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Inhibition of the Serotonin (5-Hydroxytryptamine) Transporter Reduces Bone Accrual during Growth

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Selective serotonin-reuptake inhibitors (SSRIs) antagonize the serotonin (5-hydroxytryptamine) transporter (5-HTT), and are frequently prescribed to children and adolescents to treat depression. However, recent findings of functional serotonergic pathways in bone cells and preliminary clinical evidence demonstrating detrimental effects of SSRIs on bone growth have raised questions regarding the effects of these drugs on the growing skeleton. The current work investigated the impact of 5-HTT inhibition on the skeleton in: 1) mice with a null mutation in the gene encoding for the 5-HTT; and 2) growing mice treated with a SSRI. In both models, 5-HTT inhibition had significant detrimental effects on bone mineral accrual. 5-HTT null mutant mice had a consistent skeletal phenotype of reduced mass, altered architecture, and inferior mechanical properties, whereas bone mineral accrual was impaired in growing mice treated with a SSRI. These phenotypes resulted from a reduction in bone formation without an increase in bone resorption and were not influenced by effects on skeletal mechanosensitivity or serum biochemistries. These findings indicate a role for the 5-HTT in the regulation of bone accrual in the growing skeleton and point to a need for further research into the prescription of SSRIs to children and adolescents. (*Endocrinology* 146: 685–693, 2005)

MAJOR DEPRESSIVE DISORDER (MDD) is well established to involve the monoamine neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) and the 5-HT transporter (5-HTT) (1, 2). The 5-HTT acts as a key regulator of serotonergic activity by its uptake of 5-HT from the extracellular space. By antagonizing the 5-HTT, using selective serotonin-reuptake inhibitors (SSRIs), serotonergic activity can be potentiated and the symptoms of MDD relieved. It is estimated that up to 10% of children and adolescents suffer from depression (3). On the basis of their good safety profile and established efficacy in the treatment of adults with MDD, SSRIs are routinely cited as the best available treatment option in depressed children and adolescents (4, 5). However, serious questions have been raised regarding the prescription of SSRIs to these age groups. Although these questions primarily result from suggestions that SSRIs potentially increase suicidal ideation in children and adolescents (6–9), an additional unresolved issue is the impact of SSRIs on bone mineral accrual in the growing skeleton. The later is important because the bone mass attained early in life is perhaps the most important determinant of lifelong skeletal health (10).

Questions regarding the effect of SSRIs on the growing skeleton have been stimulated by recent findings of functional serotonergic pathways in bone (11–14) and preliminary clinical evidence demonstrating detrimental effects of SSRIs on the skeleton (15–17). The 5-HTT has been found to be both present and highly specific for 5-HT uptake in all of the major bone cell types (osteoblasts, osteocytes, and osteoclasts). Inhibition of the 5-HTT in these cells using SSRIs influences their function *in vitro* (11–13). In addition to the 5-HTT, osteoblasts, osteocytes, and periosteal fibroblasts (a cell population containing osteoblast precursor cells) also possess functional receptors for 5-HT, the stimulation of which modulates bone cell activity (12–14). The presence of functional serotonergic pathways in bone cells enables SSRIs to potentially influence skeletal biology. This is supported by preliminary clinical evidence that has demonstrated SSRI use to be associated with increased bone loss at the hip in elderly women (15), decreased bone mineral density (BMD) among men (16), and decreased skeletal growth in children (17).

The aim of the current work was to investigate the impact of 5-HTT inhibition on bone mineral accrual in the growing mouse skeleton. This was achieved by assessing: 1) mice with a null mutation in the gene encoding for the 5-HTT; and 2) normal growing mice treated with a SSRI. The former animals are considered a model of chronic SSRI use (18, 19), and

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Abbreviations: BFR, Bone formation rate; BMC, bone mineral content; BMD, bone mineral density; BS, bone surface; BV, bone volume; CON, vehicle-treated control; DXA, dual-energy x-ray absorptiometry; ES, eroded surface; HIGH, high-dose (20 mg/kg) fluoxetine treated; 5-HT, serotonin or 5-hydroxytryptamine; 5-HTT, 5-hydroxytryptamine transporter; 5-HTT $^{-/-}$, 5-HTT homozygous mutant mice; 5-HTT$^{+/+}$, 5-HTT homozygous wild-type mice; I_{MAX}, maximum second moment of area; I_{MIN}, minimum second moment of area; I_P, polar moment of inertia; LOW, low-dose (5 mg/kg) fluoxetine treated; MAR, mineral apposition rate; MDD, major depressive disorder; MS, mineralizing surface; OcS, osteoclast surface; PLSD, protected least-significant difference; r, relative value (*e.g.* rBFR/BS, rMAR); SSRI, selective serotonin-reuptake inhibitor; TV, tissue volume.

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initial work has shown that these mice may exhibit a skeletal phenotype (20).

Materials and Methods

Animals

The effect of gene-mediated disruption of 5-HTT functioning on the skeleton was investigated using 5-HTT homozygous mutant (5-HTT$^{-/-}$) mice with a CD-1 background. These were generated by replacing exon 2 of the 5-HTT gene with a PGK-neo gene cassette using homologous recombination, as previously described (18). Control animals consisted of homozygous wild-type (5-HTT$^{+/+}$) littermates. Genotype was determined by PCR amplification of tail DNA. The effect of pharmacological inhibition of the 5-HTT on bone mineral accrual was investigated using 30 virgin female C57BL/6J mice purchased at 3 wk of age from The Jackson Laboratories (Bar Harbor, ME). Mice were maintained under standardized environmental conditions with *ad libitum* access to standard mouse chow and water. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Indiana University.

Effect of null mutation of the 5-HTT gene on the skeleton

5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice were either 4 (young) or 19 (adult) wk of age when killed. Dual-energy x-ray absorptiometry (DXA) was performed after death using a PIXImus II mouse densitometer (Lunar Corp., Madison, WI) to measure whole-body (minus tail) bone mineral content (BMC); and the cranium, lumbar spine (L2–L5), and femurs were harvested for further analyses (see below). In addition, serum was collected from young mice for assessment of serum biochemistry. To permit calculation of bone formation rates (BFRs) in young mice, injections of calcein (30 mg/kg body mass; Sigma Chemical Co., St. Louis, MO) were administered 7 and 3 d before death. To investigate the role of the 5-HTT in mechanotransduction, the right ulnas of adult mice were mechanically loaded before death (at 16 wk of age) using the ulna axial loading model (21). A preliminary load-strain calibration experiment was performed as previously described (22), and loads in experimental animals were manipulated to expose ulnas, within each genotype, to an identical mechanical stimulus (3800 $\mu\epsilon$). A 2-Hz haversine waveform for 120 cycles/d on 3 consecutive days was used. Left ulnas served as an internal control and were not loaded. Normal cage activity was allowed between loading sessions; ip injections of calcein (30 mg/kg body mass; Sigma Chemical Co.) were administered 4 and 9 d after the first loading day, and animals were killed 18 d after initial loading (at 19 wk of age). Ulnas were processed, and histomorphometric indices of bone formation [mineralizing surface (MS)/bone surface (BS), mineral apposition rate (MAR), and BFR/BS] were measured, as previously described (22).

Effect of inhibition of the 5-HTT on the growing skeleton

At 4 wk of age, animals were divided into three intervention groups: 1) vehicle (sterile saline)-treated controls (CON); 2) low-dose fluoxetine hydrochloride (5 mg/kg)-treated (LOW); and 3) high-dose fluoxetine hydrochloride (20 mg/kg)-treated (HIGH). Fluoxetine hydrochloride (Sigma-Aldrich, Inc.) was chosen as the SSRI for use because it has been shown to have a favorable risk-benefit profile in children and adolescents (23–25) and is currently the only SSRI that has received United States Food and Drug Administration labeling as safe and effective for the treatment of MDD in children and adolescents. The drug doses were chosen on the basis of previous reports of the antiimmobility effects of fluoxetine in mice (19, 26, 27). Fluoxetine was mixed in vehicle (sterile saline), and animals in each group received their respective intervention via ip injection (6.7 ml/kg) at the same time daily for 4 continuous wk. At baseline and at weekly intervals, *in vivo* DXA, using a PIXImus II mouse densitometer (Lunar Corp.), was performed to measure whole-body (minus tail), cranial, and whole-right-hindlimb (femur, tibia, and foot) BMC. In addition, because fluoxetine has been shown to influence food intake (28) and activity levels (27, 29) in mice, food intake per gram of body weight was assessed over a 7-d period during the third week of intervention, and activity levels were assessed during the fourth week of intervention. The latter were assessed by treating each animal with its respective intervention in its home cage and 30 min later placing it in a novel illuminated test environment (VersaMax Animal Activity Monitoring System; AccuScan Instruments, Inc., Columbus, OH) for a 10-min measurement period. The total distance traveled by each mouse was recorded from the number of laser beam breaks during the test period. Animals were killed after 4 wk of treatment (8 wk of age); and the cranium, lumbar vertebrae (L2–L5), and femurs were removed for further analyses (see below).

BMC

DXA was performed using a PIXImus II mouse densitometer (Lunar Corp.) with ultra-high resolution (0.18 × 0.18 mm/pixel) to measure *ex vivo* whole-cranial, spinal (L2–L5), and femoral BMC; whereas a Norland Medical Systems Stratec XCT Research SA+ peripheral quantitative computed tomography machine (Stratec Electronics, Pforzheim, Germany) was used to assess localized femoral volumetric BMD and BMC. The latter involved taking a transverse midshaft scan as representative of a predominantly cortical site and a transverse scan through the distal femur as a site that was predominantly trabecular. The distal femoral scans were 25.0% and 12.5% proximal from the distal end of the bone in young (including fluoxetine-treated) and adult femurs, respectively. All scans were performed using a 70-μm voxel size, and the bone edge was detected within the Stratec software using contour mode 1 with a threshold of 400 mg/cm^3. Cortical bone parameters were recorded from midshaft scans, and total (cortical and trabecular) bone parameters from distal scans. Distal scans were not separated into cortical and trabecular compartments due to the large voxel size relative to distal femoral bone thickness, and thus potential for partial-volume effects.

Bone architecture

Femoral midshaft geometry was assessed using a desktop microcomputed tomography machine (μCT-20; Scanco Medical AG, Auenring, Switzerland). A single transverse midshaft slice was acquired using a 7-μm voxel size. The slice image was imported into NIH Image 1.62 for the Macintosh (National Institutes of Health, Bethesda, MD); wherein cortical area and thickness, periosteal and endosteal perimeters, and the maximum (I_{MAX}) and minimum (I_{MIN}) second moments of area were determined using standard and customized macros. The polar moment of inertia (I_P) was derived as the sum of the I_{MAX} and I_{MIN} measurements.

Mechanical properties

Mechanical properties of the femoral midshaft were determined as described previously (30). Femurs were positioned, cranial side up, across the lower supports of a miniature materials-testing machine (Vitrodyne V1000; Liveco, Inc., Burlington, VT). The supports had a span width of 6.0 mm and 9.0 mm for young and adult femurs, respectively. Bones were fixed in place, with a static preload of approximately 0.1 N, before being loaded to failure in three-point bending using a cross-head speed of 0.2 mm/sec. During testing, force and displacement measurements were collected every 0.01 sec. From the force *vs.* displacement curves, ultimate force, stiffness, and energy to ultimate force were derived.

Histomorphometry

The femur, fifth lumbar (L5) vertebrae, and cranium from young mice, as well as adult femur, were processed for histomorphometry. Bones were fixed in 10% neutral buffered formalin before being embedded undecalcified in methyl methacrylate (Aldrich Chemical Co., Inc., Milwaukee, WI). From the young midshaft femur and cranium, thick (~50 μm) sections were taken using a diamond-embedded wire saw (Histo-saw; Delaware Diamond Knives, Wilmington, DE). Sections were mounted unstained and ground to a final thickness of approximately 20 μm to enable assessment of femoral and cranial (parietal) BFRs. From the young and adult distal femurs and young L5 vertebrae, thin (4 μm) sections were taken using a microtome (Reichert-Jung 2050; Reichert-Jung, Heidelberg, Germany). Sections from the L5 vertebrae were mounted unstained to enable determination of trabecular BFRs, whereas sections from the young and adult distal femurs were stained with McNeal's tetrachrome to enable assessment of trabecular bone structure. One section per bone was viewed on a Leitz DMRXE micro-

scope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany) and the image captured using a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Histomorphometric indices were measured using Image Pro Plus v.4.1 software (Media Cybernetics, Silver Spring, MD). From unstained sections, MS/BS, MAR, and BFR/BS were derived. From stained sections, trabecular bone volume [bone volume (BV)/tissue volume (TV)], thickness, number and spacing, and osteoclast surface (OcS)/BS and eroded surface (ES)/BS were determined. All measurements and calculations were performed according to the guidelines of the American Society for Bone and Mineral Research (31).

Serum assays

Sera were collected from young (6–10 wk old) 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice. All samples were analyzed in one assay per analyte. Serum creatinine, total calcium, phosphorus, and albumin were measured on a Roche Integra 800 Analyzer (Roche Diagnostics, Indianapolis, IN). Serum IGF-I and TSH levels were measured as described previously (32, 33). For serum C-terminal telopeptide fragments of type-I collagen cross-links, sera were analyzed in singlicate with the Rat Laps kit (Nordic Bioscience Diagnostics A/S, Herlev, Denmark), as per the manufacturer's instructions. The detection limit was 2.0 ng/ml. Serum PTH levels were measured in singlicate by the Mouse Intact PTH ELISA kit (Immutopics San Clemente, CA), according to the manufacturer's instructions. The detection limit was 3 pg/ml. Serum 17β-estradiol and testosterone were measured in duplicate by enzyme linked immunoassay (Diagnostic Automation Inc., Calabasas, CA), as per the manufacturer's instructions. The detection limit was 10 pg/ml and 50 pg/ml for 17β-estradiol and testosterone, respectively.

Statistics

Statistical analyses were performed with the Statistical Package for Social Sciences (SPSS 6.1.1; Norusis/SPSS Inc., Chicago, IL) software. All comparisons were two-tailed, with a level of significance set at 0.05, unless otherwise specified. To compare bone mass, architecture, strength, and histomorphometric measurements in 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice, two-way factorial ANOVAs (genotype $\times$ sex) were used. Mechanical loading effects on histomorphometric values from right (loaded) and left (nonloaded) ulnas were tested for significance using paired t tests. To determine genotype influences on the response to mechanical loading, individual differences in systemic factors were controlled by subtracting left ulna values from right ulna values. This generated a new set of relative (r) values for each variable (*i.e.* rMS/BS, rMAR, and rBFR/BS) which were compared using two-way factorial ANOVAs (genotype $\times$ sex). Genotype influences on serum biochemistries were compared using unpaired *t* tests. The effects of fluoxetine hydrochloride on bone mineral accrual and BFRs were determined using one-way ANOVAs followed by Fisher's protected least-significant difference (PLSD) for pairwise comparisons.

Results

Animal characterization

Life-long disruption of 5-HTT functioning via null mutation of its gene did not result in any gross morphologic abnormalities or obvious skeletal deformations. 5-HTT$^{-/-}$ mice gained weight and were equivalent in body weight to 5-HTT$^{+/+}$ littermates at 4 and 19 wks of age (Table 1). Skeletal development and longitudinal bone growth were not influenced by genotype, with no differences being observed in femoral length, distal femoral growth plate height, or cranial size between 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice. Similarly, temporary inhibition of the 5-HTT, using fluoxetine hydrochloride in C57BL/6J mice, did not significantly influence body weight gain, although a trend for decreased weight gain with increasing drug dose was evident ($P = 0.06$). This trend did not result from differences in food intake between treatment groups ($P = 0.78$); however, there was a drug influence on activity levels, with animals in the HIGH group traveling 4.04 $\pm$ 0.76 m during monitoring compared with the 5.63 $\pm$ 0.80 m and 6.31 $\pm$ 0.80 m traveled by the LOW and CON groups, respectively ($P < 0.05$). There was no effect of fluoxetine on femoral length or distal femoral growth plate height ($P = 0.34–0.61$).

Null mutation of the 5-HTT gene causes a consistent skeletal phenotype

5-HTT$^{-/-}$ mice had a consistent skeletal phenotype of reduced bone mass, altered architecture, and inferior mechanical properties compared with their 5-HTT$^{+/+}$ counterparts. Whole-body BMC was 6.4–13.0% lower in 5-HTT$^{-/-}$ mice than 5-HTT$^{+/+}$ mice (Table 2). Significantly lower cranial, spinal, and femoral BMC in 5-HTT$^{-/-}$ mice contributed to these whole-body findings. The reduction in femoral BMC resulted from genotype differences in both cortical and trabecular BMC, with a more profound phenotype being found

TABLE 1. Body weight and bone size in 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice

	Male		Female		Two-way ANOVA results[a]		
	5-HTT$^{+/+}$	5-HTT$^{-/-}$	5-HTT$^{+/+}$	5-HTT$^{-/-}$	Genotype	Sex	Interaction
Young[b]							
Body weight (g)	21.3 ± 2.6	20.2 ± 1.0	17.1 ± 1.8	16.6 ± 1.5	NS	**<0.001**	NS
Cranial length (mm)	21.9 ± 0.6	21.7 ± 0.6	21.9 ± 0.6	21.2 ± 0.6	NS	NS	NS
Cranial width (mm)	10.8 ± 0.3	11.2 ± 0.2	10.8 ± 0.2	10.8 ± 0.3	NS	NS	NS
Cranial height (mm)	8.1 ± 0.3	7.8 ± 0.2	7.9 ± 0.2	7.8 ± 0.3	0.09	0.06	NS
Femur length (mm)	13.0 ± 0.5	12.9 ± 0.5	12.8 ± 0.5	12.4 ± 0.3	NS	0.06	NS
Growth plate height (μm)[c]	230.3 ± 17.4	216.7 ± 21.7	206.8 ± 9.7	197.5 ± 26.7	NS	**<0.05**	NS
n	9	6	5	5			
Adult[d]							
Body weight (g)	30.9 ± 2.1	31.9 ± 3.3	24.8 ± 1.9	26.0 ± 4.9	NS	**<0.001**	NS
Femur length (mm)	16.4 ± 0.5	16.3 ± 0.5	16.3 ± 0.6	16.0 ± 0.3	NS	NS	NS
n	11	10	16	12			

Values are given as mean ± SD.

[a] Significant differences ($P < 0.05$) indicated by *bold face numerals*; suggestive differences ($0.05 \leq P \leq 0.1$) indicated by *lightfaced numerals*; nonsignificant differences ($P > 0.10$) indicated by NS.

[b] Four-week-old mice are indicated as young.

[c] Average of five measurements taken of the distal femoral growth plate.

[d] Nineteen-week-old mice are indicated as adult.

TABLE 2. Bone mineral parameters in 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice

	Male		Female		Two-way ANOVA results[a]		
	5-HTT$^{+/+}$	5-HTT$^{-/-}$	5-HTT$^{+/+}$	5-HTT$^{-/-}$	Genotype	Sex	Interaction
Young[b]							
Total body BMC (mg)	408.6 ± 25.3	382.6 ± 33.7	387.2 ± 46.3	350.2 ± 29.2	**<0.05**	0.06	NS
Cranial BMC (mg)	182.3 ± 17.8	160.9 ± 12.2	171.4 ± 21.1	151.6 ± 16.1	**<0.01**	NS	NS
Spinal BMC (mg)	17.1 ± 2.4	15.5 ± 2.4	17.1 ± 1.5	14.8 ± 2.3	**<0.05**	NS	NS
Femoral BMC (mg)	14.0 ± 1.3	12.6 ± 1.4	13.3 ± 2.1	11.5 ± 0.8	**0.01**	NS	NS
Midshaft femoral BMC (mg/mm)	6.62 ± 0.67	6.01 ± 0.50	6.25 ± 0.87	5.68 ± 0.26	**<0.05**	NS	NS
Distal femoral BMC (mg/mm)	3.22 ± 0.64	2.65 ± 0.40	2.72 ± 0.75	2.17 ± 0.32	**<0.05**	**<0.05**	NS
Adult[c]							
Total body BMC (mg)	1295.5 ± 139.9	1127.3 ± 109.7	1146.3 ± 127.4	1062.4 ± 57.9	**<0.01**	**0.01**	NS
Cranial BMC (mg)	336.9 ± 21.9	320.6 ± 13.2	318.3 ± 16.9	303.3 ± 16.4	**0.01**	**<0.01**	NS
Spinal BMC (mg)	27.8 ± 3.2	24.9 ± 2.2	24.4 ± 3.62	21.8 ± 2.8	**0.02**	**0.01**	NS
Femoral BMC (mg)	20.7 ± 3.2	17.9 ± 2.2	18.6 ± 2.3	18.5 ± 3.2	0.07	NS	0.09
Midshaft femoral BMC (mg/mm)	15.35 ± 1.49	13.73 ± 1.02	12.97 ± 1.15	12.61 ± 0.92	**<0.01**	**<0.001**	0.08
Distal femoral BMC (mg/mm)	5.13 ± 0.80	4.05 ± 0.79[d]	3.69 ± 0.34	3.48 ± 0.57	—[e]	—[e]	<0.05

Values are given as mean ± SD.

[a] Significant differences ($P < 0.05$) indicated by *bold face numerals*; suggestive differences ($0.05 \leq P \leq 0.1$) indicated by *lightface numerals*; nonsignificant differences ($P > 0.10$) indicated by NS.

[b] Four-week-old mice are indicated as young.

[c] Nineteen-week-old mice are indicated as adult.

[d] $P < 0.01$ *vs.* male WT (unpaired *t* test).

[e] Genotype and sex main effects were ignored in the presence of a significant genotype X sex interaction.

in the latter. Distal femoral (cortical and trabecular) BMC was 17.6–20.2% lower in 5-HTT$^{-/-}$ mice than 5-HTT$^{+/+}$ mice, whereas midshaft (cortical) BMC was 2.8–10.6% lower in 5-HTT$^{-/-}$ mice (Table 2). The low BMC in the distal femur in both age groups contributed to reduced volumetric BMD at this site (all $P < 0.05$); however, there were no differences in volumetric BMD at the femoral midshaft. Confirming a significant genotype effect on trabecular bone, 5-HTT$^{-/-}$ mice had significantly altered trabecular structure, with BW/TV being 30.6–40.0% lower than in 5-HTT$^{+/+}$ mice (Fig. 1). This resulted from the presence of less trabeculae (21.3–31.4%) (all $P < 0.02$) rather than a difference in trabeculae thickness (all $P > 0.05$). The presence of less trabeculae resulted in 48.4–65.3% greater trabecular spacing in 5-HTT$^{-/-}$ mice (all $P < 0.01$).

Although null mutation of the gene encoding the 5-HTT did not influence femoral longitudinal bone growth, as indicated by equivalent femoral lengths and growth plate heights, it did negatively impact cross-sectional architecture, with femoral midshaft cortical area being lower in 5-HTT$^{-/-}$ mice ($P < 0.01$) (Fig. 2A). This resulted from a smaller total cross-sectional area (all $P < 0.01$) rather than a reduction in cortical thickness (all $P > 0.05$). Periosteal and endosteal perimeters were both smaller in 5-HTT$^{-/-}$ mice than in 5-HTT$^{+/+}$ mice (all $P < 0.05$). Reductions in both perimeters enabled cortical thickness to be equivalent between the genotypes; however, the narrower bones in 5-HTT$^{-/-}$ mice meant that bone mineral was distributed closer to the centroid. This resulted in 5-HTT$^{-/-}$ mice having a significantly lower I_P ($= I_{MAX} + I_{MIN}$) than in 5-HTT$^{+/+}$ mice (Fig. 2B).

Consistent with the phenotypes of altered bone mass and architecture, femurs from 5-HTT$^{-/-}$ mice had altered mechanical properties. In comparison with 5-HTT$^{+/+}$ mice, ultimate force of the femur in three-point bending was 9.8–13.2% lower in 5-HTT$^{-/-}$ mice at both ages (Fig. 3). The 5-HTT$^{-/-}$ bones at both ages were also unable to absorb as much energy before breaking, with the measurements being 14.3–29.9% lower than 5-HTT$^{+/+}$ bones (all $P < 0.05$). Stiffness of adult 5-HTT$^{-/-}$ bones was 11.3–16.8% lower than in

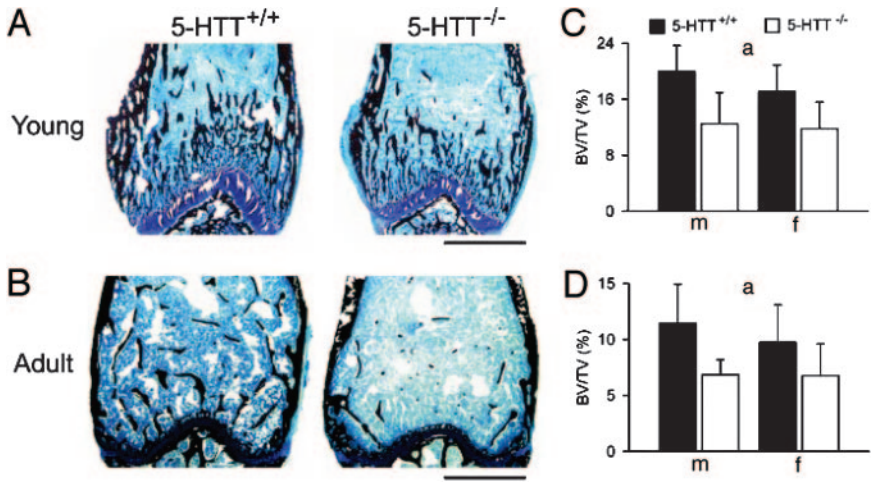

FIG. 1. Trabecular bone structure within the distal femur of 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice. Photomicrographs of representative sections taken from (A) young (4 wk old) and (B) adult (19 wk old) mice. Sections are stained with McNeal's tetrachrome, which stains bone *black*. Note the altered trabecular content and structure in 5-HTT$^{-/-}$ mice at both ages. *Scale bars*, 1 mm. Distal femoral trabecular bone volume (BV/TV) in (C) young and (D) adult mice. m, Male; f, female; *bars*, mean ± SD; a, $P < 0.05$ for genotype main effect, as determined by two-way factorial ANOVA (genotype × sex). There were no significant genotype × sex interactions (all $P > 0.05$).

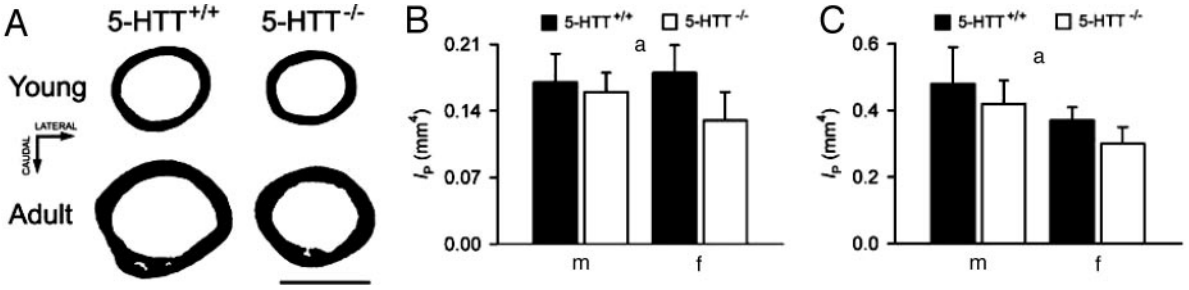

FIG. 2. Bone architecture in 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice. A, Representative microcomputed tomography images of femoral midshaft cross-sectional architecture in young (4 wk old) and adult (19 wk old) 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice. Note the narrower bones in 5-HTT$^{-/-}$ mice at both ages. *Scale bar*, 1 mm. I_P values in (B) young and (C) adult 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice. *Bars*, Mean ± SD; a, $P < 0.05$ for genotype main effect, as determined by two-way factorial ANOVA (genotype × sex). There were no significant genotype × sex interactions (all $P > 0.05$).

5-HTT$^{+/+}$ bones ($P < 0.05$). Stiffness in young mice did not differ significantly with genotype.

The skeletal phenotype in 5-HTT$^{-/-}$ mice resulted from decreased bone formation

The altered bone mass, architecture, and strength in young 5-HTT$^{-/-}$ mice was generated by a reduction in bone formation rather than an increase in bone resorption. 5-HTT$^{-/-}$ mice had significantly reduced BFRs at each of the sites assessed. These included both cortical (Fig. 4A) and trabecular (Fig. 4B) sites and weight-bearing (Fig. 4A) and non-weightbearing (Fig. 4C) bones. The reduction in bone formation at each site ranged from 12.4–37.0% (Fig. 4, D–F). In contrast, there were no significant differences between genotypes in measures of bone resorption at the trabecular site, with the percentage of bone covered by osteoclasts (OcS/BS) and the area resorbed by osteoclasts [eroded surface (ES)/BS] being equivalent between genotypes ($P = 0.31$–0.69).

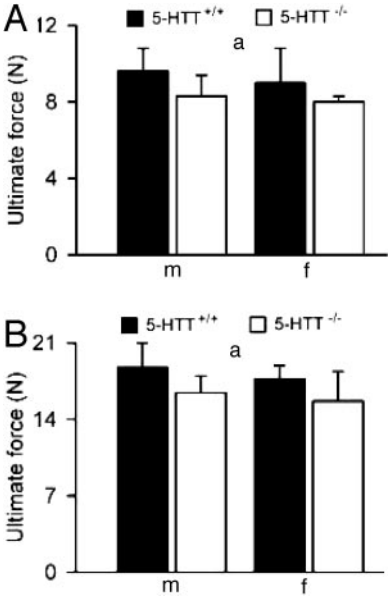

FIG. 3. Femur midshaft mechanical properties in 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice. Ultimate force in (A) young (4 wk old) and (B) adult (19 wk old) mice. *Bars*, Mean ± SD; a, $P < 0.05$ for genotype main effect, as determined by two-way factorial ANOVA (genotype × sex). There were no significant genotype × sex interactions (all $P > 0.05$).

The skeletal phenotype in 5-HTT$^{-/-}$ mice did not result from altered mechanosensitivity

Because altered mechanosensitivity can contribute to a skeletal phenotype (34) and preliminary evidence has suggested that serotonergic pathways play a role in skeletal mechanotransduction (14), we investigated the influence of genotype differences in mechanosensitivity to the observed phenotype. Using the ulna axial loading model (21), mechanical loading induced adaptation by stimulating new bone formation. This was reflected by loaded (right) ulnas in both 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice having significantly greater MS/BS, MAR, and BFR/BS than in nonloaded (left) ulnas (all $P < 0.05$). To explore genotype influences on the response to mechanical loading, individual differences in systemic factors were controlled by subtracting left ulna values from right ulna values. This generated a new set of relative (r) values for each variable (*i.e.* rMS/BS, rMAR, and rBFR/BS). 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice had equivalent rBFR/BS, indicating that genotype did not influence the response to loading (Fig. 5). Similarly, rMS/BS and rMAR did not differ with genotype (all $P > 0.05$).

The skeletal phenotype in 5-HTT$^{-/-}$ mice did not result from altered serum biochemistry

Skeletally relevant serum biochemical markers did not differ significantly between genotypes (Table 3). Mean serum testosterone levels were higher and PTH levels lower in 5-HTT$^{-/-}$ mice; however, the differences were not statistically significant compared with 5-HTT$^{+/+}$ mice. Increased testosterone is anabolic and would be expected to increase bone mass (35), whereas hypoparathyroidism results in an increase in bone volume in mice (36). These effects are contrary to the low bone mass phenotype observed in 5-HTT$^{-/-}$ mice and therefore not likely to be the cause of the phenotype.

Inhibition of the 5-HTT decreases bone mineral accrual during growth

The skeletal phenotype in mice with null mutation of the 5-HTT gene presented at an early age. To investigate whether short-term pharmacological antagonism of the 5-HTT results in a similar phenotype, normal growing mice were treated for 4 wk with a SSRI (fluoxetine hydrochloride). Animals in the HIGH group gained significantly less whole-body bone

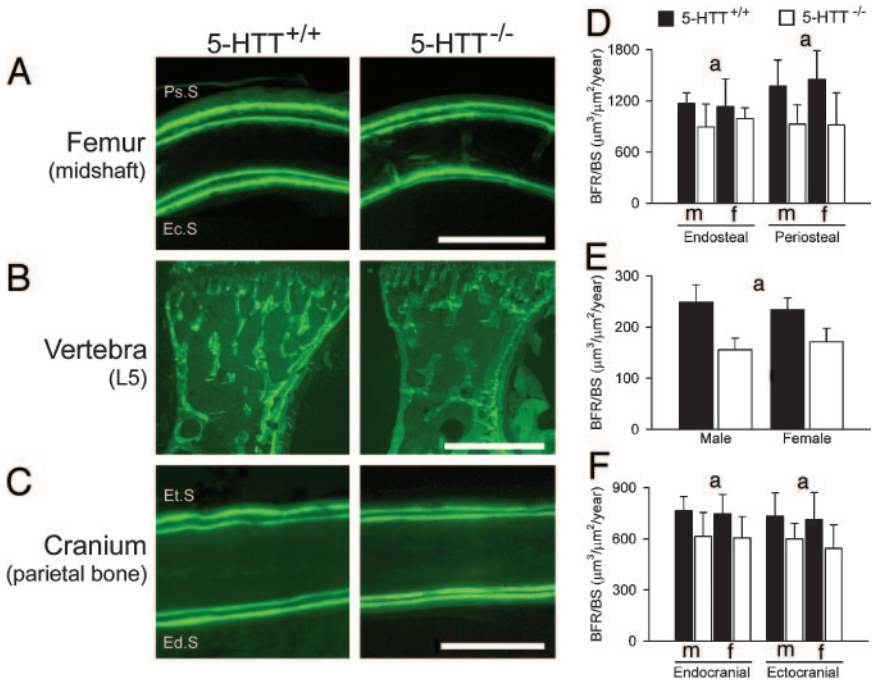

FIG. 4. BFRs in young (4 wk old) 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice. Representative photomicrographs taken from: A, the femoral midshaft (Ps.S, periosteal surface; Ec.S, endocortical surface; *scale bar*, 200 μm); B, trabecular bone within the L5 vertebrae (*scale bar*, 600 μm); and C, the parietal bone of the cranium (Et.S, ectocranial surface; Ed.S, endocranial surface; *scale bar*, 100 μm). BFR/BS in the: D, femoral midshaft; E, L5 vertebrae; and F, parietal bone of the cranium. *Bars*, Mean ± SD; a, $P < 0.05$ for genotype main effect, as determined by two-way factorial ANOVA (genotype × sex). There were no significant genotype × sex interactions (all $P > 0.05$).

mineral, over the intervention period, than those in the CON group (Fig. 6A). Animals in the LOW group did not differ from control ($P = 0.63$). Differences in bone mineral accrual between the HIGH and CON groups appeared to be restricted to weight-bearing sites. There were no differences in bone mineral accrual in the cranium (Fig. 6B), whereas animals in the HIGH group had significantly less bone mineral accrual in the whole hindlimb than did the CON group (Fig. 6C). Confirming a drug effect predominantly on weight-bearing sites, the isolated lumbar spine (L2–5) and femurs from the HIGH group had 5.9% and 9.4% lower BMC than in the CON group, respectively (all $P < 0.04$). In contrast, there were no differences between groups in BMC within harvested craniums ($P = 0.25$). The lower BMC in HIGH-group femurs resulted from differences in both cortical and trabecular bone. Distal femoral (cortical and trabecular) BMC was 9.9% lower in HIGH femurs than CON femurs ($P < 0.001$), whereas midshaft (cortical) volumetric BMD was 2.6% lower in HIGH femurs ($P < 0.05$).

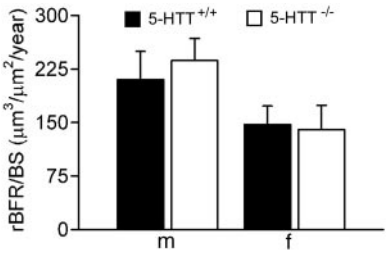

FIG. 5. Response of 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice to mechanical loading. *Bars*, Relative (right minus left) BFR (rBFR/BS) on the midshaft ulna periosteal surface in adult (16 wk old) 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice after mechanical loading. Note that rBFR/BS is greater than zero, indicating right (loaded) ulnas had greater bone formation than left (nonloaded) ulnas and, thus, the presence of an osteogenic loading response. *Bars*, Mean ± SEM. There was no significant genotype main effect or genotype × sex interaction, as determined by two-way factorial ANOVA ($P > 0.05$).

The decreased bone mineral accrual with inhibition of the 5-HTT resulted from decreased bone formation

The reduced bone mineral accrual in the HIGH-group hindlimb resulted from a deficit in bone formation at both cortical (midshaft femur) and trabecular (distal femoral metaphysis) sites. There was a drug dose effect on midshaft femoral cortical bone formation, with the HIGH group having 27.8% lower periosteal BFR/BS than the CON group (Fig. 7A). The LOW group had 16.4% lower periosteal BFR/BS than the CON group; however, this difference was not significant ($P = 0.18$). The reduced BFR in the HIGH group resulted from a reduction in MAR ($P = 0.001$) rather than a reduction in MS/BS ($P = 0.42$). At the distal femoral metaphysis, BFR/BS in the HIGH group was 27.8% lower than in the CON group ($P < 0.05$) (Fig. 7B), whereas there was no difference between the LOW group and the CON ($P = 0.35$)

TABLE 3. Serum assays in 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice

	5-HTT$^{+/+}$	5-HTT$^{-/-}$
Albumin (g/dl)	2 ± 0.1	2 ± 0.1
C-terminal type-I collagen (ng/ml)	164 ± 34	121 ± 024
Calcium (mg/dl)	8.8 ± 0.2	8.8 ± 0.1
Creatinine (mg/dl)	0.2 ± 0.02	0.2 ± 0.02
Estradiol (pg/ml)[a]	10.7 ± 4.7	5.9 ± 1.3
IGF-I (ng/ml)	353 ± 20	353 ± 55
PTH (pg/ml)	27 ± 11	15 ± 3
Phosphorous (mg/dl)	5.8 ± 0.3	6.2 ± 0.2
Testosterone (pg/ml)[b]	160 ± 49	311 ± 90
TSH (ng/ml)	437 ± 67	357 ± 70

Values are given as mean ± SEM from three to nine mice per genotype.
[a] Measured in female mice only.
[b] Measured in male mice only.

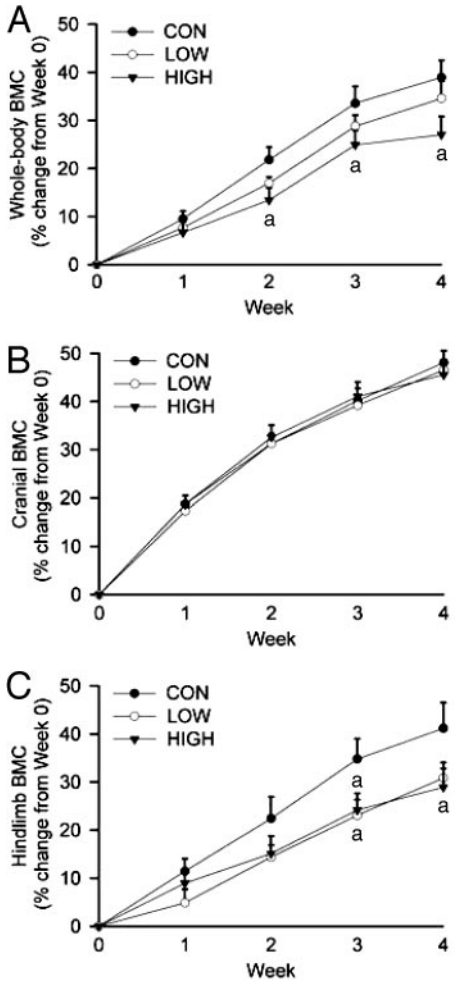

FIG. 6. Influence of fluoxetine hydrochloride on bone mineral accrual in the: A, whole-body; B, cranium; and C, hindlimb of growing mice. CON, Vehicle (saline)-treated control. *Error bars*, ± SEM; a, significantly different from CON ($P < 0.05$), as determined by one-way ANOVA followed by Fisher's PLSD for pairwise comparisons.

group. At this site, there were no differences in measures of bone resorption, with OcS/BS and ES/BS being equivalent between groups ($P = 0.44–0.54$).

Discussion

In two differing models, we found inhibition of the 5-HTT to have significant detrimental effects on bone mineral accrual in the growing mouse skeleton. Null mutation of the gene encoding for the 5-HTT resulted in a consistent skeletal phenotype of reduced mass, altered architecture, and inferior mechanical properties. This finding was confirmed in a second model wherein inhibition of the 5-HTT using a SSRI resulted in reduced bone mineral accrual during growth. In both models, the skeletal phenotype resulted from a reduction in bone formation, indicating an osteoblastic phenotype. Combining the current findings with those demonstrating the presence of functional serotonergic pathways in bone cells (11–14), a role for the 5-HTT in the regulation of bone accrual during skeleton growth is revealed.

There are several putative explanations for the observed

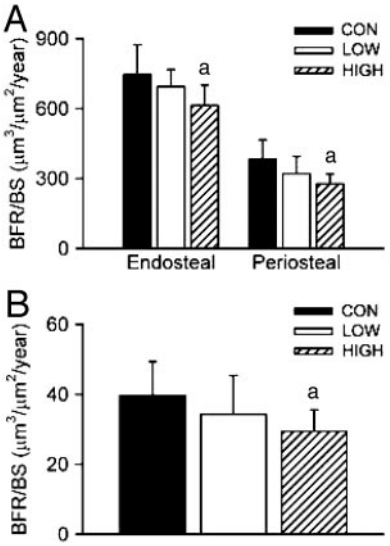

FIG. 7. Influence of fluoxetine hydrochloride on BFR/BS values in the: A, femoral midshaft; and B, distal femoral metaphysis of growing mice. CON, Vehicle (saline)-treated control. *Bars*, Mean ± SD; a, significantly different from CON ($P < 0.03$), as determined by one-way ANOVA followed by Fisher's PLSD for pairwise comparisons.

skeletal phenotypes. Previous studies of bone cell culture suggested that serotonergic pathways may influence mechanotransduction (14), so mice with inhibition of the 5-HTT could have had reduced skeletal responsiveness to mechanical loading and, thus, reduced bone mineral accrual. However, using an established loading model (21) wherein ulnas of 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice were exposed to an identical osteogenic mechanical stimulus, no influence of null mutation of the 5-HTT gene was found on skeletal mechanosensitivity. An established effect of both gene- and drug-mediated inhibition of the 5-HTT in mice is heightened anxiety-like behavior, which manifests in a hypoactive locomotor behavioral phenotype (19, 27, 29, 37, 38). Because bone tissue is sensitive to mechanical loading, a reduction in cage activity could have contributed to the reduced bone mass observed in weight-bearing bones. However, it is unlikely that reduced activity would have any effect on bone in the cranium, yet bone formation and BMC were reduced in the cranial bones of the 5-HTT$^{-/-}$ mice. This suggests that the reduced bone formation in 5-HTT null mice was not the result of reduced physical activity. Further evidence is required to confirm this in the SSRI-treated mice, because they were found to have a hypoactive phenotype and skeletal differences were restricted to weight-bearing bones. The function of the 5-HTT within the gastrointestinal tract may have contributed to the skeletal phenotypes. Here, the 5-HTT functions to uptake 5-HT into mucosal epithelial cells to regulate peristalsis (39). Although 5-HTT$^{-/-}$ mice have altered colorectal motility, the bowel continues to function (40). This was indicated in the current study by the finding of no effect of 5-HTT mutation on body weight in either young or adult mice and by previous findings showing that 5-HTT$^{-/-}$ mice gain weight and survive well (18, 19). Similarly, SSRI-treated mice gained weight and had no difference in food intake from control mice. These findings

suggest that the effect of the 5-HTT on the gastrointestinal tract was not sufficient to significantly alter the nutritional status of our mice and is therefore unlikely to have caused the observed skeletal phenotypes. Another consideration to the observed phenotypes is the effect of 5-HTT inhibition on circulating levels of skeletally relevant hormones and biochemical mediators. Serotonergic pathways directly modulate numerous hormonal and biochemical pathways, including those in the hypothalamic-pituitary-adrenal axis (41). However, our 5-HTT null mutant mice did not display significant differences in any of the hormones or biochemical markers assessed. This is consistent with previous reports that found no genotype differences between 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ mice in plasma corticosterone or adrenocorticotropic hormone levels (42, 43). We observed trends toward lower PTH and higher testosterone in 5-HTT$^{-/-}$ mice, both of which would be expected to increase bone mass (35, 36). These trends are opposite to the low bone mass phenotype observed in 5-HTT$^{-/-}$ mice, so we conclude that the phenotype was not caused by altered osteotropic hormone levels.

With putative indirect skeletal effects of 5-HTT inhibition being unable to adequately explain the observed skeletal phenotype, a possible mechanism is a direct effect of 5-HTT inhibition on osteoblasts. This is supported by findings that osteoblasts possess a functioning 5-HTT, which has high affinity for 5-HT (13), and that osteoblasts respond to stimulation of their 5-HT receptors (13, 14). Although osteoclasts also possess a functioning 5-HTT (11), there was no effect of 5-HTT inhibition on bone resorption measures in either of the skeletal models studied. Direct osteoblastic effects of 5-HTT inhibition in the 5-HTT$^{-/-}$ mice may have originated during embryonic skeletal development and in early postnatal life. The 5-HTT has widespread distribution during ontogeny (44), and it affects the embryonic development of numerous tissues (45). In terms of the skeleton, the 5-HTT has been shown to play a role in craniofacial morphogenesis (46) and postnatal craniofacial growth (47). However, 5-HTT$^{-/-}$ mice do not show any major anatomical anomalies when examined postnatally (18), and we were unable to find any differences in postnatal cranial size in 5-HTT$^{-/-}$ mice when assessed at 4 wk of age. Similarly, studies into drug-mediated antagonism of the 5-HTT have shown that *in utero* exposure does not increase the risk of birth defects or result in poor perinatal condition (48–50). We were unable to find any differences in long bone length or growth plate height between 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mice or mice treated postnatally with differing doses of a SSRI. These findings indicate that the observed skeletal phenotypes did not result from 5-HTT effects on prenatal bone development or postnatal longitudinal bone growth.

The finding of a skeletal phenotype in both of the models investigated is of interest, given the widespread prescription of SSRIs for the treatment of depression and other affective disorders in children and adolescents (4, 5). The 5-HTT is the primary target for these agents, and 5-HTT$^{-/-}$ mice are considered a model of chronic SSRI use (18, 19). Based on the current findings, SSRIs may be postulated to negatively impact bone status during growth. There is currently only preliminary case-study evidence to support this hypothesis (17). Likewise, there is limited evidence of SSRI effects on the mature skeleton. A number of studies have shown that SSRI use increases the risk for fracture (51, 52); however, these studies failed to include potentially significant contributing factors. The increased risk of fractures while taking SSRIs may be due to confounding by indication, with depression being an independent risk factor for fracture (53, 54). Similarly, the increase in fracture risk with SSRIs may be related to an increase in the risk for falls while on this medication (55, 56). Past studies have not found an association between reduced BMD and antidepressant-drug use (55, 57). However, these studies either failed to isolate the effects of different families of antidepressant drugs or had insufficient power to test the relationship between SSRI use and BMD. In contrast, SSRI use was recently found to significantly increase hip bone loss among elderly women when assessed longitudinally over an average assessment period of 4.9 yr and after controlling for possible confounders (including depressive symptoms) (15). Similarly, we recently found, in a cross-sectional study of 5995 men, that SSRI use was associated with lower BMD of the femoral neck and lumbar spine (16). These findings indicate that SSRIs do negatively impact the skeleton and that further research is required to decipher their precise influence.

In conclusion, we found inhibition of the 5-HTT via either null mutation of the 5-HTT gene or pharmacological inhibition of the transporter itself to result in the presence of a consistent skeletal phenotype. In two separate models, inhibition of the 5-HTT resulted in reduced bone mineral accrual via a reduction in bone formation. The observed changes are not considered to be due to indirect effects derived from inhibition of the 5-HTT gene in tissues other than bone and, combined with recent findings of functional serotonergic pathways in bone cells, indicate a role for the 5-HTT in the regulation of bone accrual in the growing skeleton. This finding is of interest, given the frequent prescription of SSRIs to children and adolescents for the treatment of depression and other affective disorders.

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