Central Depletion of Brain-Derived Neurotrophic Factor in Mice Results in High Bone Mass and Metabolic Phenotype

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Brain-derived neurotrophic factor (BDNF) plays important roles in neuronal differentiation/survival, the regulation of food intake, and the pathobiology of obesity and type 2 diabetes mellitus. BDNF and its receptor are expressed in osteoblasts and chondrocyte. BDNF in vitro has a positive effect on bone; whether central BDNF affects bone mass in vivo is not known. We therefore examined bone mass and energy use in brain-targeted BDNF conditional knockout mice (Bdnf2lox/2lox/93). The deletion of BDNF in the brain led to a metabolic phenotype characterized by hyperphagia, obesity, and increased abdominal white adipose tissue. Central BDNF deletion produces a marked skeletal phenotype characterized by increased femur length, elevated whole bone mineral density, and bone mineral content. The skeletal changes are developmentally regulated and appear concurrently with the metabolic phenotype, suggesting that the metabolic and skeletal actions of BDNF are linked. The increased bone development is evident in both the cortical and trabecular regions. Compared with control, Bdnf2lox/2lox/93 mice show greater trabecular bone volume (+50% for distal femur, $P < 0.001$; +35% for vertebral body, $P < 0.001$) and midfemoral cortical thickness (+11 to 17%, $P < 0.05$), measured at 3 and 6 months of age. The skeletal and metabolic phenotypes were gender dependent, with female being more affected than male mice. However, uncoupling protein-1 expression in brown fat, a marker of sympathetic tone, was not different between genotypes. We show that deletion of central BDNF expression in mice results in increased bone mass and white adipose tissue, with no significant changes in sympathetic signaling or peripheral serotonin, associated with hyperphagia, obesity, and leptin resistance. (Endocrinology 153: 5394–5405, 2012)

Brain-derived neurotrophic factor (BDNF) is a neuronal survival, differentiation, and plasticity factor, also mediating the estrogen actions in hippocampus and hypothalamic area (1, 2). BDNF emerged as an essential constituent of central neural circuits involved in regulating energy homeostasis (3, 4). Studies suggest that the metabolic actions of BDNF are under central control and that it has an anorexigenic effect, regulating food intake and energy expenditure (5, 6). BDNF acts on central and peripheral neurons through binding to the product of tyrosine kinase receptor type B/neurotrophic tyrosine kinase, receptor, type 2, a high-affinity tyrosine kinase receptor (7). Fetal or early postnatal depletion of this neurotrophin or its receptor in mice results in hyperphagic

Abbreviations: BA, Cortical bone area; BATA, bone area fraction; BDNF, Brain-derived neurotrophic factor; BV/TV, bone volume fraction; CamK, calmodulin-dependent protein kinase II; Conn.D, connectivity density; μCT, microcomputed tomography; DXA, dual-energy x-ray absorptiometry; EEF2, eukaryotic translation elongation factor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Imax, maximum inertia; Imm, minimum inertia; KO, knockout; NE, norepinephrine; pMOI, polar multiplicity of infection; SMI, structure model index; TA, total cross-sectional area; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; UCP-1, uncoupling protein 1; VMH, ventromedial hypothalamus; WAT, white adipose tissue; WBBMC, whole-body bone mineral content; WBBMD whole-body bone mineral density; WT, wild type.
behavior and dramatic obesity (6, 8, 9). In humans, similar alterations were linked to BDNF haploinsufficiency and to a de novo missense mutation in the TrkB gene (10, 11). BDNF is expressed in energy balance centers in the hypothalamus and hindbrain in the developing and mature brain (8, 12). Within the hypothalamus, it is most abundant in the ventromedial hypothalamus (VMH), a critical subpopulation of neurons important for the regulation of food intake (9). The VMH contains receptors for signals denoting energy status, including leptin, insulin, melanin-concentrating hormone, and orexin (13). BDNF is also widely expressed in nonneuronal cells such as leukocytes, chondrocyte, fibroblasts, adipocytes, and osteoblasts (7), thus suggesting that BDNF may participate in regulating the development of bony tissue and that BDNF has a positive effect on bone (14–18).

In addition to the classical pathways of bone regulation triggered by hormones, autocrine/paracrine signals, and mechanical loading, it is now emerging that bone homeostasis is under the control of other systems within the organism, including the central nervous system (19). The first clear evidence that central signaling affects bone was the finding that leptin deficient mice, despite their hypogonadism had high bone mass, and that this phenotype was reversed by intracerebroventricular injections of leptin (20). Furthermore, mice lacking the β2-adrenergic receptor are resistant to the central bone-reducing effects of leptin, demonstrating that central leptin signaling may affect bone via the sympathetic nervous system (20–22). Thus, sympathetic signaling appears to be involved in bone regulation and to have a negative impact on bone mass (20, 21). These studies have fortified the link between skeletal and neuronal biology, supporting the idea that central leptin signaling deficiency is related with high bone mass (20–22). Although central leptin has a negative impact on bone, peripheral leptin is anabolic to bone (23, 24). These data are further supported by the observations that peripheral leptin treatment increases bone mass both in rodents and in hypoleptinemic women (25, 26) and that leptin receptor-deficient db/db mice show reduced bone volume (27). Thus, leptin has opposite peripheral vs. central effects on bone. Recent reports show that bone mass and strength are reduced in the absence of leptin signaling, indicating that leptin acts in vivo as an anabolic bone hormone (28, 29). These data are further supported by the findings that both central (intracerebroventricular) and sc administration of leptin in leptin-deficient ob/ob mice partially rescues the metabolic phenotype, reducing body weight, food intake, and adiposity while stimulating bone growth in both the tibiae and vertebrae, leading to enhanced bone formation. Central (intracerebroventricular) leptin injection increases bone formation, bone mineral density, muscle mass, serum IGF-I, and the expression of osteogenic genes in leptin-deficient ob/ob mice (30, 31).

Recent studies indicate that additional mechanisms exist to mediate the actions of peripheral leptin on bone cells. The selective deletion of estrogen receptor-α in neuronal cells results in increased bone mass attributed to decreased leptin sensitivity in the hypothalamus, leading to increased secretion of leptin from white adipose tissue (WAT) and increased bone mass accrual (28). However, unlike ob/ob mice, the nestin-estrogen receptor-α−/− phenotype occurs in the absence of any change in circulating catecholamines or sympathetic tone and is proposed to be mediated by the anabolic action of peripheral leptin on bone.

This study examines whether central BDNF plays a physiological role in the regulation of bone mass in vivo. Our rationale was that selective deletion of BDNF in the brain of Bdnf<sup>2lox/2lox/93</sup> mice produces a metabolic phenotype characterized by obesity, hyperphagia, and elevated leptin (6), similar to that of the high bone mass ob/ob mouse (20). To address this question, we examined the skeletal phenotype and sympathetic tone in the BDNF conditional mutant mouse (Bdnf<sup>2lox/2lox/93</sup>). These mice lack BDNF only in neuronal cells, whereas other peripheral sources of BDNF are intact (6).

In summary we show that deletion of central BDNF expression in mice results in increased bone mass and WAT, with no significant changes in sympathetic signaling.

Materials and Methods

Ethics statement

All of the following procedures were approved by the Institutional Animal Care and Use Committee at Tufts University and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institutional Animal Care and Use Committee protocol no. B2011-60, Tufts University). The guidelines were accepted equally by all the institutions.

Bdnf<sup>2lox/2lox/93</sup> mice

Bdnf<sup>2lox/2lox/93</sup> mice were generated as previously described (6). Briefly, excision of the BDNF coding sequence in the brain was accomplished by crossing mice carrying Bdnf<sup>2lox</sup> alleles to a line of mice expressing cre recombinase under the direction of the α-calcium/calmodulin-dependent protein kinase II (CamK) promoter, which drives expression in postmitotic neurons. Mutant mice were individually housed (because they are hyperaggressive), and wild-type mice were both individually and group housed. The housing temperature was 21.6 °C.

Body weight and fat pad

Body weight and fat pad weight were measured at ages 3 and 6 months. The fat pad was measured after surgical removal of total abdominal fat from euthanized animals.
Peripheral dual-energy x-ray absorptiometry (DXA) expression: isolation of total RNA, reverse transcription, and real-time PCR

Total RNA was isolated from brown adipose tissue, WAT, and bone using RNAzolRT following the manufacturer’s protocols (Molecular Research Center, Inc., Cincinnati, OH). To obtain RNA from bone, the femur and humerus bones were mechanically disrupted in TriReagent using a homogenizer (UltraTurraxH; IKA-HWerke GmbH and Co., Staufen, Germany) as described (32). Total RNA used for BDNF expression was treated with deoxyribonuclease I (Ambion, Austin, TX) to remove any DNA contamination because the primers/probes used for BDNF expression target a coding region with no introns. Total RNA was quantified spectrophotometrically as the 260:280 ratio (NanoDrop; Thermo Scientific, Svedso bro, NJ). Total RNA was reverse transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) or Superscript II (Invitrogen-Life Technologies, Carlsbad, CA). Real-time PCR was performed using StepOne real-time PCR system and power SYBR Green PCR master mix (Applied Biosystems). A fixed amount (1%) of the reverse transcription reaction was used as template. Changes in BDNF mRNA or UCP-1 mRNA were expressed as percent of template. Real-time PCR for some samples was performed using the real-time PCR 7500 fast system (Applied Biosystems). The primers used for BDNF were: GCCCTTGAGCCTCTTCTAC (forward) and GGGGCATCCA GTGAATTTT (reverse), and primer/probe pairs Mm04230607_s1 (Applied Biosystems). The primers used for UCP-1 were: GCCC TCTACGACTCAGTCCA (forward) and TAAAGCGCGCTGAGA TTCCTGT (reverse). The results were compared with a standard curve [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] and normalized to an expression of a housekeeping gene, [eukaryotic translation elongation factor 2 (EEF2)]. GAPDH forward, CCCC TCTTGT (forward) and TAAGCCGGCTGAGA TCTTGT (reverse). The results were compared with a standard curve [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] and normalized to an expression of a housekeeping gene, [eukaryotic translation elongation factor 2 (EEF2)].

Norepinephrine (NE) and 5-hydroxytryptamine levels

Levels of NE in brain and brown fat tissues and serum levels of 5-hydroxytryptamine were determined by a specific HPLC assay performed by the Center for Molecular Neurosciences/Kennedy Center Neurochemistry Core Laboratory at Vanderbilt University. The CMN/KC Neurochemistry Core Laboratory is supported by Vanderbilt Kennedy Center for Research on Human Development, Vanderbilt Conte Center for Neuroscience Research and The Vanderbilt Center for Molecular Neuroscience. HPLC control and data acquisition are managed by Empower software (Waters Empower Software, Orlando, FL).

The hyperactive and aggressive behavior of the Bdnf2lox/2lox/93 mice was incompatible with the time-dependent sampling and collection of urine for testing the catecholamine levels and sympathetic tone in mice models as previously described (28, 33).

Peripheral dual-energy x-ray absorptiometry (DXA)

Assessment of whole-body (exclusive of the head region) bone mineral density (WBBMD; grams per square centimeter), whole-body mineral content (WBBMC; grams), and body composition (percent body fat) was performed after euthanasia using peripheral DXA (PIXImus; GE Lunar Corp., Madison, WI), as described previously (34, 35).

Specimen preparation

Femora and lumbar vertebrae were harvested at 3 and 6 months of age and cleaned of soft tissue. The right femur and L5 vertebral body were prepared for microcomputed tomography (μCT) imaging by wrapping in saline-soaked gauze and freezing at −20 C. The contralateral, left femur was fixed using 10% neutral-buffered formalin at 4 C for 48–72 h and then transferred to 70% ethanol at 4 C.

Trabecular and cortical bone morphology by μCT

Bone morphology and microarchitecture were assessed at 3 and 6 months of age using high-resolution μCT (μCT40; Scanco Medical, Bruttisellen, Switzerland), as described previously (36). In brief, the distal femoral metaphysis and L5 vertebral body were scanned using an x-ray energy of 70 KeV, integration time of 200 msec, and 12-μm isotropic voxel size. For the cancellous bone region, we assessed bone volume fraction (BV/TV; percentage), trabecular thickness (Tb.Th; micrometers), trabecular separation (Tb.Sp; micrometers), trabecular number (Tb.N; 1 per millimeter), connectivity density (Conn.D; 1 per cubic millimeters), and structure model index (SMI). Transverse computed tomography slices also were acquired at the femoral midshaft to assess the following: total cross-sectional area (TA), cortical bone area (BA), and medullary area, respectively; square millimeters; bone area fraction (BA/TA; percentage); cortical thickness (micrometers); anteroposterior and mediolateral diameters (millimeters); area moments of inertia [maximum inertia (Imax), minimum inertia (Imm)], and polar multiplicity of infection (pMOI; microcomputed tomography (41)); and bone strength index [(pMOI/d × I) × 100, where d is the midshaft diameter, and I is the bone length (millimeters)] (37).

Bone histology

Tibiae and femora were harvested from 3- and 6-month-old mice (control and Bdnf2lox/2lox/93). Bones were fixed in 10% (vol/vol) buffered formalin, decalcified in EDTA, embedded in paraffin, sectioned, and then stained with hematoxylin and eosin and tartrate-resistant acid phosphatase. A standard region of interest at least 0.5 mm below the growth plate (excluding the primary spongiosa and trabecular connected cortical bone) was selected, and this region was used for all animals, regardless of the shape of the section (38). Images were taken using an Axiovert 200M microscope (Zeiss, Jena, Germany) for at least four mice per group. Data are means ± SD of at least 20 measures per group. Standard bone histomorphometry as described by Parfitt et al. (39) was performed using the Bio-Quant image analysis software (R & M Biometrics, Nashville, TN) by the Histomorphometry and Molecular Analysis Core Laboratory of the University of Alabama at Birmingham Bone Center. The distal femoral growth plates in mice remain open and active at 6 months. Growth plate thickness was measured perpendicular to the plane of the growth plate as defined by columns of resting, proliferating, and hypertrophic chondrocytes within cartilage extracellular matrix, using Safranin-O stained femurs of 6-month control and Bdnf2lox/2lox/93 mice of both sexes. We measured 10–12 independent distal femur sections from four to six mice of each group.
Statistical analysis
Data are expressed as mean ± SEM. Statistical comparisons involved male and female Bdnf2lox/2lox/93 mice and paired BDNF+/+ (control) littersmates at 2–3 and 6 months. Statistical significance of the differences between means of two groups was assessed by Student's t test. For comparison between more than two groups, the ANOVA followed by Bonferroni's t test was used. Two-way ANOVA was used to test for the effects of genotype and gender on bone parameters. Significance was set at P < 0.05.

Results
BDNF expression in the Bdnf2lox/2lox/93 mice
Previous experiments have shown that BDNF expression is significantly reduced in various brain areas of Bdnf2lox/2lox/93 mice, and it is neuronal cell specific (6, 9). This is supported by the fact that there is no cre-mediated recombination of floxed BDNF alleles in nonbrain tissues such as kidney and heart of Bdnf2lox/2lox/93 mice (6). Expression of BDNF mRNA has been previously found in bone and WAT (14, 40). In the present work, we show that BDNF levels in bone and WAT are not significantly different between the mutant and control mice (Fig. 1). This confirms that there is no cre-mediated recombination of floxed BDNF alleles in bone or WAT, supporting the concept that the central deletion of BDNF is not affecting BDNF expression in bone and WAT.

Whole-body BMD
Both male and female Bdnf2lox/2lox/93 mice showed a significant increase in body weight at 3 and 6 months of age, compared with wild-type littermates (36–60% for males and 99–116% for females) (Fig. 2A). The increased body weight in Bdnf2lox/2lox/93 mice was accompanied by a significant increase in abdominal fat pad (2- to 3-fold increase for males and 10-fold for females), indicating that the increased body mass is mainly due to increased fat mass (Fig. 2B).

In parallel, Bdnf2lox/2lox/93 mice at 3 and 6 months of age had longer femurs (+6–10%, P < 0.01) (Fig. 2C). The increased skeletal mass was associated with increased WBBMD and WBBMC (Fig. 2, D and E). Body fat also increased in female knockout (KO) mice compared with wild type (Fig. 2F).

Bone morphology and histology: trabecular bone at distal femur and lumbar vertebrae and cortical bone at femoral diaphysis
A μCT analysis revealed that compared with control mice, Bdnf2lox/2lox/93 mice at 3 months, age had significantly greater BV/TV (P < 0.001), Tb.N (P < 0.02), and Tb.Th (P < 0.0002) along with reduced Tb.Sp (P < 0.02). These changes occurred in both males and females, with Bdnf2lox/2lox/93 having 32 and 57% greater trabecular bone parameters for males and females, respectively. Similar patterns were observed in both the fifth lumbar vertebra and the distal femur (Fig. 3A). Increases in bone microarchitecture were enhanced at 6 months of age, with a more pronounced effect in females than males (Table 2).

At 3 and 6 months of age, BA/TA was greater in the Bdnf2lox/2lox/93 mice than in the wild-type (WT) littersmates (1 and 6%, respectively, P < 0.001). Total bone area, cortical bone area, cortical thickness, and cross-sectional geometry (Imin, pMOI) also increased in Bdnf2lox/2lox/93 vs. WT mice (Table 1; 2).

The increase in cortical thickness was further confirmed by histological analysis of the femurs of Bdnf2lox/2lox/93 compared with wild-type mice (Fig. 3B). In females, osteoblast perimeters were significantly greater in femurs of 6-month-old mutant mice. As shown in Table 3, the decrease in osteoclast number was significant only in females, and only when normalized per bone slice, whereas both genders show the increased bone mass. A small but not significant increase of total osteoclast number in Bdnf2lox/2lox/93 female and male mice was observed. Therefore, the striking phenotype of increased bone mass in the absence of central BDNF cannot be explained solely by decreased osteoclast number. The growth plate thickness was greater in Bdnf2lox/2lox/93 mice compared with wild-type mice (Table 3). In mutant males at 6 months, there was no significant increase in osteoblast perimeter per unit bone surface or the number of osteoblasts per bone surface (Table 3). We infer that increased endochondral ossification contributes to the increased bone mass in the BDNF KO mice. These data confirm a stronger skeletal phenotype in females mutant than in...
males that might be related to interactions between BDNF and estrogen.

**Brain NE levels, serum serotonin, and peripheral UCP-1 expression in WT and Bdnf2lox/2lox/93 mice**

UCP-1 is a mitochondrial protein that is expressed specifically in brown adipose tissue and has a significant role in regulating energy expenditure and body temperature in rodents. Sympathetic, adrenergic activation is associated with a significant induction of UCP-1 mRNA in brown adipose tissues, and UCP-1 mRNA levels in brown adipose tissue are a useful and specific marker of the peripheral sympathetic tone (28, 30, 41, 42). We examined whether the absence of brain-derived BDNF expression is associated with a change in sympathetic tone. Our results show that UCP-1 expression is not changed in Bdnf2lox/2lox/93 mice (Fig. 4 A).

Measurements of NE levels in brain and brown fat showed no differences between BDNF mutant and control mice (Fig. 4, B and C). Similarly, serum levels of serotonin were comparable in Bdnf2lox/2lox/93 and wild-type mice (Fig. 4 D). These results exclude the possibility that the bone phenotype in Bdnf2lox/2lox/93 mice is caused by altered serotonin regulation or sympathetic tone.

**Discussion**

To determine the importance of central BDNF signaling for the regulation of bone mass, we used a mouse model in which BDNF has been selectively deleted from the brain after birth through the use of the cre-LoxP recombination under the direction of the α-calcium/CamK promoter, which drives expression in postmitotic neurons; this is a well described strategy for targeted gene deletion in the mouse central nervous system (6). We previously reported (6) that there was no cre-mediated recombination of floxed BDNF alleles in peripheral kidney and heart tissues in Bdnf2lox/2lox/93 mutant mice. In this study, we confirmed by quantitative RT-PCR analysis that the expression of BDNF in bone and WAT is retained in the Bdnf2lox/2lox/93 mutant mice. Therefore, the differences we report in the current study cannot be attributed to alterations in BDNF expression in these tissues. In addition, this result further validates the neuronal specificity of the targeting strategy used to selectively remove BDNF expression in the brain using cre recombinase 93 under the control of α-calcium/CamK promoter.
TABLE 1. Lack of central BDNF increases both midshaft cortical and distal femur trabecular bone properties (effect at 3 months of age)

<table>
<thead>
<tr>
<th></th>
<th>3 months old</th>
<th>3 months old</th>
<th>F values (two way ANOVA)</th>
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<tr>
<td></td>
<td>Female (n = 4)</td>
<td>Female (n = 5)</td>
<td>Male (n = 6)</td>
</tr>
<tr>
<td>Distal femur (trabecular and cortical)</td>
<td></td>
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<tr>
<td>BV/TV (%)</td>
<td>7.64 ± 1.13</td>
<td>15.33 ± 0.79ab</td>
<td>8.26 ± 0.51</td>
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<td>Tb.Th (µm)</td>
<td>43.6 ± 0.61</td>
<td>55.2 ± 1.41ab</td>
<td>42.0 ± 0.66</td>
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<td>Tb.N (mm/m)</td>
<td>3.47 ± 0.23</td>
<td>4.16 ± 0.07ab</td>
<td>4.03 ± 0.09</td>
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<tr>
<td>Tb.Sp (µm)</td>
<td>289.6 ± 20.38</td>
<td>234.2 ± 5.74ab</td>
<td>245.2 ± 5.74</td>
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<tr>
<td>Conn.D (mm⁻³)</td>
<td>77.39 ± 13.6</td>
<td>111.86 ± 4.86ab</td>
<td>90.49 ± 6.59</td>
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<td>SMI</td>
<td>2.96 ± 0.16</td>
<td>2.29 ± 0.05ab</td>
<td>3.06 ± 0.07</td>
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<td>Midshaft femur (cortical)</td>
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<tr>
<td>Total area (mm²)</td>
<td>0.92 ± 0.049</td>
<td>1.27 ± 0.047ab</td>
<td>1.01 ± 0.04</td>
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<td>BA/TA (%)</td>
<td>54.32 ± 1.6</td>
<td>57.81 ± 0.49a</td>
<td>58.93 ± 0.72</td>
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<td>Cortical thickness (µm)</td>
<td>187.2 ± 10.8</td>
<td>219.6 ± 8.67ab</td>
<td>214.7 ± 11.25</td>
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<td>lmax (mm³)</td>
<td>0.06 ± 0.005</td>
<td>0.11 ± 0.009ab</td>
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<td>lmin (mm³)</td>
<td>0.04 ± 0.005</td>
<td>0.09 ± 0.006ab</td>
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<td>pMOI (mm⁴)</td>
<td>0.10 ± 0.009</td>
<td>0.20 ± 0.015ab</td>
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<td>Lumbar vertebra (trabecular)</td>
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<tr>
<td>BV/TV (%)</td>
<td>14.24 ± 0.79</td>
<td>22.21 ± 0.87ab</td>
<td>17.92 ± 1.03</td>
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<td>Tb.Th (µm)</td>
<td>47.7 ± 0.47</td>
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<td>Tb.N (mm/m)</td>
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<td>Tb.Sp (µm)</td>
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<td>0.68 ± 0.07ab</td>
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Comparison of µCT parameters for trabecular and cortical bone at distal and midshaft femurs and fifth lumbar vertebrae in male and female Bdnf²/²lox/²lox/93 and wild-type mice at age 3 months. Lack of central BDNF increases both midshaft cortical and distal femur trabecular bone properties. The number in parentheses (n) indicates the number of animals used. Differences due to genotype and gender were tested using two-way ANOVA.

a Significantly different with respect to the relative wild-type for P < 0.05 or less (by Bonferroni t test following ANOVA).

b Significantly different with respect to the relative wild-type for P ≤ 0.05 (by Student’s t test).

c F values are significantly different (P < 0.001).

d F values are significantly different (P < 0.02).

e F values are significantly different (P < 0.005).

f F values are significantly different (P < 0.05).

g F values are significantly different (P < 0.01).

Deletion of BDNF in the brain resulted in a bone phenotype characterized by high bone mass, a metabolic phenotype with significantly elevated abdominal WAT, and previously described hyperphagia, obesity, and elevated leptin (6, 9). Our results show that the skeletal action of BDNF targets bones in the axial and appendicular skeleton and affects both cortical and trabecular domains. The high bone mass is due to a large increase in bone formation parameters. Compared with the wild-type, Bdnf²/²lox/²lox/93 mice have 6 and 10% increased femur length at 3 and 6 months of age, respectively; and increased WWBMD and WWBMC at 6 months of age. The µCT analysis shows that Bdnf²/²lox/²lox/93 mice show increased bone volume in both cortical and trabecular districts and increased number of trabeculae in the lumbar vertebrae and femur at 3 and 6 months. These changes occurred in both males and females, with Bdnf²/²lox/²lox/93 mice having 32 and 57% greater trabecular bone parameters for males and females, respectively. Similar patterns were observed in both the fifth lumbar vertebra and the distal femur, and these skeletal changes are gender dependent, with females being more strongly affected than males. This latter observation was further confirmed by histological analysis of femurs.

Regarding the metabolic phenotype, previous studies have shown that mice lacking BDNF in the brain exhibit obesity, high-serum leptin levels, and hyperactivity (6). Food consumption in control and mutant animals at 3 and 4 months of age, fed a standard chow diet ad libitum, was 74% higher in mutant animals than in controls (43). Previous preliminary observations would suggest that the basal metabolic rate of Bdnf²/²lox/²lox/93 mutant mice...
measured using indirect calorimetry (O₂ consumption/CO₂ production) was not different between BDNF mutants and wild-type controls (Rios, M., unpublished data). Furthermore, our findings on mutants with BDNF depletion in the VMH are consistent with previous studies showing that pair feeding of other lines of BDNF mutants with wild-type controls is sufficient to normalize their weights (9, 44), suggesting normal energy expenditure. It is inferred, therefore, that changes in body weight in BDNF mutant mice arise solely from increased food intake and not from alterations in their basic metabolic rate.

Our results further show that the increased body weight is due mainly to a significant increase in abdominal WAT. Notably, these metabolic changes occur over the same developmental time frame and show the same gender dependence as the changes in bone formation with females being more affected than males. It is known that circulating leptin is mainly adipocyte derived and is regulated by the leptin expression within WAT and the amount of WAT. In previous experiments we showed that serum levels of leptin and insulin were 15-fold and 6-fold higher, respectively, in Bdnf²lox/²lox/⁹³ mice as compared with controls (6). In younger mutants that were not yet obese, serum levels of leptin and insulin were determined to be normal, indicating that the elevated levels detected in the older obese mutants were secondary effects of obesity. Glucose levels in the conditional mutants were 70% higher than in controls. BDNF conditional mutants are leptin and insulin resistant, and this resistance is a secondary effect of obesity (6). Therefore, the observed increase in the amount of abdominal WAT in Bdnf²lox/²lox/⁹³ mice can be responsible for the previously reported elevated serum leptin level in these mice (6).

A point of interest is the fact that the BDNF conditional KO mice phenotype investigated here occurs in the apparent absence of any change in sympathetic tone. This is inferred from measurements of UCP-1 mRNA levels in brown adipose tissue that were not significantly different between the two groups as well as NE levels in brain and brown adipose tissue. Therefore, no support was obtained for the possibility that the bone phenotype seen in Bdnf²lox/²lox/⁹³ is caused by altered sympathetic tone. Brown fat is important for thermoregulation. The oxidation of lipids in brown fat cells is regulated by the direct sympathetic nervous system stimulation via NE and is accomplished by expression of UCP-1. Interestingly, it has been shown that increased sympathetic activity, due to cold stimulus and/or impaired response to cold by brown adipose tissue, can cause bone loss (30).

Oxytocin and serotonin have been implicated as regulators of both body weight and bone mass through the regulation of the leptin-sympathetic transmission (33, 42,
Histomorphometric parameters were measured in equivalent regions of interest using sections of distal femurs from wild-type and 6-month-old Bdnf<sup>2lox/2lox</sup>/93 mice (n = 4–12 sections). Obs/BS (osteoblast number) was significant only in females and only when normalized per bone slice. Obs, Osteoblast surface; Obs/BS, bone surface ratio; N.Ob/BS, number of osteoblasts in (cells/unit surface); N.Oc, number of tartrate-resistant acid phosphatase-positive osteoclasts; N.Oc/BS (in cells/unit surface), cortical thickness at mid-diaphysis (in microns)/growth plate cartilage thickness (in microns).

<sup>a</sup> Statistically significant difference from wild type (P < 0.05).
Our central finding is that targeted brain deletion of BDNF expression causes increased bone mass in parallel with the metabolic phenotype. The increased leptin level may lead to the bone accrual through a peripherally mediated anabolic action of leptin on osteoblasts (27, 28). Our data suggest that central BDNF signaling has a negative impact on bone, whereas peripheral BDNF may exert direct anabolic action on bone, also modulating differentiation and differentiation of condrocytes (15).

In this regard, BDNF regulates the pace of differentiation at the growth plate, explaining why Bdnf2lox/2lox/93 mice have longer femurs and thicker growth plates (49). Therefore, it is emerging that bone formation is the balance between the peripheral vs. central action of mediators including BDNF and hormones such as leptin, serotonin, oxytocin, and estrogen.

With the rise in obesity worldwide, an important debate has developed as to whether excess fat has a detrimental or protective effect on skeletal health in children and adults. Recent studies provide evidence that an increase in fat mass has a negative effect on the growing skeleton during childhood and adolescence (50) and that obese children are more prone to fracture because they have lower bone mass relative to body size (51). In contrast, the widely accepted opinion is that fat appears to be protective of bone in adults and adolescents (50) and that obese children are more prone to fractures, obese adults may be at increased risk of fracture because they have lower bone mass relative to body size (51). Therefore, it is emerging that bone formation is the balance between the peripheral vs. central action of mediators including BDNF and hormones such as leptin, serotonin, oxytocin, and estrogen.

FIG. 4. UCP-1, noradrenaline, and serotonin levels in Bdnf2lox/2lox/93 mice. A, UCP-1 expression in interscapular brown adipose tissue was measured by real-time quantitative RT-PCR. UCP-1 mRNA was normalized to the total mRNA used for template (0.1 ng). Differences between genotypes were not statistically significant at \( P > 0.93 \) (n = 5 control and n = 6 KO mice, females 3–6 months of age). Measurements from mice at 3 and 6 months of age were grouped after first determining that there was no age effect (\( P > 0.95 \) for interactions by age and \( P > 0.78 \) for interactions by genotype, two way ANOVA). Brain noradrenaline (B), brown fat noradrenaline (C), and serum serotonin (D) measured by HPLC shows no significant differences between the mutant and wild type. Group averages and sds were calculated for each group of four (or three in the case of WT plasma samples). A t test (two tailed, assuming equal variance of sample groups) was performed to test the probability of differences between the KO and WT groups being significant, using \( P < 0.05 \) as the criterion.
For example, in adolescence, the gradual rise in serum leptin levels before puberty, together with a decline in circulating levels of soluble leptin receptor, may serve as an initiator of puberty in response to favorable nutritional conditions and this also explain why puberty commences earlier in obese children (50). Similarly, during puberty, estrogen activates the GH-IGF-I axis, which in turn drives skeletal growth (57, 58). Understanding how alterations in obesogenic hormones, including BDNF, determine changes in bone mass from childhood to adulthood may be vital in informing future pharmaceutical interventions in obesity, which may help to improve bone mass during critical periods of bone mass accrual.

**Therapeutic implications**

An intriguing aspect of these findings relates to the possible actions of peripherally administered BDNF. This is hypothesized to initiate a positive feedback loop involving the following: 1) direct anabolic action on osteoblasts in bone; 2) BDNF crossing the blood-brain barrier to act centrally on the hypothalamus, suppressing appetite and circumventing leptin resistance (4, 59, 60); and 3) decreased food fat. We propose that peripheral administration of BDNF can have dual beneficial effects through reversal of low bone mass and obesity. Peripheral administration of BDNF to diabetic leptin receptor KO db/db mice decreases food intake and lowers blood glucose to normoglycemic levels (3, 61–64). Future studies will address the important question of whether BDNF is indeed a brain-derived pleiotropic factor that could be an effective single therapy for concurrent targeting of osteoporosis and metabolic diseases.

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