# Depression induces bone loss through stimulation of the sympathetic nervous system

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**Major depression is associated with low bone mass and increased incidence of osteoporotic fractures. However, causality between depression and bone loss has not been established. Here, we show that mice subjected to chronic mild stress (CMS), an established model of depression in rodents, display behavioral depression accompanied by impaired bone mass and structure, as portrayed by decreases in trabecular bone volume density, trabecular number, and trabecular connectivity density assessed in the distal femoral metaphysis and L3 vertebral body. Bone remodeling analysis revealed that the CMS-induced skeletal deficiency is accompanied by restrained bone formation resulting from reduced osteoblast number. Antidepressant therapy, which prevents the behavioral responses to CMS, completely inhibits the decrease in bone formation and markedly attenuates the CMS-induced bone loss. The depression-triggered bone loss is associated with a substantial increase in bone norepinephrine levels and can be blocked by the β-adrenergic antagonist propranolol, suggesting that the sympathetic nervous system mediates the skeletal effects of stress-induced depression. These results define a linkage among depression, excessive adrenergic activity, and reduced bone formation, thus demonstrating an interaction among behavioral responses, the brain, and the skeleton, which leads to impaired bone structure. Together with the common occurrence of depression and bone loss in the aging population, the present data implicate depression as a potential major risk factor for osteoporosis and the associated increase in fracture incidence.**

antidepressant | chronic depression | osteoporosis | bone formation | adrenergic signaling

In vertebrates, bone mass and shape are determined by continuous remodeling, consisting of the concerted occurrence of bone resorption by a specialized cell, the osteoclast, and bone formation by another specialized cell, the osteoblast. Osteoporosis, the most common degenerative disease in developed countries, typically exhibits reduced bone mass resulting from imbalanced bone remodeling with a net increase in bone resorption.

The bone mass status is commonly determined by using bone mineral density (BMD) measurements. Epidemiological studies implicate major depression as one of the most important medical conditions that contribute to reduced BMD and increased incidence of osteoporosis. Several studies, comparing altogether >200 patients with major depression and 240 controls, demonstrate a 6–15% lower BMD in the depressed patients. This decrease, which is indicative of osteoporosis, was found in both depressed women and men (1–4). In most studies, the association between depression and low BMD was reported in patients older than 35–40 years (4–6), an age group targeted by osteoporosis and the associated increase in fracture incidence (7). Indeed, depressed patients have a higher risk of developing osteoporotic fractures (8). However, causality between depression and bone loss has not been defined.

Stressful life events often lead to depression (9, 10). Likewise, controlled studies in both humans and experimental animals suggest that exposure to stressful stimuli induces bone loss (11–14). However, these studies used serological surrogates of bone remodeling, mainly osteocalcin, which reflects the bone remodeling rate rather than the balance between bone formation and resorption or bone structural status. In this study, we sought to establish the skeletal effects of chronic stress-induced depression. Indeed, we demonstrate that chronic mild stress (CMS), an established model for depression in rodents (15), leads to bone loss. Our data further show that CMS selectively inhibits bone formation and that this inhibition is mediated by activation of the sympathetic nervous system (SNS).

## Results

Exposure of mice to CMS resulted in progressive reduction in sucrose preference, which on the 4th week after stress initiation, reached a level <50% compared with untreated controls (Fig. 1*A*). Such a decrease reflects reduced responsiveness to rewards, comparable to anhedonia, one of the core symptoms of major depressive disorder in humans (16).

CMS also reduced the time spent in social exploration (Fig. 1*B*), another behavioral manifestation of depression-like condition in rodents (17). These behavioral changes were accompanied by substantial, generalized impairment of bone mass and structure portrayed by decreases in the trabecular bone volume density (BV/TV) and trabecular number in both the body of the third lumbar vertebra (L3) and secondary spongiosa in the distal femoral metaphysis (Fig. 1 *C* and D). The trabecular connectivity, a measure of the structural integrity of the trabecular network, was also impaired in both skeletal sites (Fig. 1*E*). The CMS-induced skeletal deficiency was accompanied by restrained bone formation rate (Fig. 1*F*) resulting from a reduced mineralizing perimeter (Fig. 1*G*), a surrogate of osteoblast number (18). The mineral appositional rate, a surrogate of osteoblast activity (18), remained unchanged (Fig. 1*H*). The osteoclast number, a surrogate of bone resorption, was slightly and insignificantly increased (Fig. 1*I*). These findings represent imbalanced bone remodeling, whereby reduction in osteoblast number leads to an absolute decrease in bone formation and thus a net increase in bone resoprtion, which, in turn, results in the CMS-induced bone loss.

To further investigate the causal relationship between depression and bone loss, mice subjected to CMS were cotreated with

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Abbreviations: BMD, bone mineral density; BV/TV, trabecular bone volume density; CMS, chronic mild stress; μCT, microcomputed tomography; NE, norepinephrine; SNS, sympathetic nervous system

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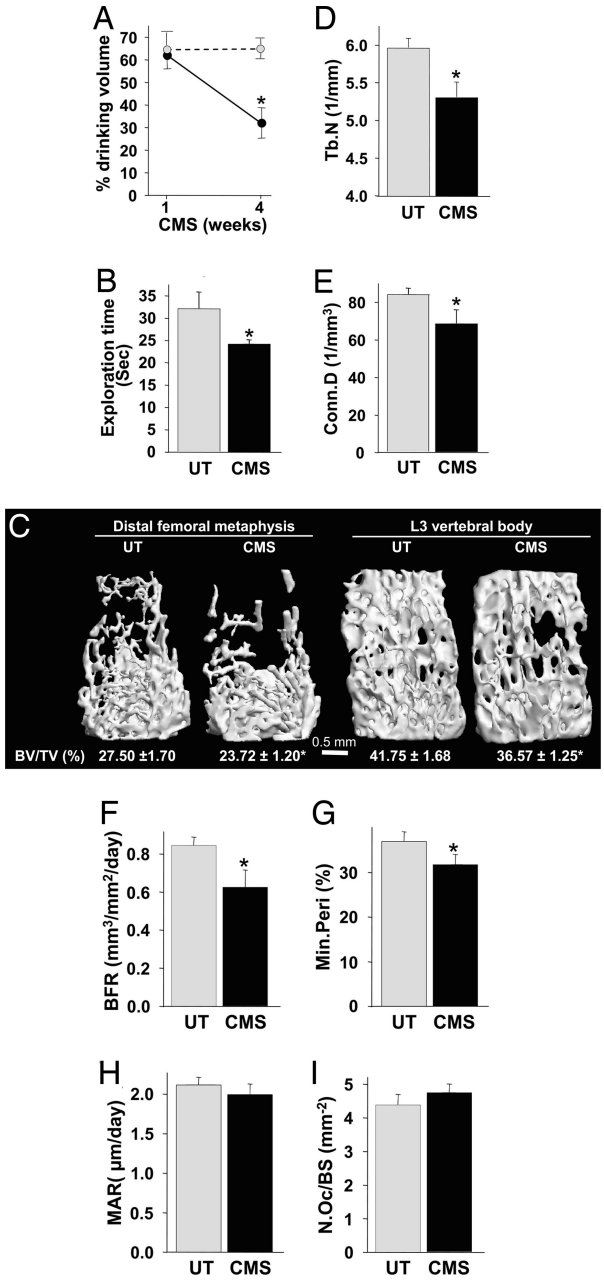


**Fig. 1.** Depressive-like behavior is associated with structural impairment of the skeleton. Mice were exposed to CMS for 4 weeks or left untreated (UT). All parameters were analyzed in the same mice. (*A*) Sucrose preference expressed as percent sucrose solution of the total drinking volume. Dashed line, UT. (*B*) Social exploration expressed as time spent in sniffing, chasing, and crawling over a juvenile conspecific. (*C*) Qualitative (3D) and quantitative $\mu$CT skeletal analyses. Trabecular bone volume density (BV/TV) is expressed as percent volume of trabecular network of total reference volume. (*D*) Trabecular number (Tb.N) per millimeter metaphyseal line. (*E*) Connectivity density (Conn.D) expressed as number of trabecular connections per cubic millimeter of reference volume. (*F*) Bone-formation rate (BFR, surrogate of overall bone formation activity) calculated as numerical product of mineralizing perimeter (Min.Peri) and mineral appositional rate (MAR). (*G*) Min.Peri, surrogate of osteoblast number, defined as percentage of trabecular perimeter with fluorescing calcein label. (*H*) MAR, surrogate of osteoblast activity, defined as the separation between double calcein labels per interlabel time interval. (*I*) Osteoclast number (N.Oc/BS), defined as the number of tartrate-resistant acid phosphatase (TRAP)-positive cells per trabecular perimeter. Quantitative data are the mean ± SEM obtained in 12 mice per condition. *, CMS vs. UT, $P < 0.05$.

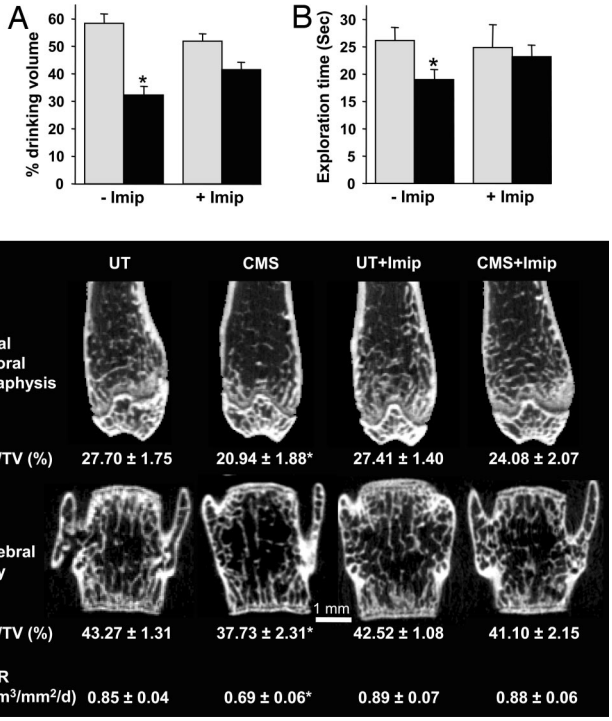


**Fig. 2.** Antidepressant treatment attenuates depressive-like behavior and bone loss. Mice were exposed to CMS for 4 weeks or left untreated (UT), and the anti-depressant drug imipramine (Imip) was administered concomitantly in drinking water to half of the mice in each category. All parameters were analyzed in the same mice. Definition of parameters is as in Fig. 1. (*A*) Sucrose preference. (*B*) Social exploration. (*C*) $\mu$CT analysis of bone volume density and dynamic histomorphometric analysis of bone formation. □, UT; ■, CMS. Data are the mean ± SE obtained in 10–12 mice per condition. *, CMS vs. UT or UT+Imip, or CMS+Imip, $P < 0.05$.

the tricyclic antidepressant drug imipramine. To avoid further stress to the animals, the imipramine treatment was administered in the drinking water. The antidepressant treatment attenuated the CMS-induced decreases in sucrose preference and social exploration, demonstrating alleviation of the stress-induced depression (Fig. 2*A* and *B*). Concomitantly, imipramine also prevented the CMS-induced bone loss, particularly in the vertebrae (Fig. 2*C*). Attenuation of the skeletal structural deterioration by imipramine was accompanied by complete inhibition of the CMS-induced restraint of bone formation, whereas bone resorption remained unaffected (data not shown), indeed confirming that inhibition of bone formation is the main process leading to CMS-induced bone loss. Further inspection of the behavioral data revealed that only half the mice exposed to the combined CMS-imipramine protocol responded to the drug (imipramine responders), displaying sucrose preference similar to that of control untreated mice. The other half displayed significantly reduced sucrose preference (imipramine nonresponders) (Fig. 3*A*). Like the sucrose preference, bone mass in the imipramine responders was similar to that of the untreated controls, whereas the imipramine nonresponders displayed a low bone mass typical of imipramine-free mice exposed to CMS (Fig. 3*B*). Taken together, these data define a cause–effect relationship whereby CMS-induced depression induces bone loss through the inhibition of osteoblast number and bone formation.

Reduced body weight and motility, often associated with low bone mass and depression in humans (19), were unaffected by CMS. Specifically, 4 weeks after the initiation of CMS, body weight in mice exposed to CMS and control animals was 29.9 ± 0.97 and 28.6 ± 0.63 g (mean ± SEM), respectively ($P > 0.1$).

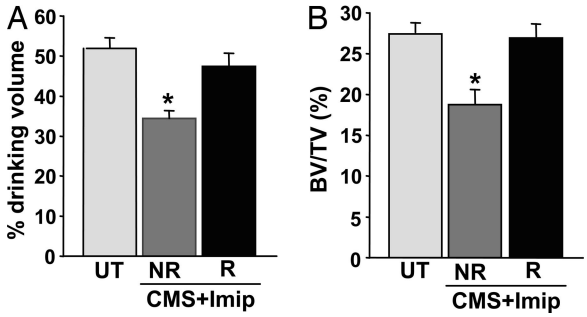


**Fig. 3.** Behavioral and skeletal responsiveness to anti-depressant treatment. Mice exposed to concomitant CMS and imipramine treatment for 4 weeks were divided to imipramine nonresponders (NR) and responders (R) based on their sucrose consumption. (*A*) Sucrose preference. (*B*) Trabecular bone volume density (BV/TV). Data are the mean ± SEM obtained in 11 untreated (UT), 5 CMS NR, and 6 R mice. *, NR vs. UT or R, $P < 0.05$.

The respective locomotor activity (number of line crossings in the open-field test) was 162.5 ± 9.3 and 149.9 ± 8.3 (mean ± SEM) ($P > 0.1$). Sexual activity and sex hormone secretion are reduced in depressed subjects (20), and sex hormones depletion is a major cause of bone loss (21). Hence, to elucidate the pathways mediating the skeletal effect of stress-induced depression, we initially measured serum testosterone levels, which remained unaltered in mice exposed to CMS as compared with nonstressed controls (Fig. 4*A*). Thus, decreased sex hormone levels are probably not involved in the CMS-induced bone loss. Corticosteroids could also mediate the effect of CMS on bone, because they are elevated in stress and depression and induce osteoporosis secondary to their inhibition of bone formation (22). Corticosteroid blood level is elevated in depressed osteoporotic patients (5, 6) and in stressed experimental animals with reduced bone turnover (12, 13). Indeed, the serum corticosterone level was significantly higher in mice subjected to CMS (Fig. 4*B*). To substantiate a role of glucocorticoids in CMS-induced bone loss, we evaluated the effects of CMS in adrenalectomized (ADX) mice. These mice showed neither CMS-induced bone loss nor an influence of CMS on sucrose preference (see Table 2, which is published as supporting information on the PNAS web site). Hence, although elevated levels of glucocorticoids may have an important role in CMS-induced depression, it is currently impossible to implicate them as direct mediators of the resultant bone loss.

Several lines of evidence suggest that inflammatory cytokines, such as IL-1 and IL-6 could be involved in mediating the effect of CMS on bone. We have recently reported that central IL-1

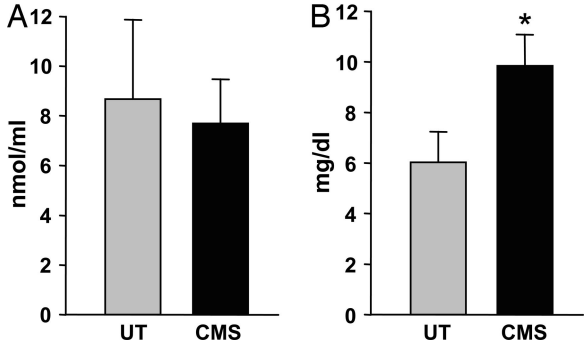


**Fig. 4.** Serum levels of bone mass-regulating steroid hormones in mice exposed to CMS for 4 weeks or left untreated (UT). (*A*) Testosterone. (*B*) Corticosterone. Data are the mean ± SEM obtained in 11 mice per condition. *, CMS vs. UT, $P < 0.05$.

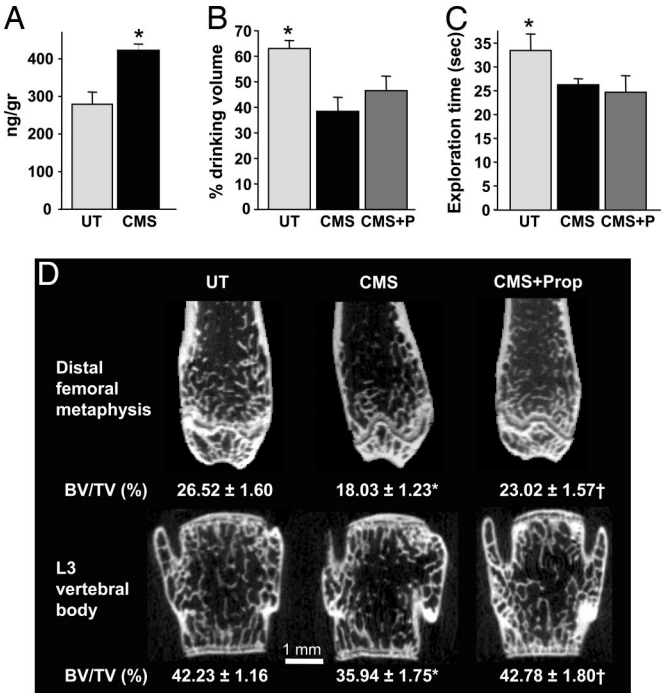


**Fig. 5.** The SNS mediates depression-induced bone loss. Mice were exposed to CMS for 4 weeks or left untreated (UT). (*A*) Norepinephrine (NE) level in trabecular bone from pooled distal femoral and proximal tibial metaphyses normalized on a per-wet-tissue basis. Data are the mean ± SEM obtained in 14 mice per condition. *, CMS vs. UT, $P < 0.05$. (*B–D*) Mice were exposed to CMS or left untreated (UT), and the β-adrenergic antagonist propranolol was administered concomitantly in the drinking water to half of the CMS mice. All parameters were analyzed in the same mice. (*B*) Sucrose preference. (*C*) Social exploration. (*D*) μCT analysis. Definition of parameters in *B–D* is as in Fig. 1. Data are the mean ± SEM obtained in 10 or 11 mice per condition. *, UT vs. CMS or CMS+P; CMS vs. UT or CMS+P, $P < 0.05$.

signaling regulates bone mass (23). However, as in the case of ADX mice, animals deficient in IL-1 receptor type I (IL-1rKO mice) do not show significant changes in sucrose preference or bone density in response to CMS (Table 2). The blood levels of IL-6 are markedly elevated in acute stressful conditions (24) and in depressed patients (25). In addition, IL-6 stimulates bone resorption and bone loss, primarily through activation of osteoclasts and its production and actions are regulated by calciotropic hormones, such as sex steroids, parathyroid hormone, and vitamin D3 (26). In this study, blood IL-6 levels in untreated mice and animals exposed to CMS were 29.9 ± 11.9 and 39.2 ± 7.5 ng/ml (mean ± SEM), respectively ($P > 0.1$), suggesting an insignificant role for IL-6 in mediating the CMS-induced bone loss.

Recently, the SNS has been implicated in the regulation of bone formation and bone mass through β2-adrenergic receptors expressed in osteoblasts (27, 28). Therefore, it could be another candidate pathway for mediating the effects of stress-induced depression on bone mass. In fact, in our CMS model, the trabecular bone level of norepinephrine (NE), the major neurotransmitter released from sympathetic nerve endings, was markedly increased (Fig. 5*A*). Hence, we further tested the effect of the β-blocker propranolol (provided in the drinking water) on CMS-induced bone loss. The propranolol cotreatment had no effect on the behavioral parameters of depression (Fig. 5 *B* and *C*). By contrast, propranolol administration to the chronically stressed mice markedly attenuated the deficits in bone mass and structure in these animals (Fig. 5*D* and Table 1) and prevented the CMS-induced inhibition of bone formation (Fig. 6 *A–C*).

**Table 1. Prevention of CMS-induced decrease in trabecular bone structural parameters by the β-adrenergic antagonist propranolol**

| | Distal femoral metaphysis | | | L3 vertebral body | | |
|---|---|---|---|---|---|---|
| Parameter | UT | CMS | CMS+P | UT | CMS | CMS+P |
| Tb.N (1/mm) | 4.71 ± 0.19 | 3.85 ± 0.24* | 4.48 ± 0.18 | 5.83 ± 0.11 | 5.26 ± 0.19* | 5.91 ± 0.11 |
| Conn.D (1/mm$^3$) | 49.50 ± 4.44 | 30.61 ± 2.75* | 48.50 ± 3.48 | 82.89 ± 4.33 | 86.09 ± 4.15 | 92.47 ± 5.61 |

Mice were exposed to CMS for 4 weeks or left untreated (UT), and the β-adrenergic antagonist propranolol (P) was administered concomitantly in the drinking water to half of the CMS mice. Trabecular number density (Tb.N) and connectivity density (Conn.D) were determined by quantitative μCT analysis. Data are the mean ± SE obtained in 10 or 11 mice per condition. *, CMS vs. UT or CMS+P, $P < 0.05$.

Propranolol had no effect on the osteoclast number (Fig. 6*D*). Thus, propranolol, a β-adrenergic antagonist, disrupts the pathway communicating the CMS-induced brain-to-bone signals.

## Discussion

This study demonstrates that experimental depression, induced by CMS, causes bone loss and impairment of bone architecture. The causative role of depression in skeletal deterioration is validated by showing that chronic antidepressant treatment, which attenuates the depressive-like behavior, also prevents the skeletal deterioration. Our data further assign a key role to the SNS in mediating the depression-induced skeletal impairment.

Bone formation and resorption are controlled by local and systemic endocrine systems, mainly gonadal hormones (21). Depression and stress inhibit the secretion of gonadal hormones (20). In particular, exposure to various chronic stressors, such as immobilization, prolonged exercise, contrast illumination, forced swimming in cold water, noise, surgery, crowding, and social stress in rats as well as intense exercise, military training, or sleep deprivation in humans, produce a general inhibitory effect on the hypothalamic–pituitary–gonadal axis, reflected by decreased serum testosterone levels (29–31). Therefore, testosterone could be involved in mediating the detrimental effects of chronic stress on the bones. However, the depressed mice had a normal testosterone blood level, suggesting that the magnitude of depression produced by CMS is insufficient to inhibit sex hormone secretion, thus excluding the involvement of these hormones in CMS-induced bone loss. Several other central systems, such as central IL-1 (23), have been recently portrayed as powerful regulators of bone remodeling. Nevertheless, our attempts to assess the specific role of central IL-1 in the depression-induced bone loss were unsuccessful because IL-1rKO mice were resistant to CMS, namely, they did not exhibit CMS-induced decreases in depressive or skeletal parameters. In addition, it has been recently suggested that depression-induced elevations in the serum levels of another cytokine, IL-6, comprise a risk factor for osteoporosis (25, 32). However, this suggestion is not supported by the present findings, which suggest that an increase in blood IL-6 levels is not critically involved in CMS-induced bone loss.

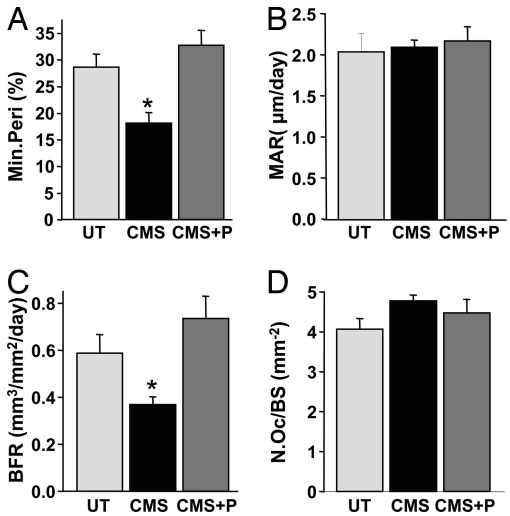


**Fig. 6.** The SNS mediates depression-induced alterations in bone remodeling. Mice were exposed to CMS for 4 weeks or left untreated (UT), and the β-adrenergic antagonist propranolol was administered concomitantly in the drinking water to half of the CMS mice. (*A*) Mineralizing perimeter. (*B*) Mineral appositional rate. (*C*) Bone formation rate. (*D*) Osteoclast number. (*A–D*) Parameters are as in Fig. 1. Data are the mean ± SEM obtained in 10 or 11 mice per condition. *, CMS vs. UT or CMS+P, $P < 0.05$.

As in many other instances of centrally regulated peripheral tissue activity, the SNS forms a pivotal link between the CNS and bone. Bone, in particular trabecular bone, is densely innervated by sympathetic fibers (36–38), and genetic ablation of adrenergic signaling leads to a high bone mass (27, 28). Like the CMS-induced depression, chronic SNS activation and sympathetic agonists inhibit bone formation mainly by decreasing osteoblast number. Conversely, sympathetic antagonists rescue bone loss by increasing osteoblast number (27, 28). Indeed, we show that the depression-like behavior and decreases in osteoblast number, bone formation, and bone mass are associated with a marked increase in the bone NE level. This finding, as well as the disruption by a β-blocker of the CMS-induced signals to the skeleton, portray the SNS as the main link transmitting depressive signals from the CNS to bone.

Recently, several mediators of brain-to-bone communication have been identified, such as NE, neuropeptide Y, CART, central IL-1, and endocannabinoids (23, 27, 28, 39–41). Linking the SNS to depression-induced bone loss, the present study constitutes a further step, adding a psychological factor to this system and setting the foundation for a discipline that we call ''neuropsychosteology,'' which is aimed at elucidating the interactions among the brain, behavior, and the skeleton. Establishing a link among depression, excessive sympathetic activation, and impaired skeletal structure is of key importance because these conditions characterize menopause and aging (42–45).

Moreover, these findings implicate depression as a potential, major risk factor for osteoporosis and the associated increase in fracture incidence. These findings also demonstrate the feasibility of concomitantly combating age-related depression and bone loss with antidepressant agents. These agents have been used in the clinic without major deleterious effects, and, therefore, such trials may become a reality in the near future.

## Materials and Methods

**Animals.** Male 129/Sv × C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were used throughout the study. The mice were 12 weeks old in the beginning of exposure to CMS. Imipramine (Sigma, St. Louis, MO) at 10 mg/kg/day or propranolol (Sigma) at 0.4 mg/day were provided in the drinking water. The number of animals was 10–12 per group. Newly formed bone was vitally double labeled with calcein (Sigma) before sacrifice as reported recently (23, 41). The experiments were approved by the Hebrew University Committee of Animal Care and Use.

**CMS.** Mice were subjected every week, over a period of 4–5 weeks, to a regimen of stressors as reported (15). Briefly, the regimen consisted of: 16-h period of water deprivation, two periods of continuous overnight illumination, two 3-h periods of 45° cage tilt, a 17-h period in a soiled cage, two 3-h periods of noise stress, and three 10-min periods of stroboscopic illumination. Nonstressed, control mice were housed in a separate room with food and water ad libitum. At the end of the 1st and 4th weeks after the initiation of CMS, the mice underwent the sucrose-preference test (17). Each mouse was placed in a measuring cage for 4 h and provided with two graduated tubes containing either 2% sucrose solution or water. Sucrose preference was defined as the percentage volume of sucrose solution consumed from the total drinking volume. One day after the last sucrose-preference test, animals were tested for social exploration. Each mouse was placed in a measuring cage and allowed to habituate for 10 min. A male conspecific juvenile was introduced into the cage for 3 min, and the time spent by the mouse in sniffing, chasing, and crawling over the juvenile was measured with a computerized event recorder using an in-house-developed software, which allows on-line recording of the incidence and duration of each behavioral component by an observer who is blind with respect to the group of the animal (46).

**Microcomputed Tomographic ($\mu$CT) Analysis.** Femora (one per mouse) and third lumbar vertebrae (L3) were fixed in phosphate-buffered formalin for 48 h and further kept at 70% ethanol. They were examined by a $\mu$CT system (Desktop $\mu$CT 40; Scanco Medical, Bassersdorf, Switzerland) as reported (41). Briefly, scans were performed at a 20-$\mu$m resolution in all three spatial dimensions. The mineralized tissues were differentially segmented by a global thresholding procedure (47). The following morphometric parameters were determined by using a direct 3D approach: BV/TV, trabecular thickness (Tb.Th), trabecular number and trabecular connectivity density (Conn.D).

**Histomorphometry.** After $\mu$CT image acquisition, the same femora were embedded undecalcified in polymethylmethacrylate (Technovit 9100; Heraeus Kulzer, Wehrheim, Germany) (23, 41). Undeplasticized 5-$\mu$m sections were left unstained for dynamic histomorphometric measurements. To identify osteoclasts, consecutive sections were deplasticized and stained for tartrate-resistant acid phosphatase (TRAP) by using an acid phosphatase kit (Sigma) and counterstained with Mayer's hematoxylin. The following parameters were determined according to the convention of standardized nomenclature (18): mineral appositional rate, mineralizing perimeter, bone-formation rate, and osteoclast number).

**Serum Testosterone Measurements.** Collection of blood samples and RIA were carried out as described (48). The sensitivity limit of the testosterone assay was 3 fmol per tube. The interassay and intraassay CV were 9.8 and 5.9%, respectively.

**Serum Corticosterone Determination.** Measurements in peripheral blood were carried out by RIA as described (49). The sensitivity limit of the assay was 0.5 mg/100 ml, and the intra- and interassay coefficients of variation were 6.4% and 7.2%, respectively.

**Serum IL-6 Determination.** IL-6 was assessed with a mouse IL-6 ELISA kit (R & D Systems, Minneapolis, MN). The reported detection limit of this assay is 1.6 pg/ml, the recovery of exogenous IL-6 is 99%, and intra-and interassay coefficients of variation were $<$7.0% and 8.9%, respectively.

**Bone NE Determination.** NE was determined in pools of one distal femoral and one proximal tibial metaphysis from each mouse as reported (50). In short, after separation, the metaphyses were weighed and homogenized in 1 ml of extraction buffer containing 0.1 mM sodium metabisulfide ($Na_2S_2O_5$) and 20 ng 3,4-dihydroxybenzylamine (DHBA) as an internal standard. The homogenates were centrifuged, and the supernatant was transferred to a 10 mM $Na_2S_2O_5$ solution containing acid-washed alumina with subsequent addition of 1 ml of 1 M Trizma Base containing 2% EDTA. The samples were vortexed and centrifuged, and the supernatant was discarded. The alumina was then washed with water, and the NE was desorbed from the alumina with 0.1 M perchloric acid containing 0.1 mM $Na._2S_2O_5$. Further NE measurement was carried out by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) using a 15-cm ion-pair chromatography column (NovaPak C18; Waters, Milford, MA). The column was eluted with 6% methanol in aqueous solution containing 0.07 M disodium hydrogen orthophosphate, 0.2 mM EDTA, and 3 mM heptanosulphonate. The flow rate was 0.7 ml/min, with electrochemical detector sensitivity at 50 nA and applied potential of 0.7 V. Measurements were made by using the internal DHBA standard as reference.

**Statistical Analysis.** Differences between animals subjected to CMS and untreated controls were analyzed by *t* test. Two-way ANOVAs were used to analyze the effects of imipramine and propranolol on CMS-induced behavioral depression and bone parameters. When significant differences were indicated by ANOVA, group means were compared by using the Fisher's LSD post hoc test for pair-wise comparisons.

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