone formation was inducible in the mature skeleton. MAR after conditional, hypothalamic Y2 deletion in adult mice was greater than controls in both distal endocortical and shaft periosteal surfaces (Fig. 5), with a 5-fold elevation in the latter region,

similar to the change in germline  $Y2^{-/-}$  mice. Of considerable interest, despite the relatively brief period after Y2 receptor deletion (5 weeks), these increases in osteoblast activity were coincident with greater bone mass. Cortical BMC was 25% greater in the tibias of hypothalamic  $Y2^{-/-}$  mice, with a more modest, but significant, change in vBMD. Importantly, these structural changes induced structural improvement, with the calculated strength index increasing 1.6-fold in the tibias of the hypothalamic  $Y2^{-/-}$  mice.

#### DISCUSSION

This study, the first to comprehensively examine cortical bone regulation by the hypothalamus, shows that loss of signaling by Y2 receptors or leptin leads to opposing effects. Cortical bone mass was increased in both germline and hypothalamic Y2 knockout mice because of elevated osteoblast activity, indicating that the  $Y2^{-/-}$  pathway has a consistently anabolic action in both cancellous and cortical compartments in bone. In contrast, leptin deficiency was associated with reduced cortical bone mass, indicating that the leptin pathway has contrasting effects on cortical and cancellous bone, with its deficiency resulting in a lowered bone mass phenotype. The opposing activities of the leptin





**FIG. 4.** Regional analysis of body weight-corrected cortical BMC and vBMD in Y2 receptor and leptin-deficient mice. (A–C) Corrected cortical BMC was elevated in Y2<sup>−/−</sup> in all regions and reduced in *ob/ob* and Y2<sup>−/−</sup>*ob/ob* in the shaft and proximal thirds compared with wildtype. (D–F) Corrected cortical vBMD was elevated in Y2<sup>−/−</sup> both shaft and proximal thirds and again consistently reduced in *ob/ob* and Y2<sup>−/−</sup>*ob/ob*. Values for BMC and vBMD in Y2<sup>−/−</sup> were greater than both leptin-deficient models throughout, with an average difference of 2- and 1.6-fold, respectively. (G–I) Corrected strength index in Y2<sup>−/−</sup> was an average of 48% greater than wildtype and 80% greater than *ob/ob* across the regions. These changes were most evident in the shaft region. Data are mean ± SE, *n* = 5–6, \**p* < 0.05 vs. wildtype, #*p* < 0.05 vs. Y2<sup>−/−</sup>, ‡*p* < 0.05 vs. *ob/ob*.

TABLE 2. FEMORAL DIMENSIONS OF Y2 RECEPTOR- AND LEPTIN-DEFICIENT MICE

Genotype	Wildtype	Y2 <sup>−/−</sup>	<i>ob/ob</i>	Y2 <sup>−/−</sup> <i>ob/ob</i>
Periosteal circumference (mm)*				
Distal	6.40 ± 0.5	7.08 ± 0.4	6.38 ± 0.9	6.68 ± 0.8
Shaft	5.09 ± 0.1	5.31 ± 0.1 <sup>‡</sup>	4.83 ± 0.1 <sup>†‡</sup>	4.78 ± 0.1 <sup>†‡</sup>
Proximal	5.67 ± 0.3	6.11 ± 0.3	5.95 ± 0.7	5.80 ± 0.5
Endosteal circumference (mm)				
Distal	4.14 ± 0.3	3.96 ± 0.2	4.17 ± 0.3	4.44 ± 0.3
Shaft	3.24 ± 0.1	3.18 ± 0.1	3.57 ± 0.1 <sup>†‡</sup>	3.60 ± 0.1 <sup>†‡</sup>
Proximal	3.10 ± 0.2	2.93 ± 0.2	3.44 ± 0.2	3.28 ± 0.2
Cortical thickness (mm)*				
Distal	0.35 ± 0.05	0.51 ± 0.07	0.27 ± 0.06 <sup>‡</sup>	0.27 ± 0.05 <sup>‡</sup>
Shaft	0.29 ± 0.01	0.35 ± 0.01 <sup>‡</sup>	0.22 ± 0.01 <sup>†‡</sup>	0.19 ± 0.01 <sup>†‡</sup>
Proximal	0.40 ± 0.03	0.52 ± 0.03 <sup>‡</sup>	0.32 ± 0.05 <sup>‡</sup>	0.33 ± 0.04 <sup>‡</sup>

Values are mean ± SE, *n* = 5–7.

\* Values corrected for body weight.

<sup>†</sup> Significantly different from wildtype.

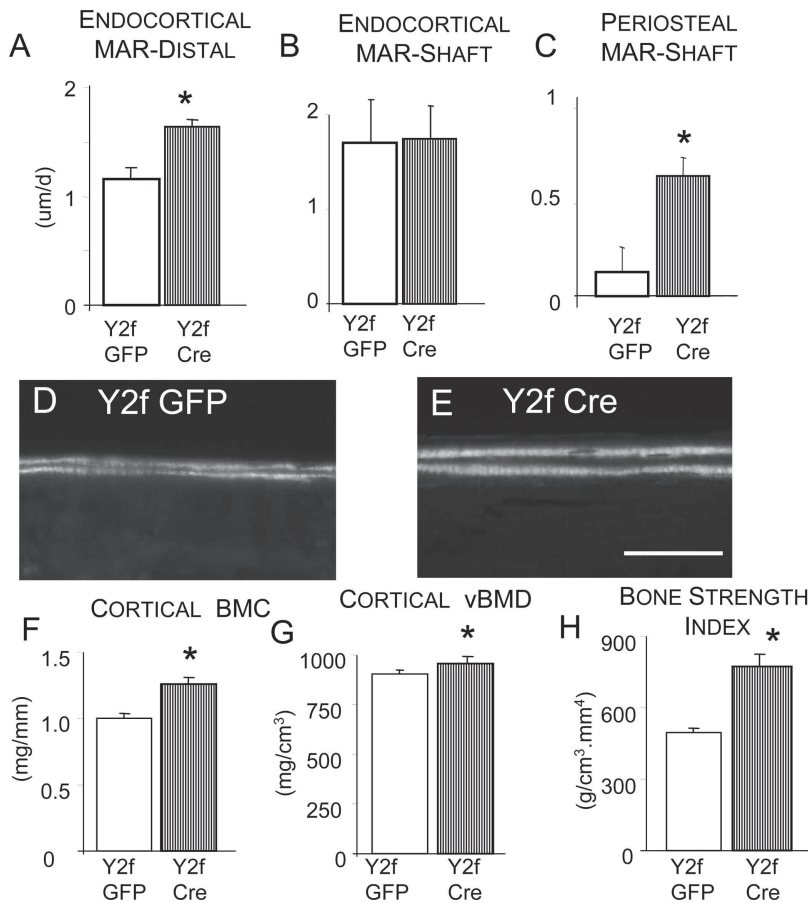
<sup>‡</sup> Significantly different from Y2<sup>−/−</sup>.

and Y2 pathways resulted in differences in cortical structure and strength index, suggesting that Y2 deletion produced significantly stronger bones than either wildtype or leptin-deficient mice.

In keeping with a generalized, centrally mediated anabolic effect of Y2 receptor deletion, osteoblast activity was elevated on cortical bone surfaces after either germline or hypothalamic Y2 deletion. Cortical bone mass was elevated in both Y2<sup>−/−</sup> models, as evidenced by QCT analysis, providing evidence of important functional outcomes of the Y2 anabolic pathway. These changes were marked, with cortical mass in Y2<sup>−/−</sup> greater than wildtype by >40% and evi-

dent along the length of the femur. The Y2-deficient changes in cortical BMD were less pronounced. Bones increased in mass and size in concert, consistent with the increase in bone circumference and periosteal bone formation. As a result, there were no differences in apparent density; however, calculations suggest that the structural changes did affect bone strength.

The induction of anabolic activity on the periosteal surface is of particular importance because periosteal circumference plays a critical role in bone strength. Strength index, a calculated estimate of in vivo bending strength, increases exponentially with increasing bone radius.<sup>(15)</sup> In keeping



**FIG. 5.** Cortical changes after viral hypothalamic Cre-recombinase expression in the hypothalamus of  $Y2^{lox/lox}$ . (A–E) Osteoblast activity in conditional, hypothalamic Y2 receptor-deficient mice. (A) Five weeks after bilateral virus injection in the hypothalamus of 11-week-old mice, distal endocortical MAR was elevated in the absence of hypothalamic Y2 receptors compared with GFP injected controls. (B) Shaft endocortical MAR was not altered. (C) Periosteal MAR in the shaft was increased 5-fold in Y2-deficient mice compared with GFP controls. (D–E) Periosteal bone apposition representing 7 days of bone accrual between serial fluorescent bands; bar represents 5  $\mu$ m. (F–H) Tibial cortical parameters in conditional, hypothalamic Y2 receptor-deficient mice. Changes after viral hypothalamic Cre-recombinase in the hypothalamus of  $Y2^{lox/lox}$  mice. Tibial (F) BMC, (G) vBMD, and (H) calculated strength index were greater in centrally Y2-deficient mice than control, with an average difference of 25% for BMC, 6% for vBMD, and 55% for strength index. Data are mean  $\pm$  SE,  $n = 7$ –9, \* $p < 0.05$  vs. control GFP virus.

with these structural changes, the increases in cortical strength index support a beneficial effect of interference of the Y2 receptor pathway, although changes in bone material properties cannot be excluded. The potential of this pathway is most apparent in the conditional, hypothalamic  $Y2^{-/-}$  mice, with changes in periosteal apposition and bone strength index evident 5 weeks after removing the Y2 receptor in adult mice. Taken together, these results clearly indicate that inhibition of hypothalamic Y2 receptor signaling exerts positive benefits on skeletal health, with increases in cortical bone mass and strength estimates, and reveal a pattern of generalized increase in osteoblast activity in both cortical and cancellous bone.

In contrast, leptin deficiency, although known to increase cancellous bone mass, exerted a negative regulatory effect in cortical bone. This study has comprehensively sampled this genotype of mice in a model unaffected by altered long bone length, revealing the marked effect leptin deficiency has on cortical bone accrual. Unadjusted cortical vBMD was modestly but significantly reduced in both leptin-deficient models, and correction of cortical BMC and vBMD values for body weight suggested an even more marked inhibitory effect on cortical bone. Corrected cortical bone mass in the obese leptin-deficient mice was 60% of wildtype in both shaft and proximal regions, those most reliant on cortical bone for strength, because of their limited cancellous bone content. Similarly, corrected vBMD

was under 75% of wildtype, clearly displaying the inhibition of cortical bone production that results from leptin deficiency.

The conflicting effects of leptin deficiency may relate to peripheral  $\beta$ -adrenergic signaling in the osteoblastic cells. The leptin-deficient stimulation of osteoblast activity in cancellous bone has been shown to result from inhibition of  $\beta$ 2-adrenergic signaling in osteoblasts.<sup>(16)</sup> However, inhibition of  $\beta$ -adrenergic signaling is associated with reduced cortical bone mass, with femoral BMC reduced in  $\beta 1^{-/-}\beta 2^{-/-}$  mice.<sup>(17)</sup> Thus, a reduction in  $\beta$ 2 signaling may inhibit cortical osteoblast activity while stimulating cancellous osteoblasts. Although the peripheral neural circuit involved in the  $Y2^{-/-}$  anabolic pathway has not yet been defined, the contrasting actions of the  $Y2^{-/-}$  and leptin-deficient models in cortical bone indicate that distinct mechanisms are likely and that the Y2-mediated effects are not mediated by  $\beta$ 2-adrenergic changes.

Cortical bone, particularly in long bones, is extremely responsive to load-bearing stimuli,<sup>(18)</sup> and femoral shaft characteristics, such as cortical size, BMD, and BMC,<sup>(19,20)</sup> are correlated with body weight. The inhibition of cortical bone production in leptin-deficient mice suggests a reduced ability to adapt to the increasing load bearing required by their greater body weight. Similarly, leptin administration has been shown to prevent disuse-induced bone loss in a model of tail suspension in rats<sup>(21)</sup> and to increase bone

strength, despite decreased body weight.<sup>(22)</sup> Osteoblast activity on the periosteum of *ob/ob* mice, however, was increased above wildtype activity, suggesting that any inhibition of the load-related response was not complete. Despite the periosteal increase, osteoblast activity was unchanged on all other cortical surfaces. As a result, strength index was similar or reduced compared with control mice, most notably in the proximal region. In addition to neural and mechanical influences, secondary hormonal changes may influence cortical bone production. The hypogonadism of *ob/ob* and *Y2<sup>-/-</sup>ob/ob*<sup>(12)</sup> mice may stimulate longitudinal growth<sup>(23)</sup> and elevate periosteal and endosteal bone formation rates.<sup>(24,25)</sup> However, testosterone and IGF-I in *ob/ob* and *Y2<sup>-/-</sup>ob/ob* mice were not different compared with *Y2<sup>-/-</sup>* mice,<sup>(5,12,13)</sup> indicating that the contrasting effects of leptin and Y2 receptor deficiency on cortical bone are unlikely to be the result of altered secondary hormonal activity.

Deletion of Y2 receptors resulted in stimulation of cortical bone formation with or without blockade of leptin action. Thus, activation of the *Y2<sup>-/-</sup>* pathway results in a stimulation of osteoblast activity on cortical surfaces, independent of circulating leptin. However, in contrast to the greater osteoblast activity in *Y2<sup>-/-</sup>ob/ob* mice, cortical bone mass was not elevated in these mice compared with *ob/ob*, suggesting that leptin-deficient processes dominate during development.

This study conclusively shows for the first time the diversity in hypothalamic control of bone homeostasis. Modulation of neuropeptide Y, Y2 receptor function is capable of marked and consistent stimulatory influence on osteoblasts. Moreover, this effect is dominated by those receptors in the hypothalamus, with conditional Y2 deletion within this region rapidly recapitulating the germline phenotype. This model highlights the magnitude and speed of skeletal changes produced by hypothalamic signaling and the therapeutic potential of the Y2-mediated pathway to strengthen the skeleton. In contrast, leptin deficiency, while enhancing cancellous bone formation, results in a low bone mass phenotype with consistent decreases in cortical bone accretion and strength index. Furthermore, these studies indicate that hypothalamic control of skeletal homeostasis is more comprehensive than initially appreciated, and the number of such bone active pathways is likely to increase, suggesting that centrally mediated modifications of peripheral homeostatic processes may present a powerful new paradigm for therapeutic intervention.

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