Journal of Cellular Biochemistry 100:1387–1394 (2007)

Fluoxetine Treatment Increases Trabecular Bone Formation in Mice (Fluoxetine Affects Bone Mass)

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Abstract Mounting evidence exists for the operation of a functional serotonin (5-HT) system in osteoclasts and osteoblasts, which involves both receptor activation and 5-HT reuptake. In previous work we showed that the serotonin transporter (5-HTT) is expressed in osteoclasts and that its activity is required by for osteoclast differentiation in vitro. The purpose of the current study was to determine the effect of treatment with fluoxetine, a specific serotonin reuptake inhibitor, on bone metabolism in vivo. Systemic administration of fluoxetine to Swiss–Webster mice for 6 weeks resulted in increased trabecular BV and BV/TV in femurs and vertebrae as determined by micro-computed tomography (μCT). This correlated with an increase in trabecular number, connectivity, and decreased trabecular spacing. Fluoxetine treatment also resulted in increased volume in vertebral trabecular bone. However, fluoxetine-treated mice were not protected against bone loss after ovariectomy, suggesting that its anabolic effect requires the presence of estrogen. The effect of blocking the 5-HTT on bone loss following an LPS-mediated inflammatory challenge was also investigated. Subcutaneous injections of LPS over the calvariae of Swiss–Webster mice for 5 days resulted in increased numbers of osteoclasts and net bone loss, whereas new bone formation and a net gain in bone mass was seen when LPS was given together with fluoxetine. We conclude that fluoxetine treatment in vivo leads to increased bone mass under normal physiologic or inflammatory conditions, but does not prevent bone loss associated with estrogen deficiency. These data suggest that commonly used anti-depressive agents may affect bone mass. J. Cell. Biochem. 100: 1387–1394, 2007.

Key words: fluoxetine; serotonin; bone

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter implicated in the etiology of many mental illnesses, including depression, anxiety, schizophrenia, eating disorders, obsessive-compulsive disorder, migraine, and panic disorder [Mann, 1999]. Disorders in serotonergic activity or storage could contribute to many of the symptoms of major depression. Abnormalities in serotonergic activity, in turn, could result from alterations in one or more of several processes, such as 5-HT synthesis, release, reuptake, metabolism, or 5-HT post-synaptic receptor abnormalities.

The serotonin transporter (5-HTT) regulates the uptake of serotonin from the synaptic space and plays a key role in the regulation of serotonin neurotransmission by controlling its synaptic levels [Blakely and Berson, 1992; Blakely et al., 1994; Chang et al., 1996]. In addition, the 5-HTT can also mediate retrograde transport, from inside the cell to the exterior, possibly to regulate cytoplasmic 5-HT concentration [Berger et al., 1992]. The 5-HTT is the target of a class of anti-depressants: the serotonin-selective reuptake inhibitors (SSRI), exemplified by fluoxetine (Prozac). SSRIs have been the treatment of choice for depression in adults [Vaswani et al., 2003], as well as children and adolescents [Ryan, 2003].

Bone destruction is characteristic of several chronic inflammatory diseases, including rheumatoid arthritis and periodontitis. Bone loss induced by inflammation results from increased numbers of osteoclasts [Kong et al., 1999]. LPS, a key constituent of Gram-negative bacteria,

Grant sponsor: NIH/NICDR; Grant number: DE007378-18.
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Received 24 July 2006; Accepted 11 August 2006
DOI 10.1002/jcb.21131

may induce osteoclast formation and promote bone resorption [Sakuma et al., 2000]. Bone resorption also results from estrogen deficiency, particularly post-menopausally. The estrogen effect on bone homeostasis can be explained by its direct effect on bone cells, as well as at the level of the adaptive immune response. Even though significant progress has been made in this field, the mechanisms involved in estrogen deficiency-induced bone loss are very complex and not completely understood [Weitzmann and Pacifici, 2006].

Numerous clinical studies have demonstrated a clear correlation between the psychophysical health status (in particular major depression) and bone mineral density (BMD) [Halbreich et al., 1995; Michelson et al., 1996; Yazici et al., 2003; Mussolino et al., 2004; Whooley et al., 2004]. In addition, several studies have reported the expression of different elements of the serotonin system in bone cells [Bliziotes et al., 2001; Westbroek et al., 2001; Battaglino et al., 2004]. Our own previous studies as well as those of others have shown that the serotonin transporter is expressed in osteoclasts and that specific blockage of the transporter affected osteoclast differentiation in vitro [Battaglino et al., 2004; Gustafsson et al., 2006].

The goal of this study was to determine the role of serotonin transport on bone metabolism in mice in vivo. To that end, we analyzed wild-type (wt) mice treated with fluoxetine under normal physiologic conditions, as well as on two conditions known to cause bone loss; LPS challenge and estrogen deficiency caused by ovariectomy.

MATERIALS AND METHODS

Reagents and Mice

Ovariectomized or sham-operated Swiss–Webster female mice (Charles River) were between 8 and 14 weeks of age during the study. Swiss–Webster mice were used because this strain has more trabecular bone (BV/TV is ~25%, compared to ~4% in C57BL/6J). Fluoxetine (F132, Sigma, St. Louis, MO) was dissolved in tissue culture grade ddH_20 (1 mg/ml) and injected (10 mg/kg/day, i.p.) daily for 6 weeks. Mice were subsequently sacrificed and the dissected femurs and vertebrae were evaluated by micro-computed tomography (micro-CT). *E. coli* serotype O55:B5 LPS (L2880; Sigma) was dissolved in phosphate-buffered saline (PBS) (5 mg/ml) by sonication for 2 min, aliquoted, and stored at −80°C until use. Before each injection, the stock solution was sonicated for 2 min again. The Institutional Animal Care and Use Committee at The Forsyth Institute approved all procedures involving animals.

Calvarial Injection

Injections were performed with a 28-G needle at a point on the midline of the skull. Prior to each injection, animals were anesthetized intraperitoneally (5 μl of anesthesia per gram of body weight) with a ketamine/xylazine/PBS (1/1/6) solution (Rompum; Fisher, Columbus, OH and Gibco BRL, Grand Island, NY). The heads of the mice were subsequently shaved. LPS (250 μg/mouse), LPS + Fluoxetine, or PBS was delivered in the space between the subcutaneous tissue and the periosteum of the skull, each in a 50-μl volume. Mice were sacrificed in a CO_2 chamber 5 days after the first injection.

Histological Analysis

Each calvarium was dissected and prepared for histologic sections by fixation in 4% paraformaldehyde at 4°C for 2 days. The specimens were washed with 5, 10, and 15% glycerol in PBS, each for 15 min, decalcified with Immunocal (Decal Chemical Corporation, Congers, NY) for 12 days and washed with Cal-arrest (Decal Chemical Corporation). Specimens were finally embedded in low melting paraffin and sectioned at 5 μ intervals. Histomorphometric analysis was carried out as described [He et al., 2004]. Data was obtained by one examiner and confirmed by a second independent examiner.

Van Gieson Staining

Van Gieson stained sections were used for histomorphometric analysis of bone. Collagen from newly formed bone (formed within 4 days of sacrifice) stains *blue*, whereas collagen from previously formed bone stains *red*. Previously formed and newly formed bone was measured between the coronal and occipital sutures, the formation area and bone length were measured by image analysis software, and the results expressed as new bone area per bone length of calvarium (mm^2/mm). Statistical significance was determined by one-way analysis of variance with significance set at the $P < 0.05$ level.

TRAP Staining

The TRAP staining solution was prepared as follows [Volejnikova et al., 1997]: 9.6 mg of naphthol AS-BI phosphate substrate (Sigma) was dissolved in 0.6 ml of *N*,*N*-dimethylformamide (Sigma) with 60 ml of 0.2 M sodium acetate buffer (pH 5.0; Sigma), which contained 84 mg of fast red-violet LB diazonium salt (Sigma), 58.2 mg of tartaric acid (Sigma), and 240 μl of 10% $MgCl_2$. Slides were incubated in the staining solution at 37°C in the dark. The slides were then washed with water for 30 min, followed by counterstaining with hematoxylin for 5–6 min. TRAP-positive multinucleated cells lining bone were considered osteoclasts. The total number of osteoclasts was counted and expressed per mm length of bone.

Micro-CT Determination of Bone Mass

For μCT imaging, fixed vertebrae, femurs, and calvariae samples were imaged and analyzed at the Institute for Biomedical Engineering, University and ETH Zurich, using a compact fan-beam-type tomograph (Micro-CT 40, Scanco Medical AG, Bassersdorf, Switzerland) as described earlier [Ruegsegger et al., 1996]. In short, samples were located in an airtight cylindrical sample holder filled with formalin. The sample holders are marked with an axial alignment line to allow for consistent positioning of the specimens. For each sample, approximately 200 micro-tomographic slices with an increment of 17 μm were acquired, covering the entire width of the bone. Three-dimensional analyses were performed to calculate morphometric indices including metric parameters such as total volume (TV), bone volume (BV), marrow volume (MV), bone surface (BS), bone volume density (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp) as well as non-metric parameter such as connectivity density (Conn.D). All indices were calculated using direct three-dimensional morphometry [Hildebrand et al., 1999].

RESULTS

Systemic Treatment With Fluoxetine Increases Trabecular Bone in Mice

To determine the effect of systemic fluoxetine treatment on bone mass in wild-type mice, we treated mice daily for 6 weeks and analyzed the femurs by micro-CT. The analysis showed no significant changes in BV or BV/TV when the bone was analyzed as a whole or when we analyzed the cortical component (Table I, Parameters: FULL and Parameters: CORTICAL). However, we observed major changes in the

TABLE I. Femur Microarchitectural Parameters After 6 Weeks of Fluoxetine Treatment

	SHAM		OVX	
Parameters (FULL)	PBS	Fluo	PBS	Fluo
Bone volume and microarchitectural parameters				
BV (mm^3)	32.0	34.8	30.5	29.9
BV/TV (%)	89.0	91.3	82.8	77.0*
Parameters (TRABECULAR)				
Bone volume and microarchitectural parameters				
BV (mm^3)	1.2	2.0*	0.59	0.54
BS (mm^2)	28.9	40.4*	16.2	17.6
BV/TV (%)	26.3	39.6*	12.6	10.3
Tb.Th (μm)	0.09	0.11	0.09	0.08*
Tb.Sp (μm)	0.54	0.51	0.28	0.23
Tb.N (1/mm)	3.71	4.66	1.89	2.07
Conn.D	258.8	367.8	113.8	128.9
Parameters (CORTICAL)				
Bone volume and microarchitectural parameters				
BV (mm^3)	1.0	1.0	0.9	0.9
BV/TV (%)	47.5	46.7	46.0	44.4
MV/TV (%)	52.4	53.2	53.9	55.5

BV, bone volume; BS, bone surface; BV/TV, bone volume/total volume, bone volume density; Tb.Th, trabecular thickness; Tb.Sp, trabecular spacing; Tb.N, trabecular number; MV/TV, marrow volume/total volume.
*$P < 0.05$.

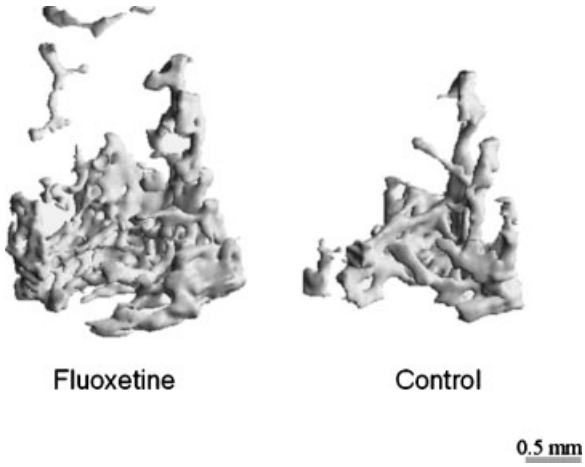

Fig. 1. Fluoxetine induces trabecular bone formation. Three-dimensional micro-CT reconstruction of the trabecular bone component of femurs in Fluoxetine-treated (**left**) and control (**right**) mice. Fluoxetine-treated animals display an increase in trabecular Bone Volume (BV, see Table I for details).

architecture of the trabecular component of bone in Fluoxetine-treated animals, compared to the untreated controls (Table I, Parameters: TRABECULAR and Fig. 1). There was a significant increase in trabecular BV/TV (+50%), with an increase in bone volume (+70%) and bone surface (+40%), which indicates a higher cellular activity. This increase in BV/TV can be best explained by an increase in trabecular number. With a stable trabecular thickness this indicates an anabolic action resulting in more and new trabeculae. There is also a trend towards increased connectivity density. We also analyzed the 4th lumbar vertebrae of these animals and observed a 22% increase in trabecular bone volume demonstrating that the effect was seen in more than one site (Table II).

Systemic Treatment With Fluoxetine Does Not Prevent Ovariectomy-Induced Bone Loss

We next determined whether fluoxetine could reduce bone loss that occurs as a result of ovariectomy. Ovariectomized (OVX) females were treated with fluoxetine or PBS for 6 weeks commencing 2 Weeks after the procedure, and femurs were analyzed by micro-CT. PBS-treated OVX animals showed significant (~50%) trabecular bone loss, as reflected by decreased BV, BV/TV, trabecular spacing, trabecular number, and connectivity density. Fluoxetine-treated OVX mice showed values that were closely similar to the PBS-treated mice, indicating that fluoxetine exerts no protective effect on bone loss in ovariectomized animals (Tables I and II).

Fluoxetine Treatment Reversed Total Bone Loss Induced by LPS, Induced the Formation of New Bone, and Resulted in Increased Numbers of Osteoclasts

We also investigated the effect of fluoxetine treatment on bone loss following an LPS-mediated inflammatory challenge. LPS or fluoxetine alone injected over the calvariae of Swiss–Webster mice for 5 days did not result in a significant increase in the numbers of calvarial osteoclasts (Fig. 2A). There was an increase in LPS-treated mice, that was not significant in this experiment. However, the combination of fluoxetine plus LPS resulted in a significant increase in the number of osteoclasts (Fig. 2A). Additionally, the combination of fluoxetine plus LPS resulted in increased new bone formation and a net gain in bone mass (Fig. 2B). These results show that Fluoxetine treatment results

TABLE II. Vertebral Microarchitectural Parameters After 6 Weeks of Fluoxetine Treatment

	SHAM		OVX	
Parameters (Trabecular)	PBS	Fluo	PBS	Fluo
Bone mass and microarchitectural parameters				
BMC (mg)	1.8	2.2*	1.8	1.8
BV (mm^3)	1.03	1.28*	1.01	1.02
BS (mm^2)	26.63	29.16	28.73	30.03
BV/TV (%)	42.06	48.69	35.42	34.96
Tb.Th (μm)	0.083	0.089	0.079	0.078
Tb.Sp (μm)	0.189	0.179	0.215	0.211
Tb.N (1/mm)	5.57	6.28	4.70	4.85

BMC, bone mineral content; BV, bone volume; BS, bone surface; BV/TV, bone volume/total volume, bone volume density; Tb.Th, trabecular thickness; Tb.Sp, trabecular spacing; Tb.N, trabecular number.
*$P < 0.05$.

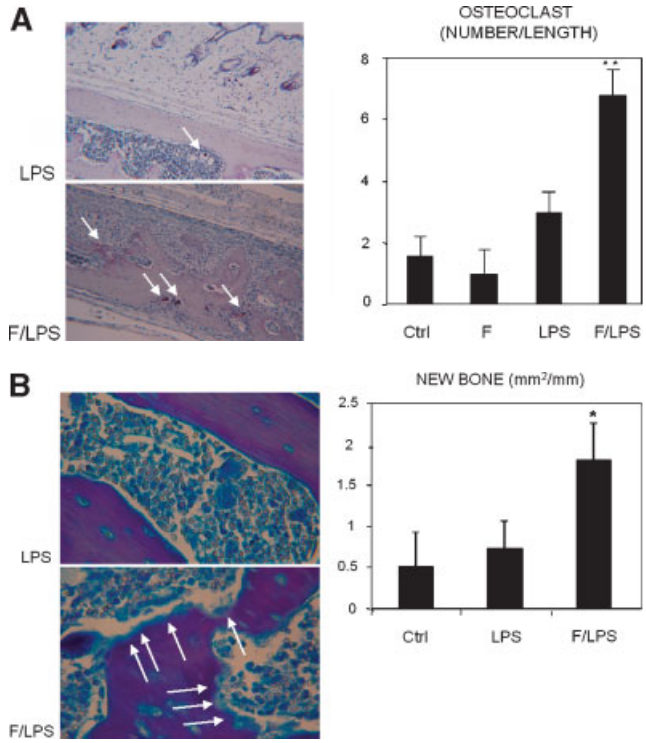

Fig. 2. Fluoxetine stimulates osteoclasts and new bone formation in vivo. LPS was injected over the calvariae of Swiss–Webster mice for 5 days alone or in combination with Fluoxetine. Calvariae were subsequently analyzed by histomorphometry. TRAP positive osteoclasts were observed (white arrows in **A**, **left panel**, magnification 200×) and counted (A, **right panel**) between the coronal and occipital sutures. (**, $P < 0.05$). Adjacent sections were stained with Van Gieson and new bone formation (blue staining area) was observed (**B**, **left panel**, magnification, 400×) and measured (B, **right panel**). Widespread blue-stained areas were observed (white arrows) in LPS/Fluoxetine-treated compared with saline-control mice. *Arrows* point to newly formed bone area. Each photomicrograph is representative of six specimens for a given group.

in increased bone mass in the context of an inflammatory challenge.

DISCUSSION

Our results show that systemic fluoxetine treatment results in a significant increase in trabecular bone mass in estrogen replete mice, as well as in response to an inflammatory challenge. These increases correspond to changes in several micro-architectural parameters. Our previous studies, [Battaglino et al., 2004] presented evidence of serotoninergic regulation of osteoclast differentiation in vitro. Earlier studies had already shown a role for serotonin transport in morphogenesis during craniofacial development. Exposure of mouse embryos to sertraline, fluoxetine, and amitriptyline caused craniofacial malformations. The observed defects resulted from both decreased proliferation and extensive cell death in mesenchymal cell and normal or elevated proliferation in the subepithelial mesenchymal layers [Shuey et al., 1992; Moiseiwitsch, 2000]. The serotonin transporter and several receptors are also expressed in osteoblasts [Bliziotes et al., 2001; Westbroek et al., 2001] as well as in osteoclasts. Since these cells contribute differently to regulate bone mass, the net regulatory effect of serotonin on bone mass is likely to be complex in vivo. Fluoxetine treatment results in decreased 5HT clearance from the extracellular compartment. Consistent with our results, Gustafsson et al. [2005] showed that rats treated with serotonin had increased BMD and altered bone architecture.

The overall effect of fluoxetine treatment on bone mass in vivo could be attributed to an effect on both bone formation and bone resorption. The micro-architectural changes that we observed (more and better-connected trabeculae) suggest that *systemic* fluoxetine treatment affects bone formation by osteoblasts. In addition, when we tested the effect of *local* fluoxetine

administration, we found that fluoxetine given together with LPS resulted in an increase in bone volume, new bone formation as well as the number of osteoclasts, all characteristic of increased bone remodeling.

In our studies, the anabolic effect of fluoxetine was not observed in ovariectomized animals, suggesting that the effect was dependent on the presence of estrogen. Estrogen is a key player in the regulation of bone loss following ovariectomy. Fluctuations in estrogen levels over the lifespan and during ovarian cycles have been correlated with the expression of the 5-HTT and 5-HT receptors, leading to the hypothesis that some of the physiological effects attributed to estrogen may be a consequence of estrogen-related changes in serotonin efficacy and receptor distribution [Fink et al., 1999; McQueen et al., 1999; Sumner et al., 1999; Rybaczyk et al., 2005]. Ovarian hormones also regulate 5-HTT protein expression and distribution, via extracellular serotonin or mRNA stability [Lu and Bethea, 2002; Lu et al., 2003]. In view of these results we propose that the fluoxetine-dependent effect on trabecular bone requires the presence of estrogen. We are currently testing that hypothesis by treating OVX mice with fluoxetine and estrogen.

It is generally accepted that the therapeutic effects of fluoxetine are due to its ability to block the 5HTT [Bengel et al., 1998; Masand and Gupta, 1999]. However, in addition, several studies have shown that even at low concentrations fluoxetine can inhibit the membrane currents mediated by activation of various types of neuronal nicotinic acetylcholine receptors [Garcia-Colunga et al., 1997; Garcia-Colunga and Miledi, 1999; Maggi et al., 1998] and the function of $5HT_{2C}$ receptors [Ni and Miledi, 1997]. These effects can be seen at fluoxetine concentrations that are reached in plasma during clinically effective treatments: 0.29–0.97 μM and in some patients up to 1.6 μM after administration of 40 mg/day during a 30-day treatment [Goodnick, 1991]. It is likely that at these levels the effect of fluoxetine on the 5HT uptake system has reached saturation [Wong et al., 1995]. Such effects could explain to a certain extent the apparent disagreement between our results using fluoxetine-treated wild-type mice and those obtained using 5HTT-defficient mice [Warden et al., 2004]. Thus, fluoxetine may have targets in vivo other that the 5HTT. In addition, studies showed that long-term treatment with SSRI's-induced downregulation of the 5HTT and that the functional consequences of 5HTT downregulation were significantly greater than those seen after acute blockade of the 5HTT by SSRIs in vivo [Benmansour et al., 2002]. Finally, fluoxetine can accumulate and persist at high concentration in the bone marrow, months after its complete disappearance from plasma and brain [Bolo et al., 2004]. These results suggest that these drugs or their metabolites may be sequestered long term in the bone marrow and possibly in surrounding tissue, raising the intriguing possibility that fluoxetine effects on bone metabolism are related to its concentration in the bone marrow micro-environment.

The effect of fluoxetine on trabecular bone in mice is furthermore likely influenced by genetic background. Several studies have reported mouse strain differences in the response to antidepressants [Holmes et al., 2003; Ripoll et al., 2003]. That phenomenon might also help explain the differences between our results and those of Warden et al. [2004] who recently reported reduced bone mass in 5-HTT KO and reduced bone accrual in fluoxetine-treated WT mice. This group used C57B1/6J mice for the Fluoxetine studies versus our use of Swiss–Webster mice. Of note, Warden et al. measured bone accrual in growing animals while we assessed changes in micro-architecture in adult bone.

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