The cannabinoid CB1 receptor regulates bone formation by modulating adrenergic signaling

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ABSTRACT **We have recently reported that in bone the cannabinoid CB1 receptor is present in sympathetic terminals. Here we show that traumatic brain injury (TBI), which in humans enhances peripheral osteogenesis and fracture healing, acutely stimulates bone formation in a distant skeletal site. At this site we demonstrate *i*) a high level of the main endocannabinoid, 2-arachidonoylglycerol (2-AG), and expression of diacylglycerol lipases, enzymes essential for 2-AG synthesis; *ii*) that the TBI-induced increase in bone formation is preceded by elevation of the 2-AG and a decrease in norepinephrine (NE) levels. The TBI stimulation of bone formation was absent in CB1-null mice. In wild-type animals it could be mimicked, including the suppression of NE levels, by 2-AG administration. The TBI- and 2-AG-induced stimulation of osteogenesis was restrained by the β-adrenergic receptor agonist isoproterenol. NE from sympathetic terminals is known to tonically inhibit bone formation by activating osteoblastic β2-adrenergic receptors. The present findings further demonstrate that the sympathetic control of bone formation is regulated through 2-AG activation of prejunctional CB1. Elevation of bone 2-AG apparently suppresses NE release from bone sympathetic terminals, thus alleviating the inhibition of bone formation. The involvement of osteoblastic CB2 signaling in this process is minimal, if any.—Tam, J., Trembovler, V., Di Marzo, V., Petrosino, S., Leo, G., Alexandrovich, A., Regev, E., Casap, N., Shteyer, A., Ledent, C., Karsak, M., Zimmer, A., Mechoulam, R., Yirmiya, R., Shohami, E., Bab, I. The cannabinoid CB1 receptor regulates bone formation by modulating adrenergic signaling. *FASEB J.* 22, 285–294 (2008)**

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IN VERTEBRATES, BONE MASS AND SHAPE are determined by continuous remodeling that consists of the concerted and balanced action of osteoblasts, cells that form bone, and osteoclasts, cells that resorb bone (1). The majority of bone diseases, such as osteoporosis, osteopetrosis, and osteosclerosis result from impaired remodeling balance. Bone remodeling, in turn, is subject to central control mainly through the sympathetic nervous system (2, 3), which is partially regulated by the endocannabinoid system (4, 5).

The endogenous cannabinoids bind to and activate the CB1 and CB2 cannabinoid receptors. Both are seven-transmembrane domain receptors that share 44% homology. They are coupled to the Gi/o subclass of G-proteins and inhibit stimulated adenylyl cyclase activity (6). CB1 is present mainly in the brain and in peripheral nerves and accounts for most of the central nervous system actions of cannabinoid drugs and endocannabinoids (7, 8). CB2 is predominantly expressed in peripheral tissues (9–11). The endocannabinoid system, through the CB1 and CB2 receptors, has been recently implicated in the regulation of bone remodeling and bone mass (12–15). Whereas CB2 is abundantly expressed in osteoblasts and osteoclasts, stimulating bone formation and inhibiting bone resorption, CB1 expression is extremely low in bone cells (12–15). Nevertheless, CB1-deficient mice, at least those with a C57BL/6J background have low bone density, in part because of a substantial decrease in their bone formation. This decrease is explainable by the absence of CB1 receptors normally expressed in bone sympathetic nerve fibers (15).

A direct clinical evidence for the central control of bone formation is the highly consistent observation of increased osteogenesis in patients with traumatic brain injury (TBI), which leads to heterotopic ossification (HO) and enhanced fracture healing mainly in the appendicular skeleton (16–18). However, the pathways mediating the TBI-induced increase in bone formation

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are not fully understood. In mice, TBI acutely stimulates central production of the major endocannabinoid 2-arachidonoylglycerol (2-AG) (19), which is physiologically present both in the central nervous system and in peripheral tissues (20, 21). In sympathetic nerve terminals, prejunctional activation of CB1 by 2-AG inhibits the release of norepinephrine (NE) (4, 5). In bone, NE derived from these terminals activates osteoblastic β2-adrenergic receptors (β2AR) and tonically inhibits bone formation (2). We have, therefore, hypothesized that the sympathetic CB1 in bone stimulates osteoblast activity by restraining NE release, thus alleviating the NE inhibition of bone formation.

To test this hypothesis, we established a mouse model in which controlled TBI induces acute stimulation of bone formation measured in trabecular bone in the distal femoral metaphysis. We also demonstrate skeletal 2-AG production and further show the absence of TBI-stimulated bone formation in mice with a CB1 null-mutation. CB2 null mice respond normally to TBI. The TBI-induced stimulation of bone formation was preceded by an increase in the metaphyseal 2-AG level and a decrease in NE. The TBI-induced inhibition of bone NE levels and enhancement of bone formation were mimicked by exogenously administered 2-AG and was prevented by pretreatment with the β-adrenergic receptor agonist, isoproterenol (ISO).

MATERIALS AND METHODS

Animals

All animals in the study were 9- to 12-week-old mice. Mice with a deletion of the *CNR2* gene (*CB2*$^{-/-}$ mice) were crossed for 10 generations to wild-type C57BL/6J mice to generate a congenic C57BL/6J CB2$^{-/-}$ strain (22). Two mutant mouse lines with deficiency in the *CNR1* gene (*CB1*$^{-/-}$ mice) were used. In one line, backcrossed for at least 10 generations to C57BL/6J mice (C57$^{CB1-/-}$), almost the entire protein-encoding sequence was removed (8). In the other line, backcrossed for 17 generations to CD1 mice (CD1$^{CB1-/-}$), the N-terminal 233 codons of *CB1* were ablated (23). The skeletal phenotype of these mice has been reported recently (15). The use of animals was approved by the Institutional Animal Care Committee of the Hebrew University of Jerusalem and by the Bezirksregierung Köln.

Traumatic brain injury

TBI was initially induced in male C3H mice (Harlan Laboratories, Jerusalem, Israel) using the closed head injury (CHI) model developed and modified in our laboratory (24, 25). Briefly, the skull was exposed by a midline incision under isofluorane anesthesia. A Teflon™ cone, 2-mm diameter, was placed ~1 mm left of the midline on the anterior frontal area. The head was fixed; the skin was retracted; and a 75 g weight was dropped from 12 cm height, which resulted in a focal injury to the left hemisphere. Sham controls received anesthesia and skin incision only. One hour after injury the animals were subjected to neurological severity score (NSS) test (26). Animals with NSS <6 or NSS >9 were excluded from further analyses. Experiments involving genetically modified mice were carried out in female mice. In each case the WT and background strain was matched.

mRNA analysis

MC3T3 E1 osteoblastic cells, newborn mouse calvarial osteoblasts (NeMCO), and osteoclast-like cells generated from the RAW 264.7 monocytic cell line in the presence of RANK ligand (RANKL) were cultured as reported previously (14). Total RNA was extracted from the cells, purified, and reverse-transcribed using routine procedures. The following primers were used for PCR: β-actin, sense, 5′-GAGACCTTCAACACCCCAGCC-3′; antisense, 5′- GGCCATCTCTTGCTCGAAGTC-3′. Diacylglycerol lipase (DAGL) α, sense, 5′-GGAGAACCCGTCCCTTAGC-3′; antisense, 5′-GCCCGAAGCAAGATTTCCT-3′. DAGLβ, sense, 5′-CAAGTCCGTGCTTATGCCT-3′; antisense, 5′-CGGGGAGTATCGGGTTAGA-3′.

Immunohistochemistry

Mice were killed by transcardial perfusion of phosphate buffered saline followed by 4% parafomaldehyde. The femora were dissected and further fixed with parafomaldehyde for 2 h at 4°C. The specimens were decalcified in 0.5 M EDTA and sections were reacted with anti-DAGLα and anti-DAGLβ antibodies kindly provided by Dr. Ken Mackie (University of Washington, Seattle, WA, USA). Further processing was carried out using the *SuperPicture*™ Polymer detection Kit (Zymed Laboratories, San Francisco, CA, USA; cat no. 87–9263) according to the manufacturer's instructions.

Ligand administration

2-AG at 10 mg/kg was injected intraperitoneally (i.p.) once as ethanol/emulphor/saline (1:1:18) solution to normal C57BL/6J and mutant *CB1*$^{-/-}$ and *CB2*$^{-/-}$ mice. This dose was selected based on an initial dose-response analysis, which showed that the stimulation of bone formation rate by 2-AG reaches a plateau at 10 mg/kg. ISO at 6 μg/g/day was administered i.p. during the 10 days preceding TBI or 2-AG injection as reported previously (27).

Histomorphometry

To study bone formation, newly formed bone was vitally labeled by the fluorochrome calcein (Sigma-Aldrich, Rechovot, Israel), 15 mg/kg intraperitoneally. In experiments designed to measure bone formation up to 8 days post-TBI, the animals received the calcein flurochrome four days and one day prior to sacrifice. In experiments measuring bone formation 24 h after TBI the first calcein dose was administered one day prior to TBI and the second one day thereafter. In all experiments the animals were sacrificed 24 h after the second calcein injection.

Whole femora were processed undecalcified as reported previously (15). Longitudinal sections through the midfrontal plane were left unstained for dynamic histomorphometry based on the vital calcein double-labeling. To identify osteoclasts, consecutive sections were stained for TRAP. Dynamic histomorphometric parameters and osteoclast counts were determined in the distal femoral metaphysis according to a standardized nomenclature (28).

Endocannabinoid determination

Bone 2-AG was measured in distal femoral and proximal tibial metaphyseal pools. Specimens were frozen in liquid nitrogen immediately after dissection and then stored at −80°C until used.

Tissue extracts in chloroform/methanol/Tris-HCl, containing d5–2-AG (Cayman Chemicals, Ann Arbor, MI, USA)

as internal standards were processed and analyzed by liquid chromatography-atmospheric pressure chemical ionisation-mass spectrometry (LC-APCI-MS) as reported previously (29).

Bone norepinephrine determination

NE was also determined in pools of distal femoral and proximal tibial metaphyses by using a modification of a previously published method (30). In short, following separation, the metaphyses were weighed and homogenized in 1 ml extraction buffer containing 0.1 mM sodium metabisulfide and 20 ng 3,4-dihydroxybenzylamine (DHBA) as an internal standard. The homogenates were centrifuged, and the supernatant was transferred to 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 were desorbed from the alumina with 0.1 M perchloric acid containing 0.1 mM $Na._2S_2O_5$. Further NE measurement was performed by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) by using a 15 cm ion-pair chromatography column (NovaPak C18; Waters Corporation, Milford, MA, USA). 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 using the internal DHBA standard as reference.

Leptin determination

Blood was obtained by cardiac puncture, and serum leptin determined by ELISA using a commercially available kit (Linco Research Co., St. Charles, MO, USA; cat. no. EZML-82K) according to the manufacturer's instructions.

Testing of 2-AG in bone cell cultures

2-AG was studied for possible effects on cell growth and activity in the MC3T3 E1 osteoblastic cell line and primary cultures of newborn mouse calvarial osteoblasts (NeMCO) according to previously reported protocols (14). Briefly, the cells were initially incubated in osteogenic medium for 10–12 days, to allow for sufficient CB2 expression, followed by 2 h serum starvation. Ligands were dissolved in DMSO and further diluted to their final concentration by using tissue-culture medium. Cell counts and alkaline phosphatase activity were determined after 48-h incubation in α-MEM supplemented with 4% BSA and ligands.

Statistics

Analyses were carried out using SigmaStat software (SPSS Science, Chicago, IL, USA). Multiple comparisons in the case of multiple time and/or treatment groups were assessed by using analysis of variance (ANOVA). When significant differences were indicated by ANOVA, group means were compared using the Student-Newman-Keuls posthoc method for multiple comparisons. We used the Student's *t* test when two groups were compared. Regression analysis was employed to assess the effect of 2-AG on osteoblasts in culture.

RESULTS

Stimulation of peripheral bone formation by TBI

Using the CHI as a mouse model for TBI (24), we demonstrate that acute brain damage induces an increase in peripheral bone formation. On day 2 after TBI in C3H males the bone formation rate (BFR) in the distal femoral metaphyseal trabecular compartment was 20% higher compared to untreated controls. Thereafter, it declined to resume baseline level on day 6 (**Fig. 1*A***). Bone formation is modulated by changes in the number of osteoblasts and/or in their activity. Here, the increase in BFR reflected stimulation of mineral appositional rate (MAR) (Fig. 1*B*), a surrogate of osteoblast activity (28), whereas the mineralizing perimeter (Min.Peri), a surrogate of osteoblast number (28) remained unchanged (Fig. 1*C*). Such a rapid bone formation response is apparently consistent with the stimulation of mature osteoblast activity, rather than an increase in osteoblasts number, which comprises a

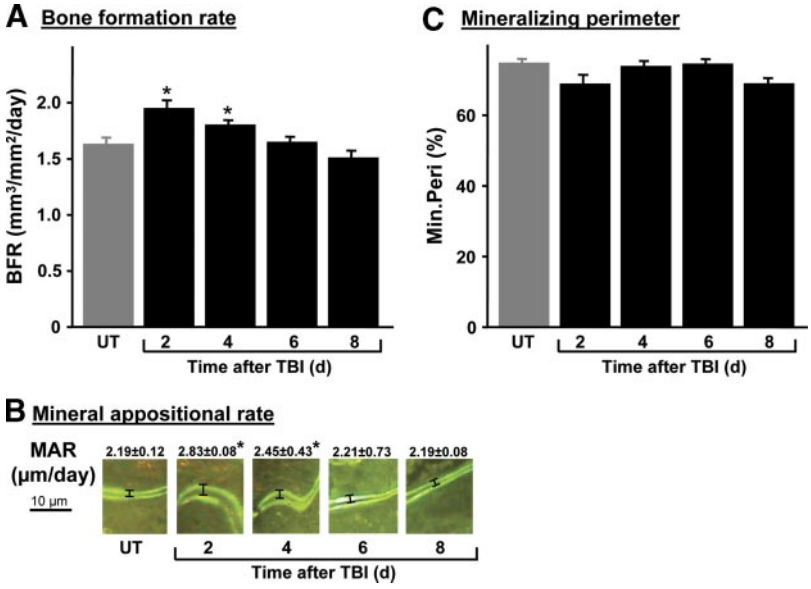

Figure 1. TBI stimulates femoral trabecular bone formation. Analysis of bone formation in distal femoral metaphysis of male C3H mice using vital fluorescent calcein staining. *A)* Bone formation rate (BFR) per unit surface area of bone trabeculae. *B)* Representative fluorescent micrographs of calcein label separation from mice with median levels of mineral appositional rate (MAR), surrogate of osteoblast activity. *C)* Mineralizing perimeter (Min. Peri, percent calcein positive trabecular surfaces), surrogate of osteoblast number. UT, untreated control mice. Quantitative data are mean ± SE from 5–10 mice per condition. $*P < 0.05$.

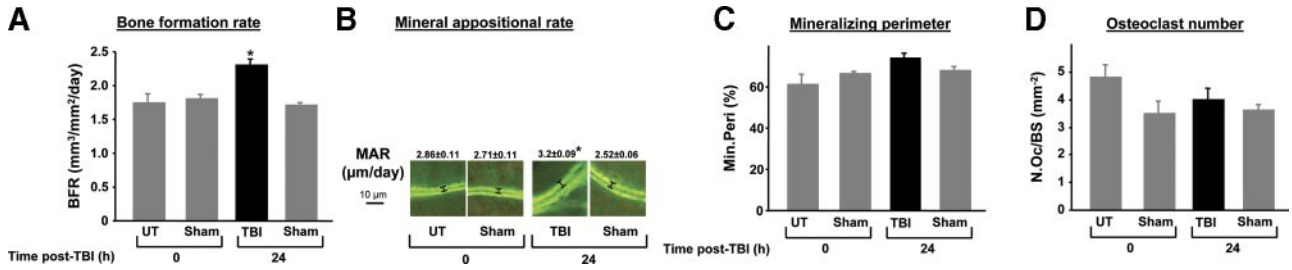

Figure 2. Acute (24-hour) systemic effect of TBI on osteoblastic and osteoclastic parameters in distal femoral metaphysis of male C3H mice. *A–C)* Analysis of bone formation using vital fluorescent calcein staining. *A)* Bone formation rate (BFR) per unit surface area of bone trabeculae. *B)* Representative fluorescent micrographs of calcein label separation from mice with median levels of mineral appositional rate (MAR), surrogate of osteoblast activity. *C)* Mineralizing perimeter (Min.Peri, percent calcein positive trabecular surfaces), surrogate of osteoblast number. *D)* Osteoclast number per unit surface area of bone trabeculae (N.Oc/BS). UT, untreated control mice. Data are mean ± SE from 5 mice per condition. $*P < 0.05$.

lengthier process of proliferation and differentiation of preosteoblasts (31). At least theoretically, the activation of fully differentiated osteoblasts could occur even earlier than two days after TBI. Indeed, the BFR measured 24 h post-TBI in the distal femoral metaphysis was ~38% higher than either the untreated or sham-TBI controls (**Fig. 2*A***). As in the case of the 2- and 4-day post-TBI stimulation in bone formation (Fig. 1), this increase reflected a statistically significant stimulation of MAR (Fig. 2*B*) accompanied by a lesser, statistically insignificant elevation of the Min.Peri (Fig. 2*C*). No differences were found in the number of osteoclasts at any of the time points studied (data not shown and Fig. 2*D*), suggesting that the early TBI-induced signals to bone are targeted only to osteoblasts.

Presence of 2-AG in bone

TBI acutely stimulates central production of the major endocannabinoid, 2-AG (19). In addition, it has been recently reported that CB1 receptors are expressed in sympathetic nerve terminals in the vicinity of trabecular osteoblasts (15) and that functional CB2 receptors are present in osteoblasts and osteoclasts (14). We show now that 2-AG is present in the trabecular bone of intact mice (3.4±0.26 nmol/g wet tissue), at levels in the same magnitude as the brain content of this endocannabinoid (6.2±1.80 nmol/g wet tissue). High levels of 2-AG (1.401±0.140 nmol/mg lipid) were also found in MC3T3 E1 osteoblasts. Because the 2-AG synthesizing enzymes DAGLα and DAGLβ (32) are expressed in bone cells (**Fig. 3**), and because the blood 2-AG level is negligible (33), local skeletal production appears to be the main source of these high levels. Together with the expression of the two cannabinoid receptors in bone, these findings implicate the occurrence of a complex skeletal endocannbinoid system.

Effect of TBI on peripheral bone formation in cannabinoid receptor deficient mice

To assess whether a direct endocannabinoid effect on bone cells is involved in the TBI-induced stimulation of bone formation, we compared post-TBI bone formation in $CB2^{-/-}$ and WT mice. Similar to WT mice, the CB2-deficient animals showed a robust increase in BFR (**Fig. 4*A***) consequent to an increase in MAR (Fig. 4*B*). Unlike the WT mice, the $CB2^{-/-}$ mice also demonstrated a significant elevation in their Min.Peri (Fig. 4*C*). By contrast, TBI failed to evoke an osteogenic response in two distinct lines of $CB1^{-/-}$ mice (**Fig. 5**) despite vast differences in their basal skeletal phenotype (15). Importantly, CB1 expression is unaltered in the $CB2^{-/-}$ mice (data not shown). Collectively, these data indicate that the TBI-induced stimulation of bone formation is critically dependent on the presence of functional CB1, but not CB2 receptors.

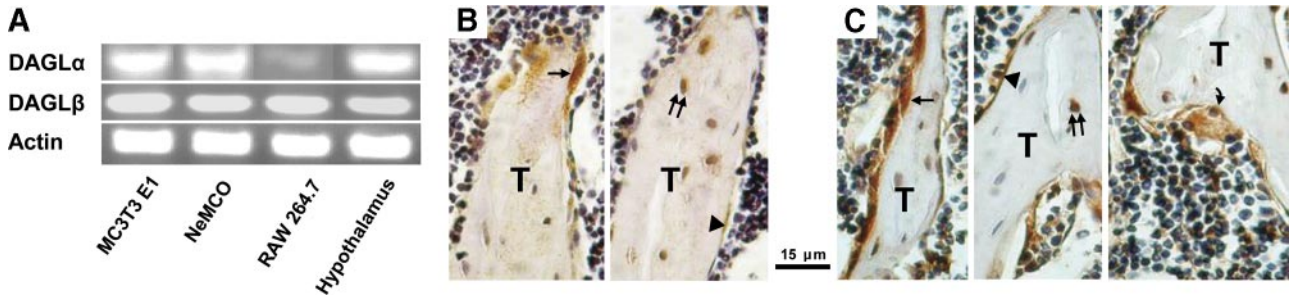

Figure 3. Expression of diacylglycerol lipases (DAGLs) in bone cells. *A)* RT-PCR analysis in cultures of MC3T3 E1 osteoblastic cells, primary calvarial osteoblasts (NeMCO), and RAW 264.7 osteoclast-like cells. Hypothalamic RNA was used as positive control. *B, C)* Immunohistochemical localization of DAGLα (*B*) and DAGLβ (*C*) in distal femoral metaphysial secondary spongiosa. T, bone trabeculae; arrows, osteoblasts; double arrows, osteocytes; arrow heads, lining cells; curved arrow, osteoclast.

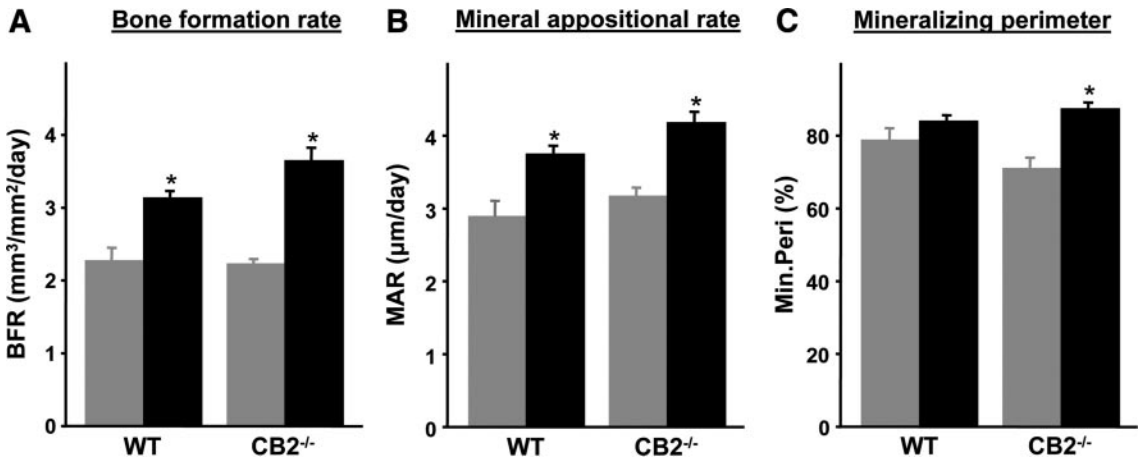

Figure 4. CB2 cannabinoid receptor is not required for TBI-induced acute stimulation of bone formation. Distal femoral metaphyses of female C57BL/6J WT and *CB2*$^{-/-}$ mice were subjected to 24 h post-TBI vital analysis of bone formation. *A)* Bone formation rate (BFR) per unit surface area of bone trabeculae. *B)* Mineral appositional rate (MAR), surrogate of osteoblast activity. *C)* Mineralizing perimeter (Min.Peri, percent calcein positive trabecular surfaces), surrogate of osteoblast number. Gray bars, Sham-TBI; black bars, TBI. Data are mean ± SE from 7 mice per condition. *$P < 0.05$.

2-AG regulates bone NE levels

To further characterize the role of CB1 in regulating bone formation, we tested whether the bone levels of 2-AG are affected by TBI. Indeed, measurements carried out in the metaphyseal trabecular compartment showed 50% increase in 2-AG content already 8 h post-TBI with a progressive decrease thereafter. The bone 2-AG returned to normal levels by 4 h later (**Fig. 6*A***). Because *i*) CB1 is present in bone sympathetic nerve terminals (15); *ii*) prejunctional activation of CB1 by 2-AG inhibits the release of norepinephrine (NE) (4, 5); and *iii*) sympathetic signaling tonically inhibits bone formation by a direct action of NE on osteoblastic β2AR (2), we measured NE levels in contralateral sites of animals subjected to 2-AG determination. As expected, the NE bone content declined after TBI, with the decrease peaking 12 h after injury (Fig. 6*A*). The 4 h time difference between the peak increase in 2-AG and peak decrease in NE is in support of a down-regulation of NE by 2-AG in trabecular bone. This temporal causal relationship between trabecular bone 2-AG and NE was substantiated by demonstrating more than 35% decreases in NE levels both 8 and 12 h after exogenous 2-AG administration (Fig. 6*B*). The causal relationship between CB1 signaling and decrease in skeletal NE is further suggested by the unaltered bone 2-AG and NE levels in the *CB1*$^{-/-}$ mice, whereas in *CB2*$^{-/-}$

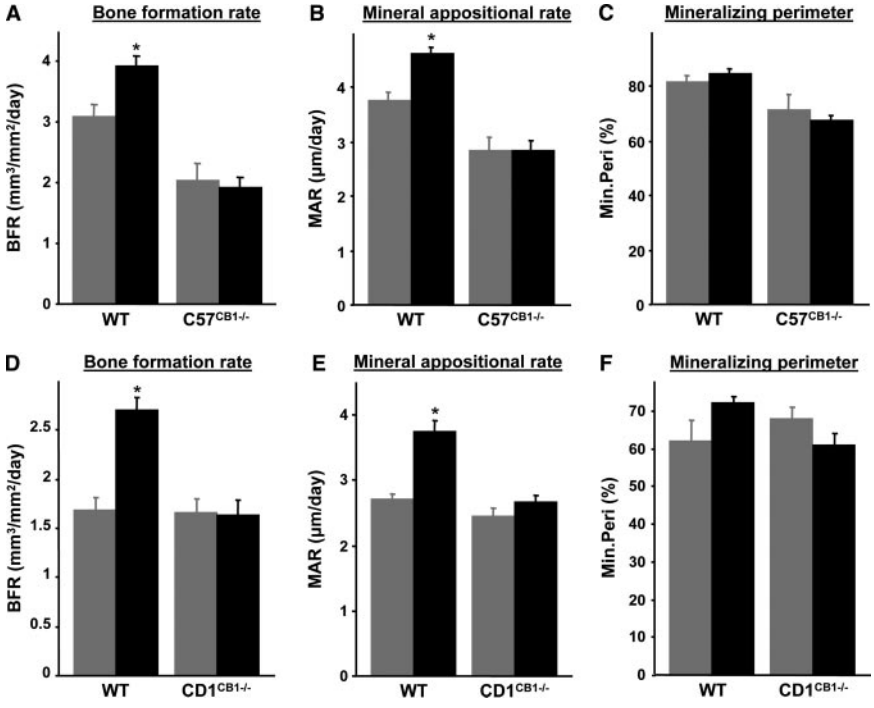

Figure 5. Acute stimulation of bone formation by TBI is dependent on CB1 signaling. Distal femoral metaphyses of female WT and *CB1*$^{-/-}$ mice were subjected to 24 h post-TBI vital analysis of bone formation. *A–C)* C57$^{CB1-/-}$ mice and WT C57BL/6J controls. *D–F)* CD1$^{CB1-/-}$ mice and WT CD1 controls. *A, D)* Bone formation rate (BFR) per unit surface area of bone trabeculae. *B, E)* Mineral appositional rate (MAR), surrogate of osteoblast activity. *C, F)* Mineralizing perimeter (Min.Peri, percent calcein positive trabecular surfaces), surrogate of osteoblast number. Gray bars, Sham-TBI; black bars, TBI. Data are mean ± SE from 5–7 mice per condition. *$P < 0.05$.

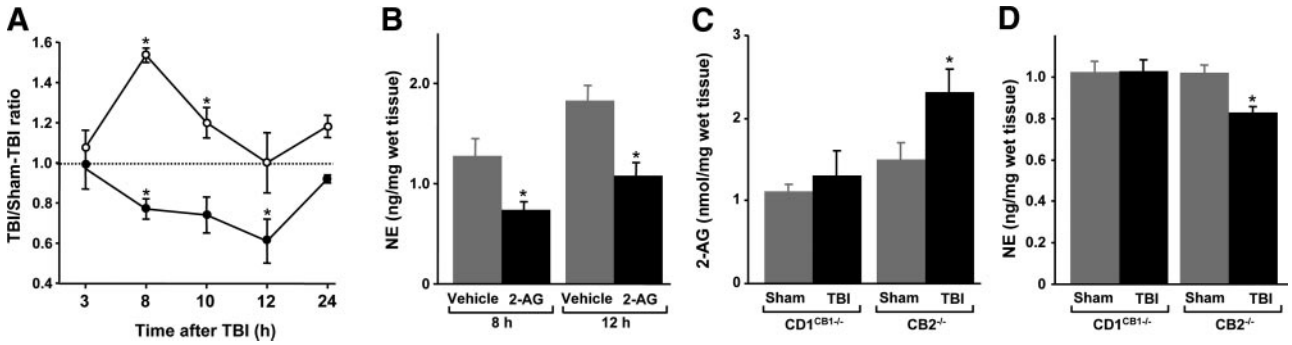

Figure 6. 2-AG suppresses bone NE levels. *A)* Mice were subjected to TBI and distal femoral and proximal tibial metaphyses from each side of mouse were pooled separately and subjected to either analysis. Open circles, 2-AG levels; filled circles, NE levels; dashed line represents levels in sham-TBI animals. *B)* Exogenously administered 2-AG decreases NE levels in distal femoral and proximal tibial metaphyseal pools. *C)* 2-AG levels in distal femoral and proximal tibial metaphyseal pools from $CD1^{CB1-/-}$ and *CB2*$^{-/-}$ mice 8 h post-TBI. *D)* NE levels in distal femoral and proximal tibial metaphyseal pools from $CD1^{CB1-/-}$ and *CB2*$^{-/-}$ mice 8 h post-TBI. Data are mean ± SE from 5–9 mice per condition. $^*P < 0.05$.

animals bone 2-AG was increased and NE was decreased similarly to WT controls (Figs. 6*C*, 6*D*).

Is leptin involved in the TBI-induced stimulation of bone formation?

Leptin is a negative regulator of bone formation (34) and 2-AG synthesis (35). It is also an activator of the sympathetic nervous system (3). The serum leptin level is chronically decreased in patients with TBI stimulated osteogenesis (36). Measuring serum leptin in the present study revealed an initial elevation 3 h post-TBI followed by normal levels determined 5 and 21 h thereafter (**Fig. 7**).

2-AG mimics the TBI-induced stimulation of bone formation

To demonstrate that 2-AG indeed regulates bone formation, and not just bone NE levels, we measured bone formation 24 h following a single 2-AG administration. Indeed, 2-AG administration to WT and *CB2*$^{-/-}$ mice enhanced bone formation rate in the distal femoral metaphysis (**Fig. 8*A***, top and middle panels) mainly consequent to an increase in MAR (Fig. 8*B*, top and middle panels). As in the case of the TBI mice (Fig. 4*C*), a statistically significant increase in the Min.Peri was found only in the absence of CB2 (Fig. 8*C*, middle panel). Like TBI in *CB1*$^{-/-}$ mice, administration of 2-AG to these animals had no significant effect on the BFR, despite a slight inhibition of the MAR (Fig. 8, bottom panels). This inhibition is consistent with the suppression of proliferation, especially in NeMCO (Fig. 10*B*). 2-AG has a relatively short life span (37); therefore, these data demonstrate that activation of CB1 by a transient systemic increase in 2-AG is sufficient to evoke an osteoblastic anabolic response that leads to increased bone formation.

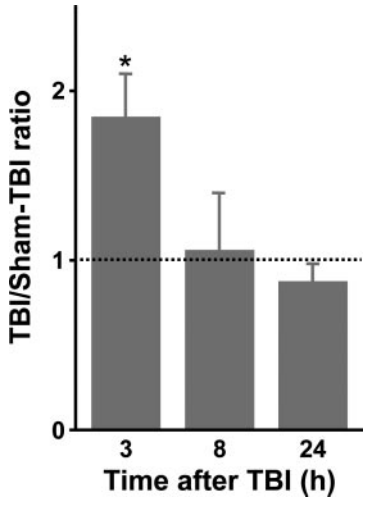

Figure 7. Post-TBI serum leptin levels determined by ELISA. Dashed line represents levels in sham-TBI animals. Data are mean ± SE from 5 mice per condition. $^*P < 0.05$.

To confirm that the TBI- and 2-AG-induced stimulation of osteogenesis are mediated by the sympathetic nervous system, we further tested the effect of the β-adrenergic receptor agonist, ISO on bone formation. Indeed, pretreatment with ISO completely blocked the effect of TBI (**Fig. *9A*, *B***, upper panels) and markedly attenuated the 2-AG-stimulated bone formation (Fig. 9*A*, *B*, bottom panels). In either case it had no effect on the Min.Peri (Fig. 9*C*). Hence, ISO reinstates the activation of osteoblastic β2AR (2) and the consequent tonic inhibition of bone formation, which had been alleviated by the 2-AG-CB1-mediated decrease in NE levels.

Does 2-AG affect osteoblasts directly?

CB2, but not CB1, is abundantly expressed in differentiating and mature osteoblasts (14) and 2-AG is perceived as an agonist for both CB1 and CB2 (38). We were, therefore, surprised that the bone anabolic response was preserved, or even increased, in the CB2 deficient mice. To clarify this point, we tested the effects of 2-AG in the osteoblastic cell line MC3T3 E1 and in the NeMCO system, comparing it to the activity

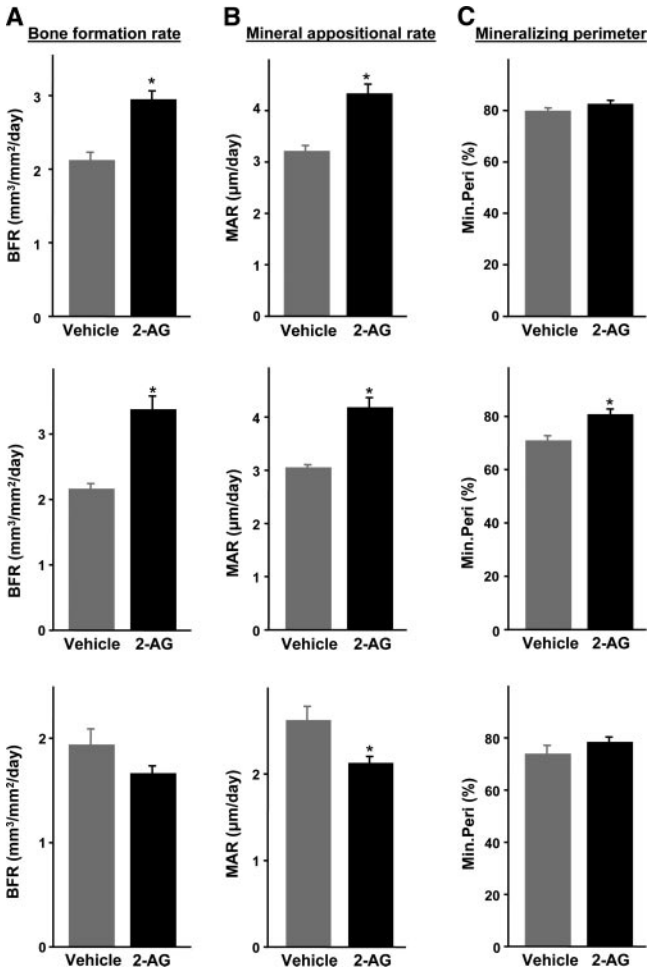

Figure 8. Exogenously administered 2-AG mimics TBI-induced stimulation of bone formation. *A–C)* Analysis of bone formation in distal femoral metaphysis of female C57BL/6J mice using vital fluorescent calcein staining. *A)* Bone formation rate (BFR) per unit surface area of bone trabeculae. *B)* Mineral appositional rate (MAR), surrogate of osteoblast activity. *C)* Mineralizing perimeter (Min.Peri, percent calcein positive trabecular surfaces), surrogate of osteoblast number. Top panel, WT mice; middle panel, *CB2*$^{-/-}$ mice; bottom panel, C57$^{CB1-/-}$ mice (17-wk-old). Data are mean ± SE from 6–10 mice per condition. *$P < 0.05$.

of the CB2-specific agonist HU-308 (39). As reported previously (14), HU-308 potently increased the cell number in both cell systems. It also stimulated the activity of alkaline phosphatase, an *in vitro* surrogate for osteoblast activity. By contrast, 2-AG had no effect on, or even inhibited cell number and alkaline phosphatase activity (**Fig. 10**). These data confirm that the stimulatory effect of 2-AG in bone is mediated by receptors present in cells other than osteoblasts, most likely sympathetic fibers (15).

DISCUSSION

We have recently reported the expression of CB1 receptors in bone sympathetic nerve terminals (15). The present study further demonstrates that the sympathetic control of bone formation is regulated by the endocannabinoid system through modulation of the CB1 activity by 2-AG, which is synthesized predominantly by bone cells. Elevation of the bone 2-AG level apparently suppresses the release of NE from the bone sympathetic nerve terminals, thus alleviating the inhibition of bone formation that results from the activation of osteoblastic β2AR (**Fig. 11**).

The involvement of 2-AG and the CB1 cannabinoid receptor in the modulation of bone sympathetic activity was targeted using the TBI stimulation of bone formation (16–18). We show here that this phenomenon can be reproduced in different mouse strains (C3H, C57BL/6J, and CD1) and is gender-independent. Although the TBI-induced changes in bone 2-AG and NE levels appear not to be sustained beyond the initial 24 h after injury, they could still trigger a prolonged bone anabolic response evident for at least 4 days in the mouse and many months in humans.

Several humoral osteoblast mitogenic factors, such as prolactin, insulin-like growth factor-1, growth hormone, and basic fibroblast growth hormone have been suggested as mediators of the TBI-induced stimulation of bone formation (16, 40–41). In addition, serum from human patients and experimental animals with TBI is mitogenic to osteoblasts *in vitro* (18, 42–45). However, at least acute skeletal response to TBI is not mitogenic and, therefore, the involvement of these factors in this response is unlikely. Leptin is known to positively regulate the sympathetic inhibition of bone formation (2) and negatively regulate 2-AG synthesis, at least in the central nervous system (35). Hence, under

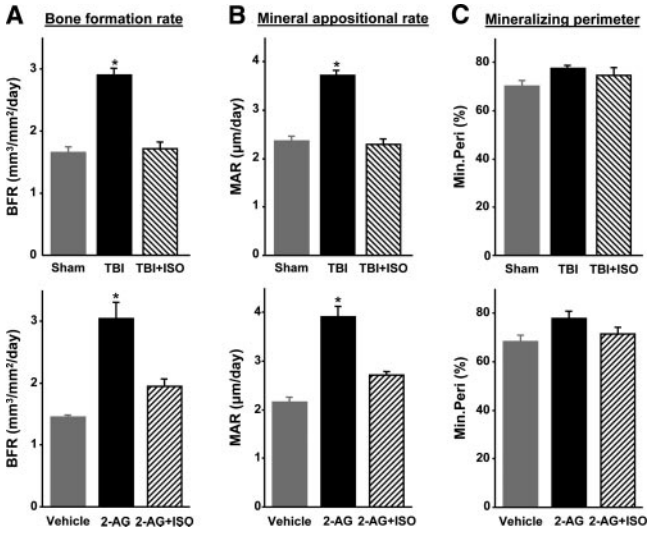

Figure 9. β2 adrenergic receptor agonist, isoproterenol (ISO) blocks TBI- and 2-AG-induced stimulation of bone formation. *A–C)* Analysis of bone formation in distal femoral metaphysis of female C57BL/6J mice using vital fluorescent calcein staining. *A)* Bone formation rate (BFR) per unit surface area of bone trabeculae. *B)* Mineral appositional rate (MAR), surrogate of osteoblast activity. *C)* Mineralizing perimeter (Min.Peri, percent calcein positive trabecular surfaces), surrogate of osteoblast number. Top panel, mice subjected to TBI; bottom panel, mice injected with 2-AG. Data are mean ± SE from 6–7 mice per condition. *$P < 0.05$.

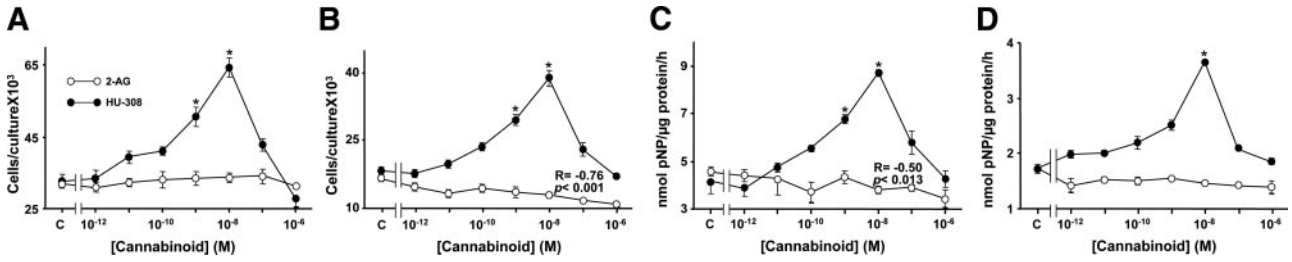

Figure 10. 2-AG does not stimulate osteoblasts directly. *A–B)* Cell number. *C–D)* Alkaline phophatase activity, surrogate of osteoblast activity. *A, C)* MC3T3 E1 osteoblastic cell cultures. *B, D)* Primary calvarial osteoblast (NeMCO) cultures. Data are mean ± SE obtained in triplicate culture wells per condition. R, correlation coefficient between 2-AG dose and osteoblastic response. $*P < 0.05$.

circumstances of increased 2-AG levels and bone formation we had expected a decrease in serum leptin. The present elevated or unchanged serum leptin levels thus suggest that it is not associated with the short-term response to TBI. Actually, the present results are in support of the involvement of a neuronal pathway in this phenomenology. Namely, using a mouse model for TBI and *CB1*$^{-/-}$ mice we show an acute enhancement of peripheral bone formation, which is mediated by the cannabinoid receptor, CB1. In this regard, it is noteworthy that we have recently reported distinct skeletal phenotypes in two mouse lines carrying different CB1 mutations (15). However, the present absence of an osteogenic response to TBI in both lines indicates that either mutation leads to nonresponsiveness to 2-AG.

We further show a high level of 2-AG in normal trabecular bone. As in the brain (19), the trabecular bone 2-AG level is stimulated by TBI, demonstrating a temporal pattern consistent with the timing of the initial increase in bone formation. One source of 2-AG in the skeleton could be local production by bone cells, suggested by the high 2-AG levels in osteoblast-like cell cultures. In addition, cells of the osteoblastic lineage express DAGLα and DAGLβ *in vitro* and *in vivo*. DAGLβ is also expressed by osteoclasts. Hence, increased DAGL expression and/or activity might be involved in processes leading to altered skeletal 2-AG levels. The involvement of enhanced gene expression in the process may also explain the 8 h delay between TBI and peak elevation in bone 2-AG level. A neural source of the skeletal 2-AG is another alternative, since it has been shown that endocannabinoids are produced by neurons in the central nervous system and in peripheral nerves. Their release from nerve cells is regulated by depolarizing stimuli (46, 47), which herein could result from the brain injury. Hence, the mechanisms underlying the increase in both brain and bone 2-AG levels may be similar, operating at pre- and/or postjunctional sites. This does not contradict the TBI-like stimulation of bone formation by exogenously administered 2-AG, inasmuch as endocannabinoids from the extracellular fluid could also access the sympathetic nerve terminal–osteoblast gap (15). Since 2-AG has a short life span (37), its inhibitory effect on NE levels 8 and 12 h after a single administration suggests that it triggers a cascade of events that maintains a several-hour-long restraint of bone NE. In fact, we have previously demonstrated such a delayed inhibitory effect of 2-AG on post-TBI transactivation of nuclear factor kappa B (NFκB) in the central nervous system (48).

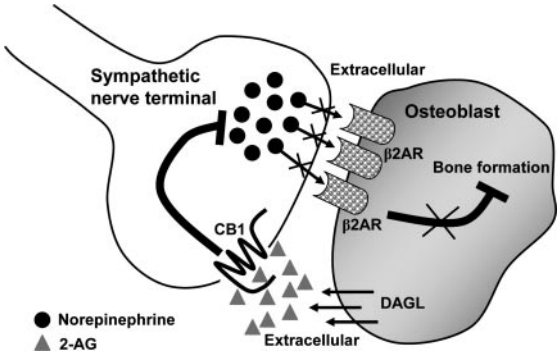

Figure 11. Model of CB1 regulation of bone formation by modulating adrenergic signaling. Activation of CB1 cannabinoid receptors in bone sympathetic nerve terminals by bone cell-derived 2-AG restrains NE release, thus alleviating the tonic suppression of bone formation by the sympathetic nervous system.

The decrease in trabecular bone NE by increased 2-AG levels is consistent with the endocannabinoid inhibition of sympathetic activity in other peripheral tissues such as blood vessels, vas deferens, heart, and lung (4, 49–54). Activation of CB1 at prejunctional axon terminals inhibits neurotransmitter release from the synaptic vesicles possibly by three mechanisms: inhibition of voltage-dependent calcium channels, activation of potassium channels, and direct interference with the vesicle release machinery (55). In turn, the consequent reduced activation of osteoblastic β2AR may lead to altered signaling events involving CRE activation and expression of transcription factors associated with the biological clock (56).

CB2 is abundantly expressed in differentiating and mature bone cells (14). Hence, the increase in osteoblast number (Min.Peri) in the CB2-deficient mice after TBI or 2-AG administration could occur secondary to changes in CB1 expression, inasmuch as a crosstalk altering receptor expression in knockout models for neuronal receptor families has been reported in other instances (57). However, the skeletal CB1 mRNA level remains unaltered following CB2 deletion. Alternatively, the increase in osteoblast number and activity in response to TBI and to exogenously administered 2-AG in the CB2-null mice could result from mitigation of a CB2-mediated, direct inhibition by

2-AG suggested by the 2-AG-induced decrease of these parameters in culture. These data further imply that in osteoblasts 2-AG acts as a CB2 inverse agonist. This type of 2-AG activity has not been shown elsewhere and should, therefore, be considered rather speculative and be the subject of a further detailed study.

In the absence of significantly increased bone 2-AG levels in CB1 deficient mice, the complete loss of sympathetic and osteogenic responses to TBI in these mice could suggest that these responses are 2-AG-dependent. However, the finding that, like TBI, 2-AG administration also failed to stimulate bone formation in the *CB1*$^{-/-}$ mice indicates that CB1 signaling *per se* is a critical link mediating the osteogenic response to TBI. As to CB2, the overall evidence presented here, including the similarity in 2-AG and NE responses to TBI between the WT and *CB2*$^{-/-}$ mice, implies that this receptor is minimally involved, if any, in the TBI stimulated osteogenesis.

Changes in bone remodeling, detectable weeks to months after endocrine manipulations that affect hormone and growth factor levels are well documented. The present and previously published findings (14, 15) demonstrate the occurrence of a skeletal endocannabinoid system consisting at least of the CB1 and CB2 receptors, the 2-AG ligand and 2-AG synthesizing enzymes. We further characterize a neuronal process whereby 2-AG, through CB1, can regulate the sympathetic nervous system, thus controlling bone formation within several hours after challenge. Given the neuroprotective role of endocannabinoids (19, 58), it is tempting to speculate that this acute skeletal anabolic activity represents a bone mass protective mechanism targeted to preventing bone loss that otherwise occurs secondary to reductions in muscle activity and mechanical stimuli commonly associated with traumatic brain and spinal cord injuries. FJ

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REFERENCES

1. Manolagas, S. C. (2000) Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr. Rev.* **21,** 115–137
2. Takeda, S., Elefteriou, F., Levasseur, R., Liu, X., Zhao, L., Parker, K. L., Armstrong, D., Ducy, P., and Karsenty, G. (2002) Leptin regulates bone formation via the sympathetic nervous system. *Cell* **111,** 305–317
3. Elefteriou, F., Ahn, J. D., Takeda, S., Starbuck, M., Yang, X., Liu, X., Kondo, H., Richards, W. G., Bannon, T. W., Noda, M., Clement, K., Vaisse, C., and Karsenty, G. (2005) Leptin regulation of bone resorption by the sympathetic nervous system and CART. *Nature* **434,** 514–520
4. Ishac, E. J., Jiang, L., Lake, K. D., Varga, K., Abood, M. E., and Kunos, G. (1996) Inhibition of exocytotic noradrenaline release by presynaptic cannabinoid CB1 receptors on peripheral sympathetic nerves. *Br. J. Pharmacol.* **118,** 2023–2028
5. Niederhoffer, N., Schmid, K., and Szabo, B. (2003) The peripheral sympathetic nervous system is the major target of cannabinoids in eliciting cardiovascular depression. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **367,** 434–443
6. Rhee, M. H., Vogel, Z., Barg, J., Bayewitch, M., Levy, R., Hanus, L., Breuer, A., and Mechoulam, R. (1997) Cannabinol derivatives: binding to cannabinoid receptors and inhibition of adenylylcyclase. *J. Med. Chem.* **40,** 3228–3233
7. Herkenham, M., Lynn, A. B., Little, M. D., Johnson, M. R., Melvin, L. S., de Costa, B. R., and Rice, K. C. (1990) Cannabinoid receptor localization in brain. *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1932–1936
8. Zimmer, A., Zimmer, A. M., Hohmann, A. G., Herkenham, M., and Bonner, T. I. (1999) Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proc. Natl. Acad. Sci. U. S. A.* **11,** 5780–5785
9. Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature* **365,** 61–65
10. Julien, B., Grenard, P., Teixeira-Clerc, F., Van Nhieu, J. T., Li, L., Karsak, M., Zimmer, A., Mallat, A., and Lotersztajn, S. (2005) Antifibrogenic role of the cannabinoid receptor CB2 in the liver. *Gastroenterology* **128,** 742–755
11. Steffens, S., Veillard, N. R., Arnaud, C., Pelli, G., Burger, F., Staub, C., Karsak, M., Zimmer, A., Frossard, J. L., and Mach, F. (2005) Low dose oral cannabinoid therapy reduces progression of atherosclerosis in mice. *Nature* **434,** 782–786
12. Idris, A. I., van't Hof, R. J., Greig, I. R., Ridge, S. A., Baker, D., Ross, R. A., and Ralston, S. H. (2005) Regulation of bone mass, bone loss and osteoclast activity by cannabinoid receptors. *Nat. Med.* **11,** 774–779
13. Karsak, M., Cohen-Solal, M., Freudenberg, J., Ostertag, A., Morieux, C., Kornak, U., Essig, J., Erxlebe, E., Bab, I., Kubisch, C., de Vernejoul, M. C., and Zimmer, A. (2005) Cannabinoid receptor type 2 gene is associated with human osteoporosis. *Hum. Mol. Genet.* **14,** 3389–3396
14. Ofek, O., Karsak, M., Leclerc, N., Fogel, M., Frenkel, B., Wright, K., Tam, J., Attar-Namdar, M., Kram, V., Shohami, E., Mechoulam, R., Zimmer, A., and Bab, I. (2006) Peripheral cannabinoid receptor, CB2, regulates bone mass. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 696–701
15. Tam, J., Ofek, O., Fride, E., Ledent, C., Gabet, Y., Muller, R., Zimmer, A., Mackie, K., Mechoulam, R., Shohami, E., and Bab, I. (2006) Involvement of neuronal cannabinoid receptor, CB1, in regulation of bone mass and bone remodeling. *Mol. Pharmacol.* **70,** 786–792
16. Wildburger, R., Zarkovic, N., Tonkovic, G., Skoric, T., Frech, S., Hartleb, M., Loncaric, I., and Zarkovic, K. (1998) Post-traumatic hormonal disturbances: prolactin as a link between head injury and enhanced osteogenesis. *J. Endocrinol. Invest.* **21,** 78–86
17. Melamed, E., Robinson, D., Halperin, N., Wallach, N., Keren, O., and Groswasser, Z. (2002) Brain injury-related heterotopic bone formation: treatment strategy and results. *Am. J. Phys. Med. Rehabil.* **81,** 670–674
18. Boes, M., Kain, M., Kakar, S., Nicholls, F., Cullinane, D., Gerstenfeld, L., Einhorn, T. A., and Tornetta, P. 3rd. (2006) Osteogenic effects of traumatic brain injury on experimental fracture-healing. *J. Bone. Joint. Surg. Am.* **88,** 738–743
19. Panikashvili, D., Simeonidou, C., Ben-Shabat, S., Hanus, L., Breuer, A., Mechoulam, R., and Shohami, E. (2001) An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. *Nature* **413,** 527–531
20. Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., Gopher, A., Almog, S., Martin, B. R., Compton, D. R., Pertweee, R. G., Griffine, G., Bayewitchf, M., Bargf, J., and Vogelf, Z. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* **50,** 83–90
21. Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., Yamashita, A., and Waku, K. (1995) 2-Arachidonoyl-

glycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun.* **215,** 89–97
22. Buckley, N. E., McCoy, K. L., Mezey, E., Bonner, T., Zimmer, A., Felder, C. C., Glass, M., and Zimmer, A. (2000) Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. *Eur. J. Pharmacol.* **396,** 141–149
23. Ledent, C., Valverde, O., Cossu, G., Petitet, F., Aubert, J. F., Beslot, F., Bohme, G. A., Imperato, A., Pedrazzini, T., and Roques, B. P. (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science* **283,** 401–404
24. Chen, Y., Constantini, S., Trembovler, V., Weinstock, M., and Shohami, E. (1996) An experimental model of closed head injury in mice: pathophysiology, histopathology and cognitive deficits. *J. Neurotrauma.* **13,** 557–568
25. Yatsiv, I., Morganti-Kossmann, M. C., Perez, D., Dinarello, C. A., Novick, D., Rubinstein, M., Otto, V. I., Rancan, M., Kossmann, T., Redaelli, C. A., Trentz, O., Shohami, E., and Stahel, P. F. (2002) Elevated intracranial IL-18 and evidence of neuroprotective effects of IL-18 binding protein after experimental closed head injury. *J. Cereb. Blood Flow. Metabol.* **22,** 971–978
26. Beni-Adani, L., Gozes, I., Cohen, Y., Assaf, Y., Steingart, R. A., Brenneman, D. E., Eizenberg, O., Trembolver, V., and Shohami, E. (2001) A peptide derived from activity-dependent neuroprotective protein (ADNP) ameliorates injury response in closed head injury in mice. *J. Pharmacol. Exp. Ther.* **296,** 57–63
27. Kondo, H., Nifuji, A., Takeda, S., Ezura, Y., Rittling, S. R., Denhardt, D. T., Nakashima, K., Karsenty, G., and Noda, M. (2005) Unloading induces osteoblastic cell suppression and osteoclastic cell activation to lead to bone loss via sympathetic nervous system. *J. Biol. Chem.* **280,** 30192–30200
28. Parfitt, A. M. (1987) Bone histomorphometry: standardization of nomenclature, symbols, and units. *J. Bone Miner. Res.* **2,** 595–610
29. Di Marzo, V., Goparaju, S. K., Wang, L., Liu, J., Batkai, S., Jarai, Z., Fezza, F., Miura, G. I., Palmiter, R. D., Sugiura, T., and Kunos, G. (2001) Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature* **410,** 822–825
30. Yao, M., Dooley, P. C., Schuijers, J. A., and Grills, B. L. (2004) The effects of hypothyroidism on nerve growth factor and norepinephrine concentrations in weight-bearing and non-weight-bearing bones of rats. *J. Musculoskelet. Neuronal. Interact.* **4,** 319–324
31. Fried, A., Benayahu, D., and Wientroub, S. (1993) Marrow stroma-derived osteogenic clonal cell lines: putative stages in osteoblastic differentiation. *J. Cell. Physiol.* **155,** 472–482
32. Bisogno, T., Howell, F., Williams, G., Minassi, A., Cascio, M. G., Ligresti, A., Matias, I., Schiano-Moriello, A., Paul, P., Williams, E. J., Gangadharan, U., Hobbs, C., Di Marzo, V., and Doherty, P. (2003) Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *J. Cell Biol.* **163,** 463–468
33. Monteleone, P., Matias, I., Martiadis, V., De Petrocellis, L., Maj, M., and Di Marzo, V. (2005) Blood levels of the endocannabinoid anandamide are increased in anorexia nervosa and in binge-eating disorder, but not in bulimia nervosa. *Neuropsychopharmacology* **30,** 1216–1221
34. Ducy, P., Amling, M., Takeda, S., Priemel, M., Schilling, A. F., Beil, F. T., Shen, J., Vinson, C., Rueger, J. M., and Karsenty, G. (2000) Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell* **100,** 197–207
35. Di Marzo, V., Goparaju, S. K., Wang, L., Liu, J., Batkai, S., Jarai, Z., Fezza, F., Miura, G. I., Palmiter, R. D., Sugiura, T., and Kunos, G. (2001) Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature* **410,** 822–825
36. Rigaux, P., Benabid, N., Darriet, D., Delecourt, C., Chieux, V., Dudermel, A. F., Sutter, B., Anselme, K., and Hardouin, P. (2005) Study of serum factors potentially involved in the pathogenesis of heterotopic bone formation after severe brain injury. *Joint Bone Spine* **72,** 146–149
37. Rouzer, C. A., Ghebreselasie, K., and Marnett, L. J. (2002) Chemical stability of 2-arachidonylglycerol under biological conditions. *Chem. Phys. Lipids.* **119,** 69–82
38. Sugiura, T., and Waku, K. (2000) 2-Arachidonoylglycerol and the cannabinoid receptors. *Chem. Phys. Lipids.* **108,** 89–106
39. Hanus, L., Breuer, A., Tchilibon, S., Shiloah, S., Goldenberg, D., Horowitz, M., Pertwee, R. G., Ross, R. A., Mechoulam, R., and Fride, E. (1999) HU-308: a specific agonist for CB(2), a peripheral cannabinoid receptor. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14228–14233
40. Wildburger, R., Zarkovic, N., Leb, G., Borovic, S., Zarkovic, K., and Tatzber, F. (2001) Post-traumatic changes in insulin-like growth factor type 1 and growth hormone in patients with bone fractures and traumatic brain injury. *Wien. Klin. Wochenschr.* **113,** 119–126
41. Wildburger, R., Zarkovic, N., Egger, G., Petek, W., Zarkovic, K., and Hofer, H. P. (1994) Basic fibroblast growth factor (BFGF) immunoreactivity as a possible link between head injury and impaired bone fracture healing. *Bone Miner.* **27,** 183–192
42. Bidner, S. M., Rubins, I. M., Desjardins, J. V., Zukor, D. J., and Goltzman, D. (1990) Evidence for a humoral mechanism for enhanced osteogenesis after head injury. *J. Bone Joint Surg. Am.* **72,** 1144–1149
43. Kurer, M. H., Khoker, M. A., and Dandona, P. (1992) Human osteoblast stimulation by sera from paraplegic patients with heterotopic ossification. *Paraplegia* **30,** 165–168
44. Renfree, K. J., Banovac, K., Hornicek, F. J., Lebwohl, N. H., Villanueva, P. A., and Nedd, K. J. (1994) Evaluation of serum osteoblast mitogenic activity in spinal cord and head injury patients with acute heterotopic ossification. *Spine* **19,** 740–746
45. Klein, B. Y., Shohami, E., Reikhinshtein, Y., Ben-Bassat, H., and Liebergall, M. (1999) Serum-mediated osteogenic effects of head injury on cultured rat marrow stromal cells. *Calcif. Tissue Int.* **65,** 217–222
46. Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J. C., and Piomelli, D. (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* **372,** 686–691
47. Stella, N., and Piomelli, D. (2001) Receptor-dependent formation of endogenous cannabinoids in cortical neurons. *Eur. J. Pharmacol.* **425,** 189–196
48. Panikashvili, D., Mechoulam, R., Beni, S. M., Alexandrovich, A., and Shohami, E. (2005) CB1 cannabinoid receptors are involved in neuroprotection via NF-kappa B inhibition. *J. Cereb. Blood Flow. Metab.* **25,** 477–484
49. Malinowska, B., Godlewski, G., Bucher, B., and Schlicker, E. (1997) Cannabinoid CB1 receptor-mediated inhibition of the neurogenic vasopressor response in the pithed rat. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **356,** 197–202
50. Deutsch, D. G., Goligorsky, M. S., Schmid, P. C., Krebsbach, R. J., Schmid, H. H., Das, S. K., Dey, S. K., Arreaza, G., Thorup, C., Stefano, G., and Moore, L. C. (1997) Production and physiological actions of anandamide in the vasculature of the rat kidney. *J. Clin. Invest.* **100,** 1538–1546
51. Pertwee, R. G., Stevenson, L. A., Elrick, D. B., Mechoulam, R., and Corbett, A. D. (1992) Inhibitory effects of certain enantiomeric cannabinoids in the mouse vas deferens and the myenteric plexus preparation of guinea-pig small intestine. *Br. J. Pharmacol.* **105,** 980–984
52. Schlicker, E., Redmer, A., Werner, A., and Kathmann, M. (2003) Lack of CB1 receptors increases noradrenaline release in vas deferens without affecting atrial noradrenaline release or cortical acetylcholine release. *Br. J. Pharmacol.* **140,** 323–328
53. Szabo, B., Nordheim, U., and Niederhoffer, N. (2001) Effects of cannabinoids on sympathetic and parasympathetic neuroeffector transmission in the rabbit heart. *J. Pharmacol. Exp. Ther.* **297,** 819–826
54. Vizi, E. S., Katona, I., and Freund, T. F. (2001) Evidence for presynaptic cannabinoid CB(1) receptor-mediated inhibition of noradrenaline release in the guinea pig lung. *Eur. J. Pharmacol.* **431,** 237–244
55. Szabo, B., and Schlicker, E. (2005) Effects of cannabinoids on neurotransmission. *Handb. Exp. Pharmacol.* **168,** 327–365
56. Fu, L., Patel, M. S., Bradley, A., Wagner, E. F., and Karsenty, G. (2005) The molecular clock mediates leptin-regulated bone formation. *Cell* **122,** 803–815
57. Contractor, A., Sailer, A. W., Darstein, M., Maron, C., Xu, J., Swanson, G. T., and Heinemann, S. F. (2003) Loss of kainate receptor-mediated heterosynaptic facilitation of mossy-fiber synapses in KA2-/- mice. *J. Neurosci.* **23,** 422–429
58. Mechoulam, R., Spatz, M., and Shohami, E. (2002) Endocannabinoids and neuroprotection. *Sci. STKE* **23,** RE5

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