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Extracellular Norepinephrine Clearance by the Norepinephrine Transporter Is Required for Skeletal Homeostasis*

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Background: The contribution of endogenous norepinephrine (NE) to skeletal homeostasis is unclear.
Results: Bone forming cells, not only neurons, express the norepinephrine transporter (NET), and blockade of extracellular NE clearance causes alterations in bone homeostasis.
Conclusion: NE clearance by NET is a component of the homeostatic machinery by which sympathetic nerves and osteoblasts control bone remodeling.
Significance: NET blockers may increase fracture risk.

Changes in bone remodeling induced by pharmacological and genetic manipulation of β-adrenergic receptor (βAR) signaling in osteoblasts support a role of sympathetic nerves in the regulation of bone remodeling. However, the contribution of endogenous sympathetic outflow and nerve-derived norepinephrine (NE) to bone remodeling under pathophysiological conditions remains unclear. We show here that differentiated osteoblasts, like neurons, express the norepinephrine transporter (NET), exhibit specific NE uptake activity via NET and can catabolize, but not generate, NE. Pharmacological blockade of NE transport by reboxetine induced bone loss in WT mice. Similarly, lack of NE reuptake in *norepinephrine transporter* (*Net*)-deficient mice led to reduced bone formation and increased bone resorption, resulting in suboptimal peak bone mass and mechanical properties associated with low sympathetic outflow and high plasma NE levels. Last, daily sympathetic activation induced by mild chronic stress was unable to induce bone loss, unless NET activity was blocked. These findings indicate that the control of endogenous NE release and reuptake by presynaptic neurons and osteoblasts is an important component of the complex homeostatic machinery by which the sympathetic nervous system controls bone remodeling. These findings also suggest that drugs antagonizing NET activity, used for the treatment of hyperactivity disorders, may have deleterious effects on bone accrual.

Bone remodeling is a tightly regulated process under the control of systemic hormones and paracrine/autocrine factors. The bone marrow environment is richly vascularized and innervated, and recent evidence support the existence of functional interactions between nerves and bone cells (1–3). Among this evidence, signaling via the β2-adrenergic receptor (β2AR)[2] has emerged as critical. The β2AR is expressed in both bone mesenchymal and hematopoietic lineages (4–6). In osteoblasts, its expression is predominant among the nine α- and β-adrenergic receptor subtypes (α1A, α1B, α1D, α2A, α2B, α2C, β1, β2, and β3) (4, 7). Moreover, the β2AR in osteoblasts is functional, as demonstrated by the accumulation of cAMP and the activation of target genes, including *Rankl*, upon exogenous stimulation by NE or isoproterenol, a non-selective β adrenergic agonist (1, 8). β2AR activation in osteoblasts stimulates the formation of osteoclast via the induction of *Rankl* expression (1) and inhibits osteoblast proliferation via *cyclin D1* and *Clock* genes (9). Genetic ablation of the β2AR, globally or specifically in osteoblasts, induces a high bone mass phenotype in mice (1, 10). Similarly, pharmacological blockade of βAR signaling using the non-selective βAR antagonist propranolol increases bone mass, whereas β2AR stimulation with various βAR agonists decreases it (11–13). As a whole, this body of knowledge strongly supports the importance and biological relevance of sympathetic nerves in the regulation of bone remodeling. However, these studies have mostly focused on post-synaptic βAR modulation and did not yet directly investigate the existence of presynaptic mechanisms regulating endogenous sympathetic outflow. Following sympathetic activation, 80–90% of the NE released in synaptic clefts is cleared by NE reuptake, the remaining extracellular NE diffusing into the circulation or being metabolized. The process of NE reuptake is

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[2] The abbreviations used are: β2AR, β2-adrenergic receptor; NE, norepinephrine; NET, norepinephrine transporter; ADHD, attention deficit hyperactivity disorder; CIS, chronic immobilization stress; BMSC, bone marrow stromal cell; DPD, deoxypyridinoline; POB, primary osteoblast; BV/TV, bone volume/tissue volume.

mediated by the norepinephrine transporter (NET), a monoamine transporter and a member of the Na^+/Cl^--dependent family of neurotransmitter transporters. NET controls the concentration of NE and duration of neurotransmission at synapses, and is located in the membrane of presynaptic neurons, although desipramine-sensitive [^{3}H]NE uptake has been observed in astrocytes as well (14) and *Net* expression has been reported in several peripheral organs in embryos (15). NET is the target of drugs used for the treatment of depression and attention deficit hyperactivity disorder (ADHD), and of drugs of abuse, including cocaine and amphetamine. In this study, we investigated whether bone cells transport NE and therefore locally control NE extracellular levels in the skeleton, and if alteration in NE reuptake caused by NE transport inhibition or *Net* deficiency in mice has consequences on bone homeostasis.

EXPERIMENTAL PROCEDURES

Animals—C57BL/6J WT mice (Jackson Laboratory) were administered reboxetine at 15 mg/kg/day via subcutaneously implanted mini-osmotic pumps (Durect Corporation 0000298). Heterozygous $Net^{+/-}$ mice (genetic background C57BL/6J) (16) were mated to produce $Net^{+/+}$ (wild-type) and $Net^{-/-}$ littermates. Chronic immobilization stress (CIS) was carried out 2 h daily by placing mice in 50-ml laboratory conical tubes perforated for adequate air supply. Murine body composition was measured using a Minispec Model mq7.5 (7.5 mHz) nuclear magnetic resonance (NMR) analyzer (Bruker Instruments). Tail suspension measurements were performed as previously described (17). All procedures were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center.

Primary Cell Culture—Calvarial osteoblasts were isolated from 3–4-day-old neonatal mice. Bone marrow stromal cells (BMSCs) were isolated from 2–3-month-old adult mice. Differentiation was induced with 50 μg/ml of ascorbic acid. Spleen-derived osteoclasts were prepared from 2–3-month-old adult mice. Differentiation was induced with 30 ng/ml of macrophage colony-stimulating factor and 50 ng/ml of receptor activator of nuclear factor κB ligand. For alkaline phosphatase-positive colony forming units (cfu-AP) assays, BMSCs were isolated from long bones, plated at a density of 1×10^7 cells/10-cm plate. One week after plating the cells, culture medium was switched to osteogenic medium containing 50 μg/ml of ascorbic acid. Two weeks following plating, alkaline phosphatase staining was performed, and the number of cfu-AP colonies was counted manually.

Semi-quantitative and Quantitative Real-time RT-PCR—Semi-quantitative RT-PCR was performed using the following primers: *Net (norepinephrine transporter)*: forward, 5′-caggcacctccattctgttt-3′, reverse, 5′-taggtgagcggcttgaagtt-3′, T_m = 60 °C; *Dbh* (*dopamine β-hydroxylase*): forward, 5′-gactcaactactgccggcacgt-3′, reverse, 5′-ctgggtgcacttgtctgtgcagt-3′, T_m = 60 °C; *Th* (*tyrosine hydroxylase*): forward, 5′-ccccacctggagtactttgtg-3′, reverse, 5′-cttgtcctctctggaactgc-3′, T_m = 60 °C; *Mao* (*monoamine oxidase*): forward, 5′-ggagaagcccagtatcacagg-3′, reverse, 5′-gaaccaagacattaattttgtattctgac-3′, T_m = 60 °C; *Comt* (*catechol-O-methyltransferase*): forward, 5′-agaccgctaccttccagaca-3′, reverse, 5′-tgagtggtcaggacttcacg-3′, T_m = 60 °C; *Emt* (*extraneuronal monoamine transporter*): forward, 5′ctgggtggtccctgagtctcc3′, reverse, 5′-tcccaggcgcatgacaagtcc-3′, T_m= 61 °C; *Ocn (osteocalcin)*: forward, 5′-accctggctgcgctctgtctct-3′, reverse, 5′-gatgcgtttgtaggcggtcttca-3′, T_m= 62 °C; *Ctr* (*calcitonin receptor*): forward, 5′-tgctggctgagtgcagaaacc-3′, reverse, 5′-ggccttcacagccttcaggtac-3′, T_m = 62 °C; *Hprt* (*hypoxanthine-guanine phosphoribosyltransferase*): forward, 5′-gttgagagatcatctccacc-3′, reverse, 5′-agcgatgatgaaccaggtt-3′, T_m = 55 °C. Real-time PCR was performed using TaqMan® or SYBR Green gene expression assays. TaqMan probes/primers were from Applied Biosystems (*Sost*, Mm00470479_m1; *Hprt1*, Mm00446968_m1; *Ucp1*, Mm00494069_m1). SYBR Green primer sequences were: *Ocn* forward, 5′-accctggctgcgctctgtctct-3′ and reverse, 5′-gatgcgtttgtaggcggtcttca-3′; *Net* forward, 5′-caggcacctccattctgttt-3′ and reverse, 5′-taggtgagcggcttgaagtt-3′; *b2 mg* (*β2-microglobulin*): forward, 5′-ttctggtgcttgtctcactga-3′ and reverse, 5′-cagtatgttcggcttcccattc-3′. Specificity of SYBR Green amplifications was verified by the presence of a single peak on the dissociation curve.

Western Blot—Western blot analyses were performed using anti-mouse NET (Mab Technologies NET05-2) or monoclonal mouse anti-β-actin antibody (Sigma A5441) and chemiluminescent detection (PerkinElmer Life Sciences).

[^{3}H]NE Uptake Assay—NE transport was assayed as described previously (18, 19).

Microcomputed Tomography Analysis—Three-dimensional microcomputed tomography analyses were performed using a Scanco μCT 40 system (Scanco Medical, Bassersdorf, Switzerland). Tomographic images were acquired at 55 kVp and 145 μA with an isotropic voxel size of 12 mm and at an integration time of 300 ms. To segment bone from non-mineralized tissue, a Gaussian noise suppression filter (σ = 0.8, support = 1) was used, and global thresholds were consistent across scans per anatomical site.

Biomechanical Test—Each femur was placed on the lower support points of a three-point bending fixture with the anterior side down and medial side forward. With the span between the lower supports set to 8 mm, the hydrated bones were loaded to failure at a rate of 3.0 mm/min using a servo-hydraulic, bench-top material testing system (Dynamight 8841, Instron, Canton, OH).

Histomorphometry—The bones were dehydrated and embedded undecalcified in methyl methacrylate. Histomorphometric measurements were performed using the Bioquant Image Analysis System (R&M Biometrics, Nashville, TN).

Bone Immunocytochemistry—Immunocytochemistry was performed in formalin-fixed long bones of neonatal mice using an antibody against NET (Mab Technologies NET05-2) or a non-immune IgG antibody, according to established protocols.

Urine DPD and Creatinine—Urinary deoxypyridinoline (DPD) and creatinine were measured by MicroVue DPD EIA Kit (Quidel Corporation 8007) and MicroVue Creatinine Assay Kit (Quidel Corporation 8009), respectively.

Statistics—All data are presented as mean ± S.E. Statistical analyses were performed using one-way analysis of variance for multiple comparisons followed by post hoc pairwise comparison with Bonferroni adjustment and unpaired two-tailed Student's *t* tests for two-group comparisons. For all analyses, $p < 0.05$ was considered significant.

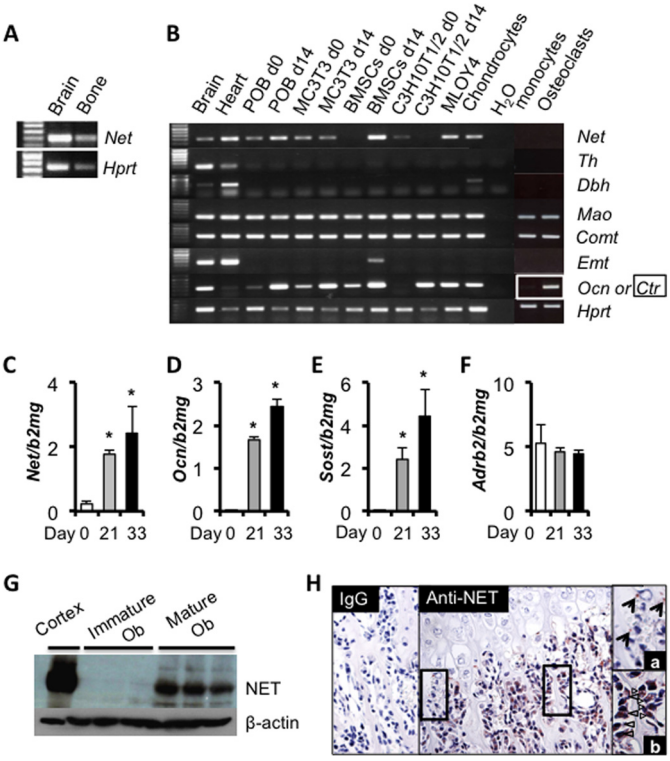


FIGURE 1. **NET is expressed in differentiated osteoblasts.** *A* and *B*, RT-PCR analysis of *Net*, *Tyrosine hydroxylase* (*Th*), *Dopamine β-hydroxylase* (*Dbh*), *Monoamine oxidase* (*Mao*), *Catechol-O-methyltransferase* (*Comt*), and *Extraneuronal monoamine transporter (Emt)*. RNA extracts from brain and heart serve as positive controls. *Osteocalcin (Ocn)* is used as a marker for differentiated osteoblasts. *Calcitonin receptor (Ctr)* is used as a marker for differentiated osteoclasts. *C–F*, real-time quantitative PCR analysis of *Net* expression in undifferentiated (*day 0*) and differentiated (*day 21* or *day 33*) primary calvarial osteoblasts. *Ocn* and *Sclerostin* (*Sost*) are used as markers for differentiated osteoblasts/osteocytes. Values are given as mean ± S.E., *, $p < 0.05$ *versus* day 0, $n = 3$. *G*, Western blot analysis of NET expression in undifferentiated (day 0) and differentiated (day 14) mouse calvarial primary osteoblasts. Protein extract from the cerebral cortex serves as a positive control. *H*, NET immunoreactivity (*brown staining*) detected by immunohistochemistry in nerves (*a*) and osteoblasts (*b*) in neonate tibia sections.

RESULTS

Osteoblasts Express Genes Required for NE Transport and Catabolism—Although the presence and functionality of the β2AR in bone cells are well described, the homeostasis of NE within the skeleton remains unknown. Surprisingly, a significant amount of *Net* mRNA were detected in bone tissues by RT-PCR, suggesting that cells distinct from neurons within the skeleton may express *Net* (Fig. 1*A*). Using RNAs from cultures of primary bone cells as well as from clonal bone cell lines, *Net* mRNA transcripts were detected by RT-PCR in BMSCs, the mesenchymal progenitor cell line C3H10T1/2, rib-derived primary chondrocytes, and in calvaria-derived primary osteoblasts (POB) as well as in the homogenous osteoblastic cell line MC3T3, with higher expression levels in *osteocalcin* (*Ocn*)-positive differentiated osteoblasts (Fig. 1*B*). In addition, these analyses showed that these cells express *Monoamine oxidase* (*Mao*) and *catechol-O-methyltransferase* (*Comt*), two genes required for NE catabolism, but do not express *Tyrosine hydroxylase* (*Th*) and *Dopamine β-hydroxylase* (*Dbh*), two genes necessary for NE synthesis. *Extraneuronal monoamine transporter (Emt)* was detected in differentiated BMSCs only. *Net* expression was

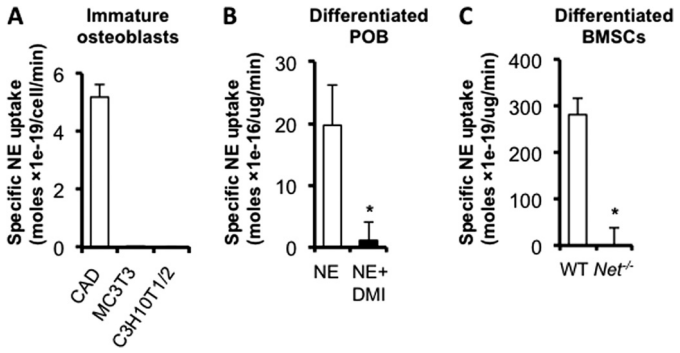
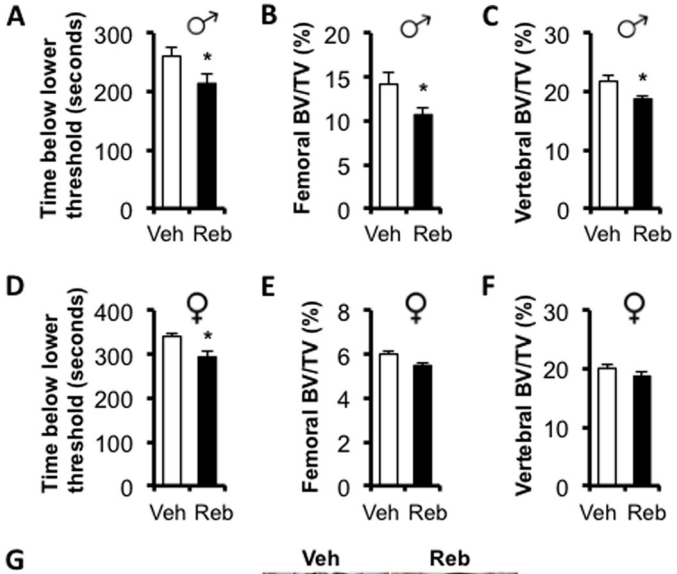


FIGURE 2. **Differentiated osteoblasts transport NE.** *In vitro* uptake assays using: *A*, non-differentiated osteoblastic cells (*CAD*: neuronal cell line stably expressing hNET, used as positive control); *B*, differentiated calvarial POB; and *C*, differentiated BMSCs. Values are given as mean ± S.E., *, $p < 0.05$, $n = 3$.

not detected in calcitonin receptor (*Ctr*)-positive primary osteoclast cultures prepared from spleen and differentiated *in vitro* with macrophage colony-stimulating factor and receptor activator of nuclear factor κB ligand.

To further characterize the expression pattern of *Net* during differentiation of the osteoblast lineage, POB were isolated from mouse calvariae, differentiated *in vitro* with ascorbic acid for 21 or 33 days and *Net* gene expression was quantified by quantitative PCR. *Net* expression increased during osteoblast differentiation, with a pattern similar to that of *Ocn* and *Sclerostin* (*Sost*, a marker of matrix-embedded osteoblasts/osteocytes) expression (Fig. 1, *C–E*). In contrast, *Adrb2* expression remained constant during differentiation (Fig. 1*F*). The expression of NET in differentiated osteoblasts was confirmed at the protein level by Western blot analysis using cultures of undifferentiated and differentiated POB (Fig. 1*G*). In addition, NET immunoreactivity was clearly detected in osteoblasts and cross-sections of nerves in long bone sections of neonatal mice (Fig. 1*H*). These results indicate that NET expression is not restricted to central and peripheral neurons, and suggest that differentiated osteoblasts have the potential to contribute to NE clearance.

Active NE Transport via NET in Differentiated Osteoblasts—The function of NET in presynaptic nerve terminals is to recapture NE from the synaptic extracellular environment following action potential and NE release. To determine whether NET in differentiated osteoblasts serves a similar function as in nerves, [^{3}H]NE uptake assays were performed using 3 independent bone cell types. [^{3}H]NE uptake was readily detectable in CAD neuronal cells transfected with *Net* (used here as positive control), but could not be detected in undifferentiated MC3T3/E1 and C3H10T1/2 mesenchymal cells (Fig. 2*A*), in agreement with the undetectable level of the transporter in undifferentiated POB measured by Western blot (Fig. 1*G*). However, significant [^{3}H]NE uptake was detected in differentiated osteoblasts, where NET is expressed (Figs. 1, *C* and *G*, and 2*B*). The specificity of this uptake activity was demonstrated by the significant reduction in NE uptake observed upon treatment of WT differentiated POB with the NET blocker desipramine (DMI) (Fig. 2*B*) and upon using differentiated BMSCs genetically deficient for *Net* (Fig. 2*C*). These results indicate that differentiated osteoblasts are equipped with a functional NE transporter and thus may contribute to skeletal NE clearance *in vivo*.



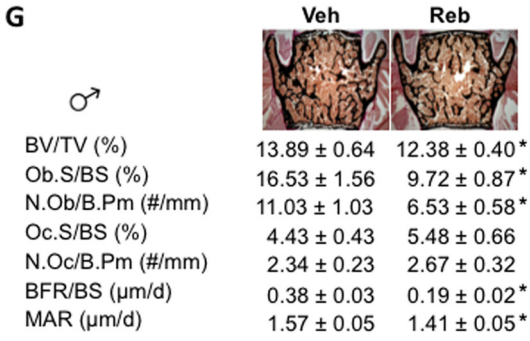


FIGURE 3. **Chronic reboxetine treatment reduces bone mass.** Behavioral tail suspension test in 14-week-old male (*A*) and female (*D*) mice treated with vehicle (*Veh*) or reboxetine (*Reb*). Micro-CT analyses of femoral (*B* and *E*) and vertebral (*C* and *F*) trabecular bone in male (*B* and *C*) and female (*E* and *F*) mice. *G*, histomorphometric analyses of vertebrae in 14-week-old male mice. *ObS/BS*, osteoblast surface/bone surface; *N.Ob/B.Pm*, number of osteoblast per bone perimeter; *OcS/BS*, osteoclast surface/bone surface; *N.Oc/B.Pm*, number of osteoclast per bone perimeter; *BFR/BS*, bone formation rate/bone surface; *MAR*, mineral apposition rate. Values are given as mean ± S.E., *, $p < 0.05$ *versus* vehicle, $n = 8–10$ per group.

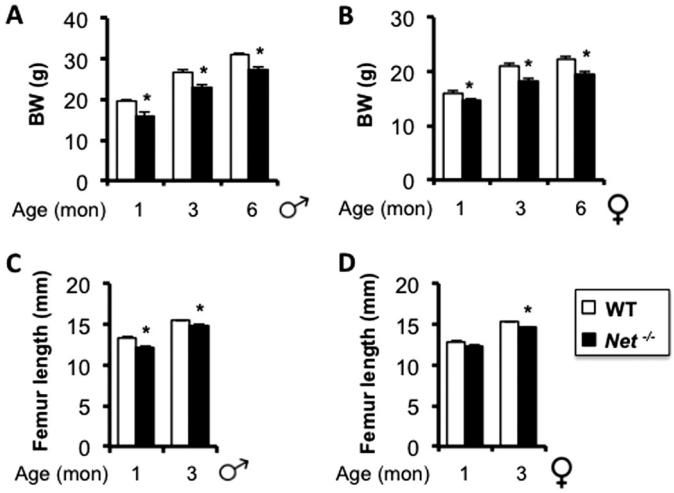


FIGURE 4. ***Net*$^{-/-}$ mice are leaner and shorter than WT littermates.** *A* and *B*, body weight (*BW*); *C* and *D*, femur length. Values are given as mean ± S.E., *, $p < 0.05$ *versus* WT, $n > 8$ per group.

NET Pharmacological Blockade Reduces Bone Mass—To gain insights into the *in vivo* relevance of our *in vitro* findings, we treated WT mice chronically for 8 weeks with reboxetine (15 mg/kg/day), a selective NET blocker, using 28-day releasing subcutaneous mini-osmotic pumps. Pumps were changed once, 28 days following implantation of the first pump. To assess the long-term potency of this chronic treatment, tail suspension tests were performed 21 days following pump implantation, as NET blockade is known to have anti-depressant activity in this behavioral test (20). As expected, a significant reduction in mouse immobility was observed in both male and female reboxetine-treated mice compared with controls (Fig. 3, *A* and *D*) indicating that the drug remained active in the pumps. Two months of chronic reboxetine treatment significantly decreased bone mass in both femurs and lumbar vertebrae (Fig. 3, *B* and *C*), as assessed by three-dimensional micro-computed tomography (μCT) and two-dimensional static and dynamic bone histomorphometric analyses (Fig. 3*G*), but surprisingly did so in males predominantly (Fig. 3, *B*, *C*, *E*, and *F*). Bone formation was significantly decreased in reboxetine-treated males compared with vehicle control mice, as demonstrated by a significant decrease in osteoblast surface/bone surface, bone formation rate/bone surface, and mineral apposition rate (Fig. 3*G*). Reboxetine treatment induced a trend toward increased osteoclast number and a significant increase in urinary DPD/creatinine (an indicator of osteoclast activity) (control, 8.8 ± 0.6 nmol of DPD/mmol of creatinine *versus* reboxetine, 10.8 ± 0.5 nmol of DPD/mmol of creatinine, $p < 0.05$, $n = 9$). These results indicate that pharmacological blockade of NE transport *in vivo* induces deleterious effects on bone mass.

Net Deficiency Results in Low Peak Bone Mass and Weakened Bone Mechanical Properties—The use of reboxetine to investigate the contribution of NE reuptake to bone remodeling has several advantages, including its clinical relevance and absence of developmental disturbances, but also drawbacks, including possible stimulation of other receptors and partial potency. Therefore, we embarked in the characterization of *Net*-deficient (*Net*$^{-/-}$) mice through the analysis of multiple architectural, biomechanical, and histological parameters before and after sexual maturity. *Net*$^{-/-}$ mice weighed less than their wild-type (WT) littermates in both genders (Fig. 4, *A* and *B*). This decrease in body weight was not accompanied by changes in body fat or muscle mass over body weight (Fig. 5, *A–D*). However, a slight but significant reduction in femur length was detected at 3 months of age in male and female *Net*$^{-/-}$ mice compared with WT controls (Fig. 4, *C* and *D*). Distal femoral cancellous bone μCT analyses revealed a significant 15 and 24% decrease in femoral BV/TV in *Net*$^{-/-}$ mice at 3 and 6 months of age, respectively, compared with WT mice. A similar phenotype was observed in lumbar vertebrae, and it reached significance in males only (Fig. 6, *A–D*), similarly to what was observed in reboxetine-treated mice. The femoral mid-shaft of *Net*$^{-/-}$ mice was more slender and the medullary volume was smaller in comparison to WT mice (Table 1, 2). Similar to the trabecular compartment phenotypes, the difference in cortical bone structure (cross-sectional area and moment of inertia) was statistically signif-

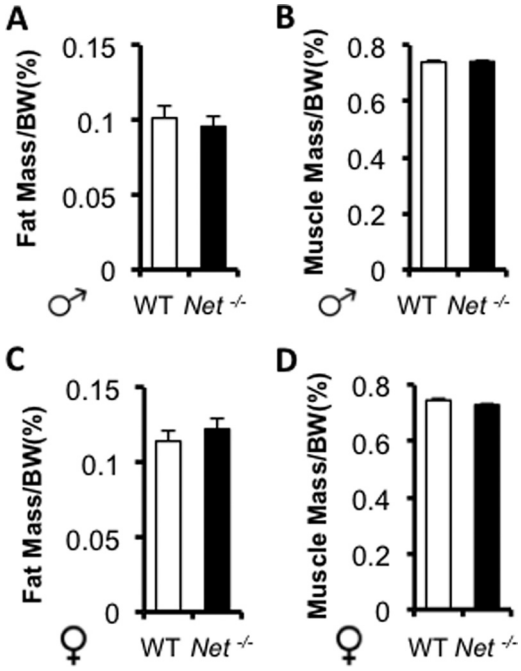


FIGURE 5. **Normal body fat or muscle mass over body weight in 3-month-old *Net*$^{-/-}$ mice.** *A* and *B*, males; *C* and *D*, females. Values are given as mean ± S.E., *, $p < 0.05$ *versus* WT, $n > 8$ per group.

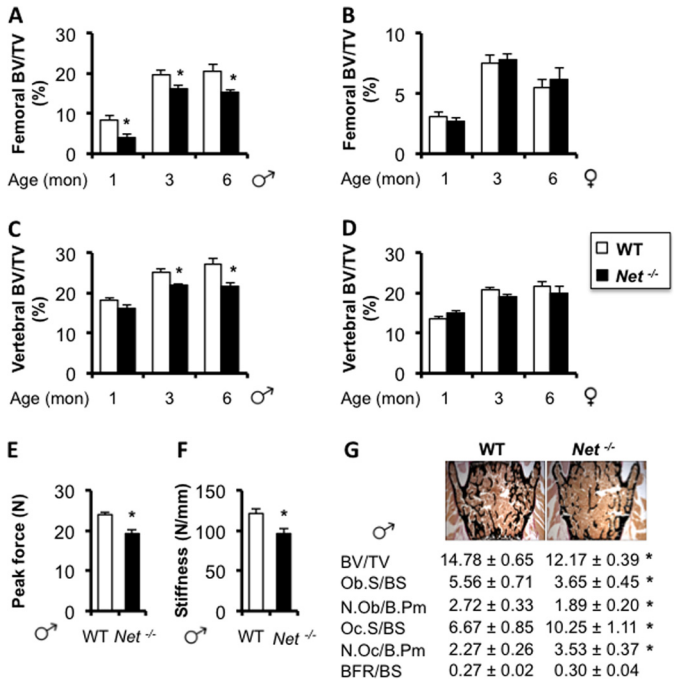


FIGURE 6. **Net deficiency causes a low peak bone mass and reduced bone strength.** Micro-CT analyses of femoral (*A* and *B*) and vertebral (*C* and *D*) trabecular bone in males (*A* and *C*) and females (*B* and *D*) at 1-, 3-, and 6-month-old ages (*BV/TV*: bone volume/tissue volume). Peak force (*E*) and stiffness (*F*) measured by biomechanical three-point bending tests of femurs from 3-month-old males. *G*, histomorphometric analyses of vertebrae in 6-month-old male mice. *ObS/BS* (%), osteoblast surface/bone surface; *N.Ob/B.Pm* (#/mm), number of osteoblast per bone perimeter; *OcS/BS* (%), osteoclast surface/bone surface; *N.Oc/B.Pm* (#/mm), number of osteoclast per bone perimeter; *BFR/BS* (μm/d), bone formation rate/bone surface. Values are given as mean ± S.E., *, $p < 0.05$ *versus* WT, $n = 8$ per group.

icant in males only. Consistent with the alterations in bone structure, biomechanical testing indicated that the femurs from male *Net*$^{-/-}$ mice had weaker mechanical properties than WT littermates, as demonstrated by a significant 20% decrease in bone stiffness and peak force (Fig. 6, *E* and *F*).

TABLE 1
Bone cortical structural properties determined by μCT analysis of the femur mid-shaft from 3-month-old male mice (mean ± S.E., $n \geq 7$)

Property	Units	WT	*Net*$^{-/-}$	*p* value
Ct.Th[a]	mm	0.187 ± 0.003	0.181 ± 0.007	0.425
Ma.V	mm^3	1.42 ± 0.03	1.09 ± 0.03	<0.0001
Ct.Ar	mm^2	0.867 ± 0.018	0.741 ± 0.038	0.006
I_{min}	mm^4	0.151 ± 0.006	0.106 ± 0.008	0.0005
Slenderness	mm^{-1}	7.25 ± 0.2	8.68 ± 0.4	0.004

[a] Ct.Th, cortical thickness; Ma.V, medullary volume; Ct.Ar, bone cross-sectional area; I_{min}, moment of inertia; Slenderness, ratio of femur length to total cross-sectional area (within periosteal perimeter).

TABLE 2
Bone cortical structural properties determined by μCT analysis of the femur mid-shaft from 3-month-old female mice (mean ± S.E., $n \geq 7$)

Property	Units	WT	*Net*$^{-/-}$	*p* value
Ct.Th[a]	mm	0.16 ± 0.014	0.155 ± 0.012	0.442
Ma.V	mm^3	1.08 ± 0.08	0.96 ± 0.09	0.008
Ct.Ar	mm^2	0.646 ± 0.075	0.585 ± 0.054	0.076
I_{min}	mm^4	0.084 ± 0.014	0.073 ± 0.01	0.079
Slenderness	mm^{-1}	9.2 ± 0.66	9.81 ± 0.69	0.083

[a] Ct.Th, cortical thickness; Ma.V, medullary volume; Ct.Ar, bone cross-sectional area; I_{min}, moment of inertia; Slenderness, ratio of femur length to total cross-sectional area (within periosteal perimeter).

In agreement with the μCT results, we observed a significant (17%) reduction in bone volume/tissue volume (BV/TV) in L3/L4 lumbar vertebral bodies of *Net*$^{-/-}$ mice compared with control littermates using two-dimensional bone histomorphometry. Osteoclast surface/bone surface was significantly increased in *Net*$^{-/-}$ mice, whereas osteoblast surface/bone surface was significantly decreased. Osteoclast and osteoblast number/bone perimeter followed the same pattern (Fig. 6*G*). Bone formation rate, however, did not significantly differ between genotypes. These results indicate that NET is necessary for the acquisition of a normal peak bone mass in both appendicular and axial skeletons, and for optimal bone mechanical properties.

Net$^{-/-}$ *Mice Have Reduced Sympathetic Outflow but High Circulating Norepinephrine Levels*—The increased osteoclast and decreased osteoblast number observed in *Net*$^{-/-}$ mice were reminiscent of what had been previously observed upon pharmacological activation of β2AR signaling in mice or rats receiving daily βAR agonist injections (1, 11, 21). These observations suggested that lack of NET leads to overt stimulation of the β2AR expressed in osteoblasts, leading to bone loss. However, independent studies have shown that NE content in nerve terminals in the heart and other sympathetically innervated organs were diminished in *Net*$^{-/-}$ mice, whereas NE serum levels were increased. These studies supported the notion that extracellular NE level increases and intracellular NE is depleted, in response to lack of NE reuptake and reduced repackaging (16). Consistent with these findings, we could detect a significant decrease in *Uncoupling protein 1* (*Ucp1*) mRNA expression (used here as a readout for reduced sympathetic outflow to peripheral tissues) in brown adipose tissue from *Net*$^{-/-}$ mice (Fig. 7, *A* and *B*) and, most importantly, in bone NE content in 3-month-old *Net*$^{-/-}$ males and females compared with WT controls (Fig. 7, *C* and *D*). These results indicate that low sympathetic outflow is not systematically accompanied by a high

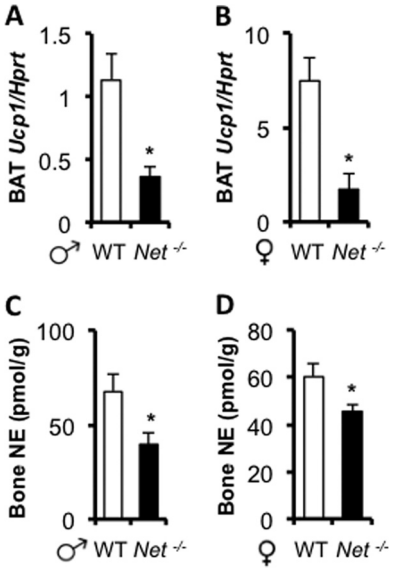


FIGURE 7. **Low sympathetic outflow in *Net*$^{-/-}$ mice.** *A* and *B,* brown adipose tissue (*BAT*) *Ucp1* expression measured by quantitative PCR is decreased in *Net*$^{-/-}$ mice. *C* and *D*, Decreased NE content in *Net*$^{-/-}$ bones. Values are given as mean ± S.E., *, $p < 0.05$ *versus* WT, $n \geq 8$.

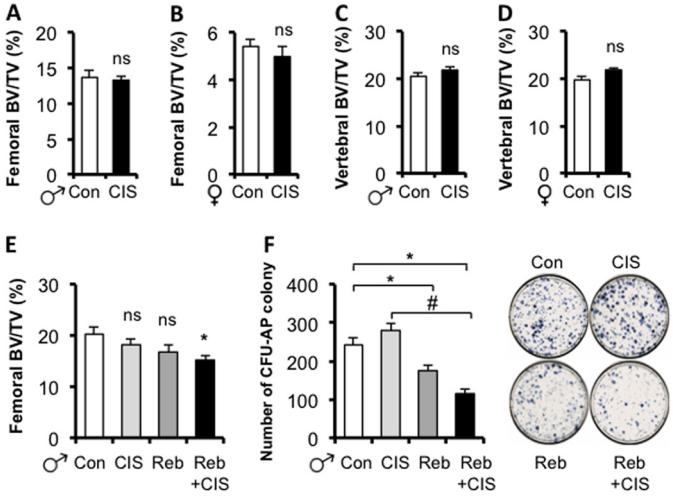


FIGURE 8. **NET blockade is required for chronic immobilization stress-induced bone loss.** *A–D*, CIS does not induce bone loss in 3-month-old WT mice. Micro-CT analyses of femoral (*A* and *B*) and vertebral (*C* and *D*) trabecular bones in males and females. *E,* CIS induces bone loss in male WT mice only when NE reuptake is inhibited. *F,* concurrent CIS and NET blockade reduces the number of bone marrow osteoprogenitors (assessed by the number of Cfu-AP colonies, *right panel*) compared with the control or CIS groups, following 14 days of *in vitro* differentiation in osteogenic condition. Values are given as mean ± S.E., *, $p < 0.05$ *versus* control; #, $p < 0.05$ *versus* CIS, $n \geq 8$.

bone mass phenotype and suggest that the bone loss observed in *Net*$^{-/-}$ mice may stem from a rise in serum NE levels resulting from the general lack of NE reuptake by NET.

Endogenous Sympathetic Activation by Chronic Immobilization Stress Causes Bone Loss Only When NE Reuptake Is Inhibited—Based on the above *in vitro* and *in vivo* observations, we hypothesized that endogenous sympathetic activation may have different repercussions on bone remodeling compared with the administration of exogenous pharmacological βAR agonists (which are not substrates for NET reuptake), and that NE reuptake by differentiated osteoblasts/osteocytes and/or neurons may contribute to the regulation of NE homeostasis and βAR signaling within the skeleton. To address this hypothesis, we subjected 2-month-old WT C57BL6J mice to CIS (daily) for 4 weeks, a time frame long enough to induce bone loss in several conditions such as ovariectomy (22) or in response to isoproterenol treatment (13). CIS has been widely used as a model of transient endogenous sympathetic activation and as an anxiety/depression-like model in mice (23). In contrast to what was observed using daily treatment with βAR agonists, a daily increase in endogenous NE serum levels induced by CIS (24) did not decrease bone volume compared with controls (no CIS), in both femurs and vertebrae, and in both genders (Fig. 8, *A–D*). These data distinguish the effect of exogenous pharmacological βAR agonists from endogenous NE on bone remodeling, and further support the existence of a homeostatic system that protects the skeleton from bone loss induced by sympathetic activation.

To further address whether NET plays a significant role in this homeostatic system, 5-week-old male WT mice were treated chronically with reboxetine, using subcutaneous mini-osmotic pumps 1 week before mice were subjected to CIS. Following 3 weeks of CIS with or without reboxetine infusion, femoral trabecular bone mass was quantified by μCT, and bone marrow stromal cells were extracted and grown under osteogenic conditions to assess the number of osteoprogenitor cells. As observed before, CIS alone did not induce a significant bone loss, nor did the 4-week-long treatment with reboxetine alone. However, NET blockade by reboxetine in mice subjected to CIS led to a significant 25% bone loss compared with vehicle-treated control mice (no CIS), and to a 16% bone loss (trend only) compared with CIS mice (Fig. 8*E*). Similarly, the number of bone marrow osteoprogenitor cells was not affected by CIS, but was significantly reduced upon reboxetine or CIS + reboxetine treatment, compared with control or CIS groups (Fig. 8*F*). These results suggest that concurrent CIS and NET blockade increases adrenergic signaling in bone and further support the notion that NE reuptake by NET is required to conserve a normal bone mass in conditions characterized by chronic sympathetic activation.

DISCUSSION

The regulation of bone remodeling by sympathetic nerves has mainly been investigated by alteration in post-synaptic βAR signaling, pharmacologically or genetically (1, 8, 10–12, 25–33). This study, in contrast, addressed the contribution of *endogenous* sympathetic signaling and NE homeostasis to the control of bone remodeling. We show that differentiated osteoblasts, like sympathetic presynaptic neurons, can transport and catabolize NE, and thus may contribute to NE clearance within the richly vascularized bone marrow microenvironment. In addition, we report that pharmacological NE transport blockade by reboxetine induces bone loss, and that *Net* genetic ablation leads to a low peak bone mass. These findings indicate that the control of NE reuptake by NET is an integral part of the homeostatic system whereby bone remodeling is regulated. Along with independent studies suggesting a role of sympatho-inhibitory presynaptic α_2ARs and endocannabinoids in the control of bone remodeling (4, 7, 34, 35), these findings also

point to the existence of multiple endogenous regulatory pathways modulating bone remodeling via the control of both NE release and clearance. Last, these results suggest that drugs blocking NET activity, which are used for the treatment of depression and ADHD, may have a deleterious effect on the skeleton.

In *Net*$^{-/-}$ mice, the up-regulation of central NE extracellular levels and α_2AR expression, and the subsequent increase in central sympatho-inhibition (36) leads to low sympathetic outflow but increased serum NE levels and low bone mass. Similarly, reduced sympathetic outflow is observed in patients and rats treated with the NET blockers reboxetine or desipramine (37–39). On the other hand, $\alpha_{2A/C}$AR$^{-/-}$ mice have elevated serum NE levels and increased sympathetic outflow associated with a high bone mass (4). The notion that conditions characterized by low sympathetic outflow systematically lead to bone gain is thus not correct. Collectively, these data warrant further investigations to understand how NE homeostasis in the central nervous system (CNS) and in the skeleton control bone accrual and maintenance. The deleterious effect of *Net* deficiency on bone mass indicates that NET is required for normal bone homeostasis and for the acquisition of normal bone size and peak bone mass. However, the global and non-inducible nature of this mouse model hampers the characterization of the precise mechanisms by which *Net* deficiency affects skeletal homeostasis. In addition, it remains unclear whether NET activity in CNS neurons or osteoblasts regulates bone remodeling. A number of observations from the analysis of *Net*$^{-/-}$ mice, however, provide initial mechanistic clues. First, although sympathetic outflow and NE tissue content are low in *Net*$^{-/-}$ mice, serum NE levels are increased due to NE spill over triggered by lack of NET activity (16). It is thus possible that the low bone mass of *Net*$^{-/-}$ mice is caused by increased circulating NE levels, which is in line with the increased bone resorption observed in patients with pheochromocytoma (40) and with the richly vascularized nature of the bone microenvironment. However, the observation that $\alpha_{2A/C}$AR$^{-/-}$ mice exhibit a high bone mass with elevated serum NE levels would argue against a major role of circulating NE in the regulation of bone remodeling, although a bone cell-autonomous role of the $\alpha_{2A/C}$AR suggested by this study or developmental phenotypes might explain the bone phenotype of these mutant mice (4). Second, *Net*$^{-/-}$ mice are characterized by increased central α_2AR signaling (due to central accumulation of NE), low sympathetic outflow, and a low bone mass, whereas $\alpha_{2A/C}$AR$^{-/-}$ mice lacking α_2AR signaling display a high bone mass with increased sympathetic outflow (4). Although these two models are limited by the global nature of the gene deletion that characterizes them, their analysis supports the hypothesis that increased central NE levels and central α_2AR chronic stimulation causes bone loss. The relative contribution of $\alpha_{2A/C}$AR and NET in the CNS *versus* bone cells will thus need to be clarified to select optimal pharmacological αAR andβAR drugs that could increase bone mass. Third, a peripheral and extraneuronal role of NET in bone maintenance is supported by the rich vascularity of the bone microenvironment (hence high blood supply), the uptake of NE by differentiated osteoblasts *in vitro*, and the uptake of a norepinephrine analog in bone in rats *in vivo* (41). To what extent skeletal *versus* CNS or peripheral neuron NET activity contributes to the regulation of bone remodeling remains to be addressed. In addition, the gender specificity (male preferentially) of the bone phenotypes caused by genetic ablation of *Net* or NE transport blockade by reboxetine suggests that the function of NET may be linked to the biology of sexual hormones. This is in line with a number of clinical studies reporting gender differences in response to NET blockade. For instance, NET inhibition results in more pronounced changes in cardiac regulation in men than women (42), and is associated with higher frequency for sexual and genitourinary treatment-emergent adverse events in males than females (43). There is also evidence of gender difference in sympathetic nervous system regulation, which is in part controlled by the activity of NET in the CNS or in the periphery (44–47). Last, whereas administration of exogenous pharmacologic βAR agonists caused bone loss in multiple studies (8, 12, 13), daily endogenous sympathetic activation induced by mild chronic immobilization stress and the associated intermittent increase in sympathetic outflow and catecholamines did not lead to bone loss in nude immunodeficient mice (48) nor in C57BL6 mice (this study). It is only upon NET blockade that CIS and the associated increase in adrenergic signaling could induce bone loss. Collectively, these data are the first to dissociate the effect of exogenous pharmacologic βAR agonists from endogenous NE on bone remodeling and support the existence of homeostatic systems that protect the skeleton from bone loss induced by increased plasma NE levels and/or overt endogenous sympathetic activation.

From a clinical point of view, the inhibitory effect of chronic NET blockade by reboxetine on bone mass observed in this study lends credence to the hypothesis that children prescribed NET blockers may be at risk of reduced bone accrual during development and pubertal growth, suboptimal peak bone mass in young adults and premature fracture upon aging, because the peak bone mass attained in young adulthood is known as a predictor of later adult bone health (49, 50). ADHD is one of the conditions for which children are prescribed NET blockers. The prevalence of ADHD in the United States is 4.4%. It is more common in males (51) and tends to decrease with age (52). The most commonly prescribed medications include amphetamines (*e.g.* Adderall®), methylphenidate (*e.g.* Ritalin® and Concerta®) and atomoxetine (Straterra®), a selective norepinephrine reuptake inhibitor approved by the FDA for the treatment of ADHD in children older than 6 and adults. Atomoxetine is a non-stimulant drug prescribed to patients in case of non-response to other classes of drugs used for the treatment of ADHD, including stimulant drugs. It was put in the United States market in 2002 and possible deleterious effects on the skeleton were not expected and hence not scrutinized. Studies reporting the effect of NET-selective blockers on bone are scarce, include a very limited number of adult patients, and did not detect any significant effect on bone mineral density (53). Observational studies of the effect of NET blockers on fracture risk in patients with ADHD are also challenging as ADHD itself could be associated with increased fracture risk due to the risk-taking behavior associated with ADHD. Although the benefits of NET blockers use to treat ADHD remain undeniable, our

observations support the need for assessing bone parameters in children and adults taking NET blockers.

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