Prostaglandin E2 mediates sensory nerve regulation of bone homeostasis

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Whether sensory nerve can sense bone density or metabolic activity to control bone homeostasis is unknown. Here we found prostaglandin E2 (PGE2) secreted by osteoblastic cells activates PGE2 receptor 4 (EP4) in sensory nerves to regulate bone formation by inhibiting sympathetic activity through the central nervous system. PGE2 secreted by osteoblasts increases when bone density decreases as demonstrated in osteoporotic animal models. Ablation of sensory nerves erodes the skeletal integrity. Specifically, knockout of the EP4 gene in the sensory nerves or cyclooxygenase-2 (COX2) in the osteoblastic cells significantly reduces bone volume in adult mice. Sympathetic tone is increased in sensory denervation models, and propranolol, a β2-adrenergic antagonist, rescues bone loss. Furthermore, injection of SW033291, a small molecule to increase PGE2 level locally, significantly boosts bone formation, whereas the effect is obstructed in EP4 knockout mice. Thus, we show that PGE2 mediates sensory nerve to control bone homeostasis and promote regeneration.

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Sensory nerves are innervated in peripheral tissues, including skin, joint, respiratory, and gastrointestinal tissues, to sense stimuli inside or outside the body, such as pain, temperature, odor, and taste\(^1\). The signals collected from sensory nerve endings are processed in the central nervous system to initiate physiological responses. Bone is the largest organ, accounting for more than 80% of body weight. Bone is also an endocrine organ that regulates calcium and mineral metabolism, glucose, fatty acids, and even cancer metastasis by interacting with other tissues\(^2\). The skeleton has abundant sensory and sympathetic innervations\(^3\), and interacts with the central nervous system\(^4\). Sympathetic nerves induce catabolic activity in bone through serotonin and CAMP-response element binding protein (CREB) signaling in the hypothalamus\(^5\). Specific deletion of sensory nerves in bone impairs bone mass accrual\(^6\), while patients with sensory nerve malfunction or loss have an increased bone fracture rate and significantly diminished post-injury bone regeneration\(^7\). These observations indicate that sensory nerves sense changes in bone density, mechanical stress, and metabolic activity to control bone homeostasis.

Based on the evidence, one or more molecules should transmit signals of changes in bone to sensory nerve fibers. Cyclooxygenase activity and prostaglandins are known to mediate skeletal metabolism and inflammation\(^8\). Among prostaglandins, prostaglandin E2 (PGE2) is a multifunctional molecule whose production is controlled by the limiting enzyme cyclooxygenase (COX)\(^9\). Evidence shows that PGE2 can elicit primary pain and prolong nociceptor sensitization\(^10\). Non-steroidal anti-inflammatory drugs and COX2 selective inhibitors are the current major medications to treat musculoskeletal pain. A multicenter study revealed that COX2 selective inhibitor is associated with lower bone mineral density (BMD) in men, whereas, in postmenopausal women it promotes BMD\(^11\), implicating PGE2 in the regulation of bone.

The 15-hydroxyprostaglandin dehydrogenase gene (HPGD) encodes a NAD\(^+\)-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which catalyzes PGE2. Mutation of this gene impairs the degradation of PGE2. HPGD mutant mice showed an increased PGE2 level in vivo, which can effectively promote regeneration in different tissues\(^12\). Interestingly, patients with HPGD mutation have presented with subperiosteal new bone formation\(^13\). PGE2 is also potent in stimulating osteoblastic bone formation in adult mice. Elevation of PGE2 by inhibiting 15-PDGH promotes new bone formation\(^14\). PGE2 is also potent in stimulating bone regeneration\(^15\). These observations indicate that sensory nerves sense changes in bone density, mechanical stress, and metabolic activity to control bone homeostasis.

Here in this study, we report that bone density regulates the level of PGE2 secreted by osteoblasts. Deletion of EP4 in sensory nerves or COX2 in osteoblasts significantly decreases bone mass. Elevation of PGE2 by inhibiting 15-PDGH promotes bone regeneration. PGE2 regulates osteoblast bone formation by activation of sensory nerves in a sympathetic nerve feedback manner.

### Results

#### Sensory denervation reduces osteoblastic bone formation.

To investigate the effect of sensory nerve in bone, we created a sensory denervation mouse model (TrkA\(^{+/−}\)) by crossing sensory nerve-specific cre (Adillin-cre) mice with nerve growth factor (NGF) receptor TrkA floxed (TrkA\(^{wt}\)) mice. Quantitative polymerase chain reaction (qPCR) and immunofluorescent staining of TrkA in the dorsal root ganglion (DRG) neurons and the other tissues isolated from the TrkA\(^{+/−}\)mice validated the knockout efficiency and specificity of the TrkA gene in the TrkA\(^{+/−}\)mice (Supplementary Figure 1). Furthermore, immunostaining of femur sections showed that most calcitonin gene-related peptide (CGRP)\(^+\) sensory nerve fibers were eliminated in the TrkA\(^{+/−}\)mice (Fig. 1a). Significant bone loss was observed in 12-week-old TrkA\(^{+/−}\)mice relative to their wild-type (WT) littermates in \(μ\)CT analysis (Fig. 1b), while no significant bone volume decrease was found of 4-week-old age (Supplementary Figure 2), indicating an essential role of sensory nerve for bone homeostasis in adults. The number of osteocalcin\(^−\) osteoblasts was significantly lower in TrkA\(^{+/−}\)mice relative to their WT littermates; whereas, the number of tarratic acid phosphatase (TRAP)\(^+\) osteoclasts was not different (Fig. 1c). Trichrome staining showed decreased osteoid in TrkA\(^{+/−}\) mice (Fig. 1d). Accordingly, the serum level of osteocalcin, a marker of osteoblastic bone formation, was significantly lower, and the level of osteoclast bone resorption marker carboxy-terminal collagen crosslinks (CTX) was not different in TrkA\(^{+/−}\) mice (Fig. 1e). Calcein double labeling confirmed the reduced bone formation and mineral apposition rate (Fig. 1f). We also evaluated the sensory innervations and bone architecture in the vertebrae of TrkA\(^{+/−}\) mice, and similar results were observed (Fig. 1g−i and Supplementary Figure 3, A–C). These results indicate that sensory nerve regulates osteoblastic bone formation in adult mice.

To examine whether sensory nerves maintain bone homeostasis through bone remodeling in adult mice, we established inducible sensory denervation in iDTR\(^{+/−}\) mice by crossing Adillin-cre mice with iDTR\(^{wt}\) mice. Sensory denervation was effectively induced in adult iDTR\(^{+}\)/− mice by injection of 1 ug per kg diphtheria toxin (DTX) three times a week for four weeks (Fig. 2a). Significant bone loss was observed using \(μ\)CT (Fig. 2b). Similarly, the number of osteoblasts, amount of osteoid, and serum osteocalcin level were significantly decreased; whereas, the number of osteoclasts and serum CTX level were unchanged relative to the vehicle group (Fig. 2c, e). The calcein double-labeling experiment confirmed that bone formation and mineral apposition rate were reduced (Fig. 2f). Moreover, sensory innervations and bone mass also decreased in the vertebrae of iDTR\(^{+}\)/− mice injected with DTX relative to the vehicle-treated mice (Fig. 2g, i and Supplementary Figure 3, D–E). Because neural changes other than those in the sensory nervous system can affect bone metabolism indirectly, we performed pole tests and grip strength tests with TrkA\(^{−}\)/− mice and iDTR\(^{+}\)/− mice injected with DTX. No motor neural activity was influenced in these two mouse models (Supplementary Figure 4). Thus, sensory nerve regulates bone homeostasis through osteoblasts during bone remodeling.

Knockout of EP4 receptor in sensory nerve induces bone loss.

Because PGE2 is known to stimulate osteoblastic bone formation, we measured PGE2 levels in the serum of both global and inducible sensory denervation mice. Interestingly, PGE2 levels increased significantly in all the denervation mouse models (Fig. 3a). The results prompted us to examine whether PGE2 mediates sensory nerve in regulation of osteoblast bone formation. We found that bone density was negatively correlated with PGE2 levels, and that PGE2 levels increased in aged or the other osteoporotic mice (Fig. 3a). Immunohistochemical analysis also showed that expression of COX2 in femur osteoblasts, the PGE2 production-limiting enzyme, increased in the sensory denervation
was expressed in bone sensory nerves, confirming that EP4 was efficiently deleted from sensory nerves in the bone marrow of EP4Avil−/− mice (Fig. 4a).

OVX and aged mice (Fig. 3b, c). As EP4 is the primary receptor of PGE2 for bone formation34, we further co-immunostained of EP4 or CGRP in OVX and aged mice femurs. In OVX mice, we observed a significant reduction of CGRP+ sensory fibers two weeks post OVX surgery (Fig. 3d, e). Interestingly, loss of EP4 expression in the sensory fibers of aged mouse bone marrow with no significant decrease of the CGRP+ nerve fibers (Fig. 3f, g). We then induced ablation of EP4 in sensory nerves using adult EP4Avil−/− mice by crossing Advillin-cre mice with EP4Avil−/− mice to validate of EP4 function in sensory nerves. qPCR analysis and immunostaining of EP4 confirmed that EP4 deletion was specifically in nerve (Supplementary Figure 5). Co-immunofluorescent staining of EP4 with CGRP showed that EP4 was expressed in bone sensory nerves, confirming that EP4 was efficiently deleted from sensory nerves in the bone marrow of EP4Avil−/− mice (Fig. 4a).

Both trabecular bone and cortical bone decreased significantly in 12-week EP4-ablated mice (Fig. 4b, c, Supplementary Figure 6). Pole tests and grip strength tests showed no changes in motor neural activity (Supplementary Figure 7). PGE2 levels in the serum and COX2 expression in osteoblasts increased (Fig. 4d, e), suggesting a compensatory increase of the ligand PGE2 in response to EP4 knockout in sensory nerves. Similar to the two sensory denervation models, in EP4Avil−/− mice, the number of osteoblasts decreased (but the serum osteocalcin level showed no statistical significance), with no changes in osteoclast number or bone degradation marker CTX (Fig. 4f, g). Importantly, PGE2 induced significant bone formation in wild-type mice was abolished in EP4Avil−/− mice, as demonstrated in trichrome and double-labeling experiments (Fig. 3h, i). As EP4 expression is also known to express in osteoblasts, osteoblastic cell-specific knockout EP4 mice were generated by crossing EP4Avil−/− mice with
PGE2 mediates sensory nerve induced osteogenesis. To examine whether PGE2 is secreted primarily by osteoblastic cells for sensory nerve regulation of bone, we further generated conditional knockout COX2 mice in the osteoblastic cells (COX2OC−/−) by crossing COX2wt mice with OC-cre mice to eliminate PGE2 secretion by osteocytes. Pole tests and grip strength tests showed no effect on motor activity, indicating that knockout of COX2 did not affect global neural activity (Supplementary Figure 9). As in EP4 knockout mice, trabecular and cortical bone decreased while body weight remained unchanged over time in COX2OC−/− mice relative to their COX2wt littermates (Fig. 5a, b, Supplementary Figure 10). COX2 staining of the mouse femurs confirmed the expression of COX2 and its ablation in osteoblasts (Fig. 5c). Interestingly, CGRP+ nerve fibers were located in the active bone remodeling areas with Ocn+ osteoblasts in co-immunostaining (Supplementary Figure 11). This suggests that PGE2 secreted by osteoblastic cells in active bone remodeling sites is essential because the bone marrow PGE2, instead of the serum PGE2 levels, was significantly different between these two groups of mice (Fig. 5d). Thus, PGE2 secreted by osteoblastic cells in active bone remodeling sites mediates sensory nerve–stimulated bone formation. Again, the number of osteoblasts and serum osteocalcin level decreased significantly, whereas TRAP+ osteoclast number and serum CTX level were unchanged (Fig. 5e).

We also deleted COX2 in osteocytes embedded in the bone matrix from terminal differentiation of osteoblasts to examine PGE2 in the osteoblastic bone-forming microenvironment essential for sensory nerve-induced osteogenesis. Crossing DMP1-cre mice with COX2wt mice generates COX2DMP1−/− mice to eliminate PGE2 secretion by osteocytes. Interestingly, bone phenotype was unchanged in COX2DMP1−/− mice relative to their COX2wt littermates (Fig. 5f). Taken together, these results show that PGE2 in the active bone-forming microenvironment,
largely secreted by osteoblasts, mediates sensory nerve-regulated osteoblastic bone formation.

PGE2 induces hypothalamic CREB signaling for osteogenesis. CREB signaling in the hypothalamus is crucial for the regulation of skeletal homeostasis. To examine whether PGE2 could activate EP4 in sensory nerves through the ventromedial nucleus of the hypothalamus (VMH), we examined the effect of PGE2 on DRG neurons and the phosphorylation of CREB in the VMH of EP4Avil−/− mice. Calcium imaging showed more illuminated DRG neurons in those pre-treated with PGE2 relative to vehicle-treated neurons, whereas DRG neuron activation was reduced significantly in EP4Avil−/− mice with or without PGE2 pre-treatment (Fig. 6a). Western blot analysis of the hypothalamus showed that phosphorylation of CREB increased gradually and peaked at 6 h after injection (Supplementary Figure 12, A). Immunostaining of VMH sections showed that phosphorylation of CREB decreased significantly in EP4Avil−/− mice relative to their WT littermates (Fig. 6b). PGE2 was then injected into EP4Avil−/− mice and their WT littermates to further test whether the central regulation is sensory nerve-dependent. Immunostaining of hypothalamus sections showed CREB phosphorylation increased significantly in mouse VMH 6 h after injection, but this was not observed in EP4Avil−/− mice (Fig. 6c).
whereas, PGE2-induced CREB phosphorylation in VMH was abolished in EP4Avil−/− mice (Fig. 6b). Then, EP1/3 and EP4 agonists were injected to examine whether EP4 receptor is responsible for PGE2-induced CREB signaling in VMH. EP1/3 agonist did not increase phosphorylation of CREB in VMH, whereas EP4 agonist significantly increased pCREB level relative to vehicle-treated mice (Supplementary Figure 12, B), indicating that EP4 in sensory nerves is specific for PGE2-induced CREB phosphorylation in VMH.

The activation of CREB signaling in the hypothalamus has been shown to suppress sympathetic tone10,13. Indeed, uncoupling protein 1 gene (UCP1) expression in adipose tissue and epinephrine concentrations in urine increased significantly in the EP4Avil−/− mice and COX2−/− mice relative to their WT littermates, indicating higher sympathetic tone in these two mouse models (Fig. 6c, d). Immunostaining of the femur sections showed small, flattened osteoblasts on the bone surface (Fig. 6e, f) and reduced Ki67 expression in osterix+ cells in both EP4Avil−/− and EP4Avil+/− mice relative to EP4wt mice (Fig. 6i, j).
Bone fraction (Tb. BV/TV) and trabecular number (Tb. N) of the femurs from 12-week-old EP4wt or EP4Avillox/− mice using antibodies against EP4 (red) and CGRP (green). DAPI stains nuclei blue. Scale bar, 50 μm. Figure 4: Representative μCT images of femurs from 12-week-old EP4wt and EP4Avillox/− mice. Quantitative analysis of trabecular bone fraction (Tb. BV/TV), trabecular number (Tb. N), cortical thickness (Ct. Th), and cortical bone volume (Cor. BV). Figure 5: Deletion of COX2 in osteoblasts leads to reduced bone formation. a Representative μCT images of the femurs of 12-week-old EP4wt and EP4Avillox/− mice. Scale bar, 1 mm. b Quantitative analysis of cortical thickness (Ct. Th) and cortical bone volume (Cor. BV). c Representative images of immunostaining and quantitative analysis of osteoid surface per bone surface (OBS/BS) in femoral bone tissue of 12-week-old EP4wt and EP4Avillox/− mice. Scale bar, 20 μm. d ELISA analysis of serum PGE2 level in 12-week-old EP4wt and EP4Avillox/− mice. e ELISA analysis of serum OCN and CTX levels in 12-week-old EP4wt and EP4Avillox/− mice. f ELISA analysis of serum CTX and OCN levels in 12-week-old EP4wt and EP4Avillox/− mice. g Calcium content (mm) of femoral bone from 12-week-old EP4wt and EP4Avillox/− mice. h Representative images of calcein double labeling of femoral trabecular bone with quantitative analysis of the trabecular bone fraction (Tb. BV/TV) and trabecular number (Tb. N). Scale bar, 20 μm. i, j Ten-week-old EP4wt and EP4Avillox/− mice were injected with vehicle or 3 mg per kg per day PGE2 for 3 consecutive days, and bone samples were harvested 12 days after injection. Calcein was injected 5 days and 1 day before sacrifice. Representative images of calcein double labeling of femoral trabecular bone with quantification of mineral apposition rate (MAR) and bone formation rate (BFR). Scale bar, 20 μm. N ≥ 5 per group. *P < 0.05, **P < 0.01 and N.S. means not significant (Student t-test, except j with ANOVA).
COX2OC−/− and EP4Avil−/− mice, indicating that increased sympathetic tone suppress osteoblastic activity (Fig. 6g, h).To confirm that the increased sympathetic activity leads to bone loss, we injected propranolol, a β2-adrenergic antagonist, into EP4Avil−/− and COX2OC−/− mice. Propranolol partially rescued bone phenotype of these two knockout mice (Fig. 6i and Supplementary Figure 13). These results indicate that sympathetic tone regulates osteoblast differentiation through EP4 activation of sensory nerve.

We also tested if PGE2 secretion is regulated by mechanical loading as mechanical loading has been shown to regulate bone homeostasis through central regulation of sympathetic tone.35,36 Mechanical loading was applied to C57BL/6J mice and bone marrow PGE2 levels were measured. The result showed that PGE2 levels significantly increased in the loading group compared with the control group (Supplementary Figure 14A). COX2 expression in osteoblasts was examined in osteoblastic MC3T3-E1 cells cultured on high-extension silicon rubber dishes with applied compression force to mimic mechanical loads on bone. Western blot analysis showed that COX2 expression increased when the compression force applied (Supplementary Figure 14B). In addition, bone marrow PGE2 levels in COX2OC−/− mice and their littermates with unloading by tail suspension were measured. The results showed that bone marrow PGE2 significantly decreased in wild-type mice in tail suspension conditions while no change was observed in COX2OC−/− mice with (Supplementary Figure 14C). These results suggest that osteoblasts secrete PGE2 secretion in responsible to mechanical loading.

PGE2 promotes skeletal regeneration through sensory nerves. PGE2 has been reported recently to potentiate regeneration of multiple tissues.23 To investigate whether PGE2 induces tissue regeneration through sensory nerves, we assessed whether PGE2 induces bone regeneration. 15-PGDH inhibitor SW033291 was injected into EP4Avil−/− and EPwt mice that had undergone surgical ablation of trabecular bone to examine the effects of an increase in local PGE2 on bone regeneration. Elevation of local PGE2 boosted trabecular bone regeneration significantly in EP4wt.
mice injected with SW033291 relative to vehicle-treated controls, as shown by μCT (Fig. 7a) and hematoxylin-eosin (HE) staining (Fig. 7b). However, the regeneration of trabecular bone by SW033291 was obstructed in EP4Avil−/− mice (Fig. 7a, b).

As known that CD31hi Endomucinhi type H vessel couples with active new bone formation37,38, we further evaluated its expression and found that the type H vessel growth was significantly increased in the regeneration area of EP4wt mice treated with SW033291. However, type H vessels were almost undetectable in EP4Avil−/− mice with or without injection of SW033291 (Fig. 7b). Thus, PGE2 stimulates bone regeneration through sensory nerves.

To further investigate whether PGE2 induces regeneration by sensory nerves of tissues other than bone, we performed partial hepatectomy in TrkAAvil−/− mice and their WT littermates treated with SW033291 or vehicle. BrdU and Ki67 staining of liver sections with partial hepatectomy showed that the regeneration rate decreased significantly in TrkAAvil−/− mice treated with SW033291 relative to their WT littermates (Supplementary Figure 15). Staining of CGRP in the regeneration areas confirmed that sensory innervation in the liver was significantly reduced in TrkAAvil−/− mice. These results show that PGE2 induces bone regeneration dependent on sensory nerves, and that sensory nerves are likely involved in regeneration of various tissues.

**Discussion**

As the largest organ, bone mechanically supports the body. Changes in bone can increase the risk of bone fracture. It is imperative to monitor changes in bone density to maintain bone homeostasis. We have found that PGE2 levels are elevated during decline in bone density in various animal models, including osteoporotic mice. The current study shows that sensory nerves sense bone density through the concentration of PGE2. The signals from PGE2, by bonding with EP4 in the sensory nerves, regulate sympathetic nerve activity for osteoblastic bone formation through the central nervous system. High sympathetic tone is
known to stimulate osteoclastic bone resorption by increasing osteoblast secretion of Rankl (receptor activator of nuclear factor kappa-B ligand). Sensory nerve activated by PGE2 promotes osteoblast proliferation and differentiation by tuning down sympathetic nerve activity. Therefore, the sympathetic nerve can regulate both osteoclastogenesis for bone resorption and osteoblast differentiation for bone formation, depending on the levels of its tone. Interestingly, deletion of sensory nerves did not affect bone development but did reduce bone volume in adult mice. Importantly, sensory nerves regulate bone formation by osteoblast secretion of PGE2 in the bone remodeling microenvironment. Thus, the primary function of sensory nerves in bone is to maintain and protect bone homeostasis by sensing PGE2 in bone. PTH and mechanical loading are known to stimulate the bone remodeling and TGFβ couples the process. These factors of bone remodeling have been reported to increase PGE2 levels in the bone marrow or cultured osteoblasts39–42, indicating that factors in regulation of bone remodeling also promotes osteoblast secretion of PGE2. Moreover, our data and previous report demonstrate that mechanical loading stimulates PGE2 levels in the skeletal system35,36,43. In osteoporotic conditions, the relative mechanical load per bone remodeling unit area increases, which promotes osteoblasts on the bone surface to secrete more PGE2 to stimulate sensory nerves. A recent study showed that an increase in PGE2 through inhibition of its degradation enzyme activity with a small molecule (SW033291) promotes tissue regeneration in various tissues, including liver, intestine, and hematopoietic cells in the bone marrow.25 We too found that the increase in PGE2 caused by SW033291 promotes bone regeneration. Importantly, the effect on bone regeneration was eliminated when EP4 in the sensory nerves was knocked out in EP4Avil/−/− mice. Similarly, liver regeneration was also diminished in TrkAAvil/−/− mice with or without injection of SW033291. It is documented that sensory and sympathetic nerves are both innervated in the liver44, and active neural sprouting was observed after partial hepatectomy, suggesting an essential role of sensory nerves in liver regeneration.45 However, we observed liver regeneration rate was not altered in the EP4Avil/−/− mice in compare with their wild type littermates, neither does SW033291 treatment show any difference between these two groups, indicating that sensory nerve is indispensable in liver regeneration, but the mechanism is not through PGE2-EP4 signaling on sensory nerves. These results suggest that PGE2-EP4 signaling on sensory nerve seems to maintain the integrity of skeletal system specifically.

Clinical studies have reported decreased BMD in patients with long-term use of non-steroidal anti-inflammatory drugs, which inhibit COX2 activity to reduce the production of PGE2.23 Evidence shows that PGE2 stimulates osteoblast differentiation by activating cAMP signaling through G protein–coupled EP4 receptor.18 However, knockout of EP4 receptor in osteoblasts with no skeletal phenotype indicates that PGE2-induced bone formation is not caused by direct signaling through osteoblasts. Our data show that deletion of EP4 receptor in peripheral sensory nerves leads to decreased bone density, and that injection of PGE2 is no longer able to stimulate bone formation. It has been well documented that sympathetic tone was tuned down with PGE2 injection peripherally, and sympathetic nerve action on bone metabolism has been shown through hypothalamic regulation12,13,16,46,47. Indeed, we show that injection of PGE2 stimulated phosphorylation of CREB in the hypothalamus, which was inhibited by knockout of EP4 in the peripheral sensory nerves (EP4Avil/−/−), which is the evidence of CNS involvement. More than 10% of clinical patients with central nervous system injury were observed to have heterotopic ossification.48 These findings show that sensory nerve signals from PGE2 in bone remodeling sites are circled back through regulation by sympathetic nerves. However, it is still unclear whether PGE2 signaling from the bone remodeling sites regulate sympathetic tone through DRG, hypothalamus or both and feedback regulation of sympathetic activity is spatially specific. Our data suggest that the feedback is likely involved both DRG and hypothalamus. It is documented that sensory signals from the viscera are carried to the central nervous system by spinal and cranial afferents.49 Sensory afferents from visceral and somatic sensory nerves converge onto common second-order neurons within the spinal dorsal horn, and a subset of these neurons convey the convergent signals to the diencephalon via the anterolateral spinothalamic tract50,51. This viscerotopically organized pathway provides direct and relayed inputs to the hypothalamus and other central nervous locations52,53. Therefore, the similar pathway is possibly employed in the PGE2-induced CREB phosphorylation in the hypothalamus. Herein, sensory nerve regulation appears to involve precise temporal-spatial action controlled by sympathetic nerves in the bone remodeling microenvironment, and the central nervous system likely coordinates the activities of different tissues for energy and calcium metabolism of bone formation.

**Methods**

**Mice and in vivo treatment.** The iDTRfl/− and Dentin matrix acidic phosphoprotein 1-Cre (DMP1-Cre) mice were purchased from the Jackson Laboratory. The Advillin-Cre (Avil-Cre) mouse strain was kindly provided by Xinzhi Dong (The Johns Hopkins University). The osteocalcin-Cre (OC-Cre) mice were obtained from Thomas J. Clemens (The Johns Hopkins University). The TrkAfl/− mice were obtained from David B. Ginty (Harvard Medical School). The COX2fl/− mice were provided by Harvey Herschman (University of California, Los Angeles). The EP4fl/− mice were obtained from Brian L. Kelsall (National Institutes of Health). Hypozygous male Avil-Cre mice (female Avil-Cre mice were not used to breed in case for the leakage of TrkA protein into the eggs) were crossed with a TrkAfl/−, EP4fl/−, or iDTRfl/− mouse. The offspring were intercrossed to generate the following genotypes: wild type (referred as WT in the text), Avil-Cre (Cre recombinase driven expressed by Advillin promoter), TrkAfl/− (mice homozygous for TrkA flox allele are referred to as TrkAfl/− in the text), EP4fl/− (referred to as EP4fl/− in the text), iDTRfl/− (referred to as iDTRfl/− in the text), Avil-Cre: iDTRfl/− (mice referred to as iDTRfl/− in the text). Hypozygous OC-Cre or DMP1-Cre mice were crossed with a COX2fl/− mouse; the offspring were intercrossed to generate the following genotypes: WT, OC-Cre, DMP1-Cre, COX2fl/− (referred as COX2fl/− in the text), OC-Cre: Avil-Cre (referred to as OC-Cre: Avil-Cre in the text), and DMP1-Cre:COX2fl/− (referred to as COX2fl/− in the text). Mice homozygous for TrkAox/− (mice homozygous for TrkAox/− in the text) were intercrossed to generate the following genotypes: WT, OC-Cre, DMP1-Cre, COX2fl/− (referred to as COX2fl/− in the text), OC-Cre: Avil-Cre (referred to as OC-Cre: Avil-Cre in the text), TrkAox/− (referred to as TrkAox/− in the text), and DMP1-Cre:COX2fl/− (referred to as COX2fl/− in the text). Mice homozygous for EP4fl/− mouse, the offspring were intercrossed to generate the following genotypes: WT, OC-Cre, DMP1-Cre, COX2fl/− (referred to as COX2fl/− in the text), OC-Cre: Avil-Cre (referred to as OC-Cre: Avil-Cre in the text), and DMP1-Cre:COX2fl/− (referred to as COX2fl/− in the text). Mice homozygous for EP4fl/− mouse, the offspring were intercrossed to generate the following genotypes: WT, OC-Cre, DMP1-Cre, COX2fl/− (referred as COX2fl/− in the text). The genotypes of the mice were measured by PCR analyses of genomic DNA, which was extracted from mouse tails within the following primers. Avil-Cre: forward: CTCCTGTTCTGTTGCATGTTG, Reverse: CGGATCCCTAACTATGCTCAT, WT: GACTGATCTGGTTAGCTCCAG, OC-Cre: forward: CAAATAAGCC CTCCTGAGATCC, Reverse: TGATACAGGAGCAATCTTC, DMP1-Cre: forward: GGAGCGCACATCAGTCTGGC, Reverse: TCTTGGAAGGAGGACTTTATG, iDTR: forward: CTGCCACCGGGTTCTTCAC, Reverse: GCCACGCTTTCTCTCCCAAGGAACOX2loxP allele forward: ATTATTGCTGTGAAAGCCACC, Reverse: GAATTCTCGAGAACGTCAGGACTTTATG, iDTRloxP allele forward: AACAGTGTGGACATTCCTTTCTATGG, Reverse: CAAAGAAGAACGAGAATTAATAC, iDTRloxP allele forward: GAGGAGAAGAAGTTTCAATAC, Reverse: AAAGTTGCTGCTTCAGTTTAT. 8 to 12-week-old C57BL/6 female mice (Jackson Lab) were anesthetized and underwent bilateral O VX or a sham operation from back age. The aged mice (12 months old) were purchased from The Jackson Laboratory. All animals were maintained at the animal facility of The Johns Hopkins University School of Medicine. All animal experimental protocols were complied with all relevant ethical regulations and approved by the Animal Care and Use Committee of The Johns Hopkins University, Baltimore, MD, USA. We obtained whole blood samples by cardiac puncture immediately after euthanasia. Serum was collected by centrifuge at 200 x g for 15min and stored at −80 °C before analyses. Femurs, tibias, and urine of the mice were also collected.

The drugs and compounds used in this study are as follows: diphtheria toxin (DTX, Sigma-Aldrich, D0564); PGE2 (Cayman Chemical, 14010); EP1/3 agonist (Cayman Chemical, 14810); EP4 agonist (Cayman Chemical, 10586); propranolol (Sigma-Aldrich, A7257); norepinephrine (Sigma-Aldrich, A8255); and SW033291 (Selleck, S7900). Dosages and time courses are noted in the corresponding text and figure legends.
Behavioral analysis. Pole tests and grip strength tests were performed to evaluate motor neural activity changes in TrkAAvilex-/-,
EP4KO-/-, and COX2KO-/- mice. All tests were performed between 16:00 and 18:00. In the 180°-landing test, the mice were placed on the edge of a hollow cylinder (diameter 12 cm, height 20 cm). The animals were placed on the edge of the cylinder, facing away from the center. At the signal, each animal was released from the edge, and the latency to fall was recorded. The peak holding strength was recorded digitally and displayed in grams.

μCT analyses. The femurs were harvested from mice, and the soft tissue around the bone was removed, followed by fixation overnight using 4% paraformaldehyde. μCT analyses were performed by using a high-resolution μCT scanner (SkyScan, 1174). The voltage of the scanning procedure was 65 kV with a 153-μA current. The resolution was set to 8.7 μm per pixel. Reconstruction software (NIRecon, v1.6, SkyScan), data analysis software (CTAn, v1.9, SkyScan), and 3D modeling visualization software (CTVid, v2.0, SkyScan) were used to analyze the diaphyseal cortical bone and the metaphyseal trabecular bone parameters of the femurs. We created cross-sectional images of the femur to perform 2D analyses of the cortical bone and 3D analyses of the trabecular bone. The region of interest (ROI) of the trabecular bone was drawn beginning from 5% of the femur length proximal to the distal metaphyseal growth plate and extending proximally for another 5% of the total femur length. The trabecular bone volume fraction (BV/TV), trabecular thickness (Tb. Th), trabecular number (Tb. N), and trabecular separation (Tb. Sp) were measured.

Immunohistochemistry, immunofluorescence and histomorphometry. The femurs were collected and fixed in 4% paraformaldehyde overnight and decalcified by using 10% EDTA (pH 7.4) (Amresco, 1105) for 21 days. The samples were then dehydrated with 30% sucrose for 24 h and embedded in paraffin or optimal cutting temperature compound (Sakura Finetek). We prepared 4-μm-thick coronal-oriented sections of the femur for hematoxylin and eosin staining. The femurs were fixed for 4 h with 4% paraformaldehyde at 4°C and then decalcified at 4°C using 0.5M EDTA (pH 7.4) for 24 h with constant shaking. The samples were dehydrated in 20% sucrose and 2% polyvinylpyrrolidone (PVP) solution for 24 h and embedded in 8% gelatin (Sigma-Aldrich, G1900) in the presence of 20% sucrose and 2% PVP. Forty-μm-thick coronal-oriented sections of the femurs were obtained. For brain section preparation, the whole brain was collected from euthanized mice and fixed with 4% paraformaldehyde for 30 min. Then, the tissue was dehydrated with 20% sucrose for 24 h andsnap-frozen.

Bone and liver regeneration models. Mice underwent general anesthesia. The bone regeneration model was established as described below. A longitudinal incision was made on the back of each mouse. The rib, sternum, and diaphragm were retracted, and a 1-cm-long window was made on each rib to expose the ribs. The rib margin was resected, and the rib was retracted to expose the abdominal cavity. A 1-cm-long window was made on the abdominal wall to expose the liver. The remnant livers were harvested after mice sacrificed. All data analyses were performed using SPSS, version 15.0, software.

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