Supporting Information

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SI Materials and Methods

Animals. Targeted mutagenesis of the *Tph1* (tryptophan hydroxylase 1) gene was described previously (1). Briefly, exon 2 of the *Tph1* locus was substituted by the nlslacZneopolyA cassette. Male mice with an Sv129 background were studied. These mice are viable, and have normal levels of serotonin or 5-hydroxytryptamine (5-HT) in the brain. $TPH_1^{-/-}$ mice had markedly decreased whole-blood serotonin levels and identical size and weight as WT mice. The mice were housed under controlled conditions at 24 °C with 24 h of light, and were allowed free access to food and water in full compliance with French government animal welfare policy. All of the experiments were approved by the Ethics Committee of Paris Diderot University. To evaluate the dynamic bone formation parameters histomorphometrically, the mice were given two fluorochrome markers by i.p. injection: 20 mg/kg tetracycline (Sigma-Aldrich) and 20 mg/kg calcein (Sigma-Aldrich), at an interval of 2 d.

Measurement of Bone Mineral Density by Dual-Energy X-Ray Absorptiometry. Dual-energy X-ray absorptiometry (DXA) analysis was carried out under anesthesia. Total-body, whole-femur, and caudal vertebral bone mineral density (BMD) (mg/cm^2) were measured using a PIXImus Instrument (Lunar; software version 1.44) in ultra–high-resolution mode (resolution 0.18×0.18 mm). The precision and reproducibility of the instrument had previously been evaluated by calculating the coefficient of variation of repeated DXA measurements. The coefficient of variation was <2% for all of the parameters evaluated. A phantom was scanned daily to monitor the stability of the measurements.

Histomorphometry and Microcomputed Tomography Analysis. The animals were killed by exsanguination under anesthesia and the left femur was excised, and the surrounding soft tissue was cleaned off. After fixation in 70% ethanol at 4 °C, femurs were trimmed and the distal halves of bones were postfixed in 70% ethanol, dehydrated in xylene at 4 °C, and embedded without demineralization in methyl methacrylate. Histomorphometric parameters were measured in accordance with American Society for Bone and Mineral Research nomenclature (2, 3) on 5-mm-thick sections using a Nikon microscope interfaced with a software package from Microvision Instruments. For tartrate-resistant acid phosphatase (TRAP) detection, sections were stained with 50 mM sodium tartrate and naphthol AS-TR phosphate (Sigma-Aldrich). Measurements of the trabecular bone were performed in a region of the secondary spongiosa. Sections were stained with aniline blue. In particular, we measured the trabecular bone volume (BV/TV), trabecular bone thickness (Tb.Th), and trabecular separation (Tb.Sp). For cortical bone, we measured the average bone and marrow diameters at the femoral metaphysis and calculated the cortical thickness (Cort.Th). Dynamic parameters were determined on two 10-mm-thick unstained sections examined under UV light (4).

Resorption and Formation Marker Measurements. Accurate deoxypyridinoline (DPD) measurement requires overnight fasting before urine collection. DPD was measured with IMMULITE Pyrilinks-D in vitro diagnostic reagent (Diagnostic Products). To correct for flow variations, the DPD results were normalized to the urinary creatinine concentration (ADVIA System; Siemens). Quantitative determination of osteocalcin and TRAcP5b levels in serum were performed by immunoradiometric assay and by an ELISA from IDS Immunodiagnostic Systems, respectively.

In Vitro Osteoclastogenesis. In vitro osteoclastogenesis was performed according to Biosse-Duplan et al. (5), with modifications. Briefly, spleen cells were isolated from 6-wk-old WT and $TPH_1^{-/-}$ male mice and a cell suspension was obtained using a 40-μm nylon mesh cell strainer. After a red blood cell lysis step, cells were washed twice with α-MEM and seeded in 0.4 mL α-MEM with 8% fetal bovine dialyzed serum in eight-well plates (1.10^6 cells/cm^2). After being allowed to attach overnight, the cultures were exposed to M-CSF for 4 d (Peprotech) (25 ng/mL), and then supplemented with M-CSF and receptor activator for NF-KB-ligand (RANKL) (Peprotech) (50 ng/mL) for a further 5 d of culture (day 0 was the day of addition of RANKL). Cultures were fed every 3–4 d by replacing the medium with fresh medium. In this system, splenocytes proliferate, differentiate into mononuclear TRAP-positive cells, and fuse to form osteoclast-like cells (OCLs) after 5 d in the presence of RANKL. Osteoclastogenesis was also assessed from bone marrow macrophages cultured for 4 d with M-CSF and RANKL at the same concentration, according to Takeshita et al. (6). For resorption assays, spleen cells (1.10^5 cells per well) or bone marrow macrophages were plated on dentin slices. Briefly, the slices were cleaned in α-MEM and then placed in 96-well plates. Spleen cells were added to each well, cultured in complete medium with M-CSF (25 ng/mL) and RANKL (100 ng/mL), and maintained for 14 d. The last 24 h of culture were carried out in the presence of 10% CO_2. Bone marrow macrophages were plated and cultured under the same conditions for 4 d. The slices were then recovered and either stained for TRAP cell determination or cleaned by ultrasonication and stained with toluidine blue for pit measurement. The number of resorption lacunae (pits) and TRAP-positive cells on each slice was counted under a light microscope. The ratio pit area:osteoclast was determined.

RNA Extraction and Real-Time Quantitative PCR. For the preparation of RNA from long bones, the soft tissues surrounding the bones were stripped off. The epiphyses were cut off, and the bone marrow was flushed out with PBS. Total RNAs were isolated using TRIzol reagents (Invitrogen) according to the manufacturer's instructions. For mRNA extraction from cell cultures, we used an RNeasy Lipid Tissue Mini Kit (Qiagen). Total RNAs were then reverse-transcribed into cDNA using a cDNA Verso Kit (Abgene). Quantitative real-time PCR expression analysis was performed on a Light Cycler 480 (Roche Diagnostics) using Absolute SYBR Green Capillary Mix (Abgene) at 60 °C for 40 cycles. Primers were designed from the online mouse library probes of Roche Diagnostics: for the bone resorption markers: TRAP (forward: 5′-cgtctctgcacagattgcat-3′; reverse: 5′-aagcgccaaacggtagtaagg-3′), Cathepsin K (forward: 5′-cgaaaagagcctagcgaaca-3′; reverse: 5′-tgggtagcagcagaaacttg-3′), NFATc1 (forward: 5′-tccaaagtcattttcgtgga-3′; reverse: 5′-ctttgcttccatctcccaga-3′), and RANK (forward: 5′-gtgctgctcgttccactg-3′; reverse: 5′-agatgctcataatgcctctcct-3′); for the serotonin system: TPH_1 (forward: 5′-cacagttcagatccccctctaca-3′; reverse: 5′-gaacgtggcctaggagttca-3′), and 5-HT transporter (SERT) (forward: 5′-ctgtttctcctgttcatcatttgcag-3′; reverse: 5′-cagtagcccaagatgatactccagtg-3′). mRNA levels were normalized using Aldolase (forward: 5′-tgaagcgctgccagtatgtta-3′; reverse: 5′-ggtcgctcagagccttgtaga-3′), HPRT (hypoxanthine guanine phospho ribosyl transferase) (forward: 5′-gttggatatgcccttgactataatga-3′; reverse: 5′-caacatcaacaggactcctcgtatt-3′), SDH_2 (succinate dehydrogenase 2) (forward: 5′-tggacatcaagactggcaagg-3′; re-

verse: 5′-cagtaggagcggatagcaggag-3′), and TBP (Tata box binding protein) (forward: 5′-cacggacaactgcgttgattt-3′; reverse: 5′-gctgctag-tctggattgttcttca-3′) as housekeeping genes. These primers were designed by R. Olaso (Centre National de Génotypage, Evry, France). The mRNA expression levels of the different 5-HT receptors and vesicular monoamine transporters (VMATs) were analyzed by means of a Taqman assay; the probes were purchased from Applied Biosystems.

Ex Vivo Treatments. Cells were treated with 5-HT (5, 10, and 20 nM), paroxetine (10 nM), reserpine (50, 100, and 200 nM), and various antagonists of 5-HT receptors such as ritanserin (100 nM), ketanserin (25, 50, and 100 nM), and SB224289 (10, 50, and 100 nM). All these chemicals were purchased from Sigma-Aldrich. RS127445 (20 nM) was a generous gift of M. McNamara (Syntex, Palo-Alto, CA).

1. Côté F, et al. (2003) Disruption of the nonneuronal tph1 gene demonstrates the importance of peripheral serotonin in cardiac function. *Proc Natl Acad Sci USA* 100: 13525–13530.
2. Parfitt AM (1988) Bone histomorphometry: Proposed system for standardization of nomenclature, symbols, and units. *Calcif Tissue Int* 42:284–286.
3. Parfitt AM, et al. (1987) Bone histomorphometry: Standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 2:595–610.
4. Collet C, et al. (2008) The serotonin 5-HT2B receptor controls bone mass via osteoblast recruitment and proliferation. *FASEB J* 22:418–427.
5. Biosse-Duplan M, Baroukh B, Dy M, de Vernejoul MC, Saffar JL (2009) Histamine promotes osteoclastogenesis through the differential expression of histamine receptors on osteoclasts and osteoblasts. *Am J Pathol* 174:1426–1434.
6. Takeshita S, Kaji K, Kudo A (2000) Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. *J Bone Miner Res* 15:1477–1488.

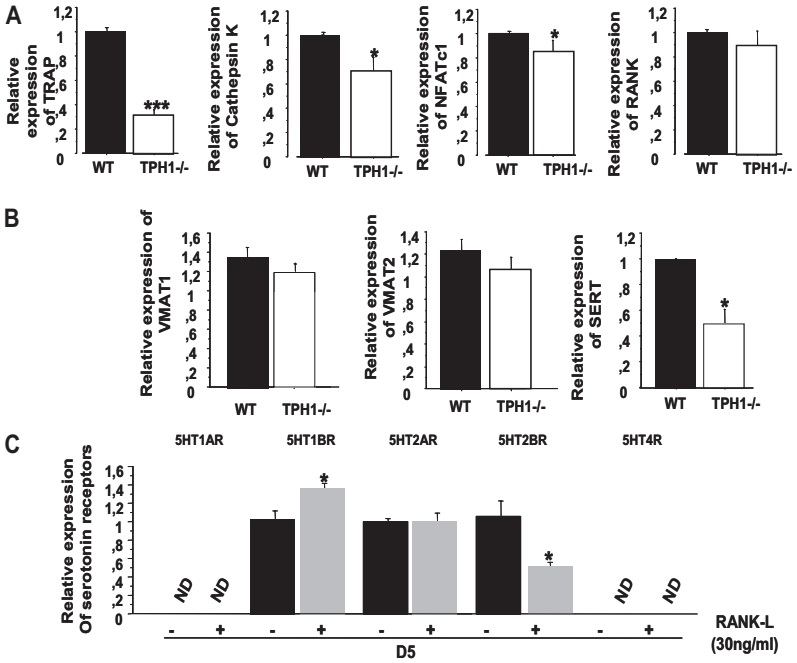

Fig. S1. Analysis of real-time PCR. (*A*) Total RNA was isolated at the end of the culture. Relative expression levels of TRAP, Cathepsin K, NFATc1, and RANK were determined by real-time PCR. Only the expression of RANK was the same in both groups, whereas TRAP, NFATc1, and Cathepsin K expression levels were all significantly lower in $TPH_1^{-/-}$ (white bars) than in WT (black bars). (*B*) Relative mRNA expression levels of VMAT1 and VMAT2 were determined by RT-PCR at the end of the culture in the presence of M-CSF and RANKL. No significant differences were found between the two genotypes. Relative mRNA expression of SERT was measured in WT and $TPH_1^{-/-}$ cells at the end of culture in the presence of M-CSF and RANKL. A significant decrease of SERT mRNA expression was observed in $TPH1^{-/-}$ culture. (*C*) Total RNAs were isolated at the end of the culture in the presence of M-CSF with or without RANKL, and the relative expression of several serotonin receptors was measured. 5-HT_{1A} and 5-HT_4 receptors were not detected (ND). 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2B} receptors were present in the WT cell cultures, in the absence (black bars) or presence (gray bars) of RANKL. $5HT_{1B}R$ was significantly increased in the presence of RANKL. $*P < 0.01$ versus WT, $**P < 0.001$ versus WT, $***P < 0.0001$ versus WT.

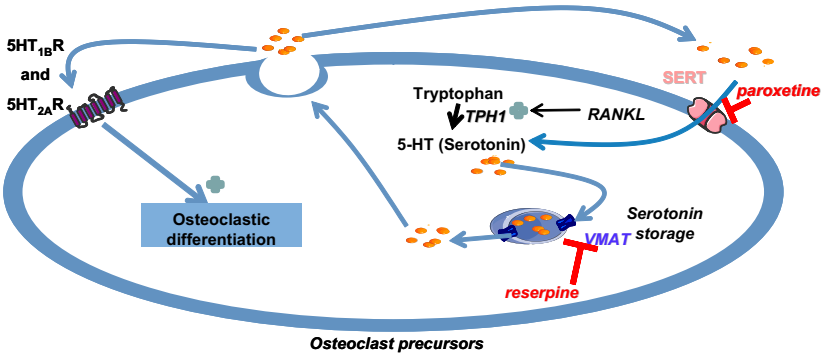

Fig. S2. A working hypothesis concerning the role of serotonin in osteoclast differentiation. Serotonin is synthesized in osteoclast precursors, and then stored in intracellular granules before being released outside the cell. Serotonin can act via an autocrine/paracrine pathway by activating 5-HT_{1B} and 5-HT_{2A} receptors leading to an increase in osteoclast differentiation.