Journal of Cellular Biochemistry 97:1283–1291 (2006)

Long-Term Serotonin Administration Leads to Higher Bone Mineral Density, Affects Bone Architecture, and Leads to Higher Femoral Bone Stiffness in Rats

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Abstract New evidence suggests a control of bone mass by the central nervous system. We have previously shown that functional serotonin receptors are present in bone cells and that serotonin stimulates proliferation of osteoblast precursor cells in vitro. In the present study we investigated the effects of serotonin on bone tissue in vivo. Ten, 2-month-old female Sprague–Dawley rats were injected with serotonin subcutaneously (s.c.) (5 mg/kg) once daily for 3 months, controls received saline. Using microdialysis and HPLC, free circulating serotonin levels were measured. DXA scans were made after 3 months of serotonin administration. Bone architecture and mechanical properties were investigated by micro-computed tomography (μCT), histomorphometry, and mechanical testing. A long-lasting hyperserotoninemia with a >10-fold increase in serotonin appeared. Total body BMD was significantly higher (0.1976 ± 0.0015 vs. 0.1913 ± 0.0012 g/cm^2) in rats receiving serotonin. Cortical thickness (Ct.Th) measured by μCT analysis was also higher, whereas trabecular bone volume (BV) was lower. Interestingly, the perimeter and cross-sectional moment of inertia (MOI), a proxy for geometrical bone strength, were the same in both groups. These data suggest that serotonin reduces resorption or/and increases apposition of endosteal bone. Mechanical testing showed that femoral stiffness was higher in serotonin-dosed animals. The energy absorption also seemed slightly, but not significantly higher. In conclusion, hyperserotoninemia led to a higher BMD, altered bone architecture and higher femural bone stiffness in growing rats, demonstrating that serotonin may have important effects on bone in vivo. J. Cell. Biochem. 97: 1283–1291, 2006.

Key words: serotonin; bone metabolism; DXA; micro-CT; biomechanics

Skeletal remodeling is a highly regulated process that involves both formation and resorption of bone. Increasing interest has been directed towards peptide and amine hormones, and their effects on bone cellular growth and differentiation. Studies on nerve terminals innervating bone have demonstrated the presence of several neuropeptides, including calcitonin gene related peptide (CGRP), vasoactive intestinal polypeptide (VIP), substance P, and neuropeptide Y [Hill and Elde, 1991]. Functional receptors for leptin and VIP have been

Grant sponsor: Nycomed Pharma AS; Grant sponsor: Norwegian Osteoporosis Foundation; Grant sponsor: Medical Research at Trondheim University Hospital.

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Received 29 August 2005; Accepted 6 October 2005
DOI 10.1002/jcb.20733

demonstrated in osteoblasts and in osteoblastic cell lines [Lundberg et al., 2001]. Serotonin (5-hydroxytryptamine or 5-HT) is a well-known amine neurotransmitter. Outside the central nervous system serotonin is mainly produced by the enterochromaffin cells of the gut and participates in the regulation of intestinal motility, fluid secretion, and regional blood flow [Gershon, 1999]. After release, serotonin is rapidly transported by the cell membrane bound serotonin transporter (5-HTT) into a number of cell types, with platelets serving as the major reservoir. Serotonin mediates its actions via multiple serotonin receptor subtypes [Hoyer et al., 2002]. Until now seven serotonin receptor families ($5\text{-}HT_{1\text{-}7}$) have been characterized, and each is further divided into several subtypes. Studies on cell cultures have shown that serotonin has mitogenic effects on fibroblasts [Seuwen et al., 1988], smooth muscle cells [Nemecek et al., 1986], and vascular endothelial cells [Pakala et al., 1994] mediated through $5\text{-}HT_2$ receptors. We recently demonstrated that long-term administration of high serotonin doses leads to a carcinoid heart-like condition with myofibroblast proliferation and plaque formation on heart valves in rats [Gustafsson et al., 2005a]. In 2000, we were the first to demonstrate functional serotonin receptors in both osteoblasts and osteocytes [Westbroek et al., 2001]. Furthermore, the expression of the serotonin transporter (5-HTT) has been demonstrated in rat osteoblasts and osteoclasts [Bliziotes et al., 2001; Battaglino et al., 2004]. Later, we showed that serotonin induced proliferation of osteoblasts and osteoclasts in vitro as well as increased BMD in rats in vivo [Gustafsson et al., 2003].

As serotonin can affect bone cells via different mechanisms in vitro, we hypothesized that it would also have effects on bone in vivo. In order to investigate the long-term impact of serotonin on BMD, architecture, and mechanical properties we injected 2-month-old female rats with serotonin subcutaneously (s.c.) daily for 3 months, and then performed DXA scans, micro-computed tomography (μCT), histomorphometry, and mechanical testing.

MATERIALS AND METHODS

Animals

The Animal Welfare Committee at Trondheim University Hospital approved this study. Twenty, 2-month-old Sprague–Dawley female rats (200 g) were housed solely in wire-top cages with aspen woodchip bedding from B&K Universal Ltd. Room temperature was $24 \pm 1^{\circ}C$ with a relative humidity of 40%–50% and a 12-h light/dark cycle. The Rat and Mouse Diet of B&K, and tap water were provided ad libitum. Before all procedures (except for s.c injections), the animals were anesthetized with 2 ml/kg body weight of a combination of fluanison (2.5 mg/ml), fentanyl (0.05 mg/ml), and midazolam (1.25 mg/ml). Serotonin (5-hydroxytryptamine creatinin sulfate complex) purchased from Sigma-Aldrich was freshly dissolved in physiological saline (5 mg/ml) before injection. Ten rats were given daily serotonin injections s.c (5 mg/kg) for 3 months; 10 controls received saline. To avoid trauma and ulcers of the neck skin, the animals were immobilized in a specially built cage during the injections, which were given strictly subcutaneously with a 30-G BD syringe. At euthanization, the animals were weighed and decapitated.

In order to perform microdialysis, an additional experiment on seven rats was carried out.

Microdialysis

To describe the pharmacokinetics of the serotonin administration protocol, a short-term study on seven animals was performed. Four animals were given daily serotonin injections (5 mg/kg s.c) for 10 days, three controls were given saline. Microdialysis was performed to determine the free fraction of circulating serotonin. We assumed that the interstitial serotonin level (collected from the femoral muscle) would reflect free circulating serotonin. Two hours before the final 10th serotonin injection, a microdialysis probe (CMA 20, 10 mm membrane length, 0.5 mm outer diameter, 20 kDa cutoff; CMA Microdialysis AB, Stockholm, Sweden) was implanted in the femoral muscles. The microdialysis probes were perfused with PBS at a flow rate of 1 μl/min using a microinfusion pump (CMA 107, CMA Microdialysis AB). After a 30-min equilibration period, baseline samples were collected for 60 min. Then serotonin was injected s.c followed by microdialysate sampling in 60-min fractions for 5 h. The samples were protected from light during the whole procedure and immediately frozen at $-80^{\circ}C$ until further analysis. In vitro recovery of serotonin was $59.3 \pm 3.2\%$ (mean $\pm$ SD).

High-Performance Liquid Chromatography (HPLC)

The microdialysis samples were analyzed with an Agilent 1100 SL LC/MS-system consisting of a G1354A quaternary pump with degasser, a G1367A well plate autosampler; a G1316A thermostatted column compartment and a G1956B single quadropol mass selective detector. Serotonin was monitored using selected ion monitoring on m/z 160. The samples were transferred to vials and added 10% of the sample with 1% formic acid to stabilize serotonin. Fifteen microliters of sample were injected on a 50 × 2.1 mm Agilent Eclipse XDB-C18 with 1.8 micron particle size. The mobile phase was made up of 5% Methanol and 95% 25 mM of formic acid. Quantitation was done with external standard using the Agilent Chemstation software. The detection limit for serotonin in microdialysis samples was found to be 1.1 nM. Mean for a sample with theoretical value of 5 nM was 4.94 with a standard deviation of ± 0.32 nM.

Dual X-Ray Absorptiometry (DXA) Measurements In Vivo

The femur and total body BMD (g/cm^2) were measured in anesthetized animals by means of DXA, using a Hologic QDR 4500A with a small animal software. BMD measurements were performed in duplicate at the start and end of the study. The coefficients of variation (CV) were: total body BMD (<0.52%), femur BMD (<1.29%), area (<0.53%), BMC (<0.55%), body fat content (1.41%), and lean body mass (0.20%).

Bone Architecture

Bone architecture was analyzed by means of μCT scanning. Femoral head and part of the metaphysis (Fig. 3E) of the dissected femurs were scanned in a SkyScan 1072 microtomograph (SkyScan, Antwerp, Belgium), with a voxelsize of 11.89 μm. Scans were processed and three-dimensional morphometric analyses of the femurs were done using free software of the 3D-Calculator Project (http://www.eur.nl/fgg/orthopaedics/Downloads.html). The data sets were separated in femoral head and metaphysis. Cortical volume (Ct.V), cortical thickness (Ct.Th), trabecular bone volume (BV), endocortical bone volume (EV), trabecular bone volume fraction (BV/EV), trabecular thickness (Tb.Th) [Hildebrand and Ruegsegger, 1997b], connectivity density (CD) [Odgaard and Gundersen, 1993], and structure model index (SMI) were determined [Hildebrand and Ruegsegger, 1997a]. Cross-sectional moment of inertia (MOI) was determined over the complete data set (femoral head + metaphysis). The mean of the periosteal perimeter was calculated for part of the metaphysis.

Bone Histomorphometry

In order to investigate bone resorption, tartrate-resistent acid phosphatase (TRAP) staining was used to stain osteoclasts, as described [Cole and Walters, 1987]. Eight sequential, longitudinal sections from each animal were used for TRAP stainings. To study bone formation, Goldner staining was performed to stain unmineralized matrix.

Mechanical Testing

The left femurs were thawed in Ringers® solution for mechanical testing of the diaphysis. The diaphyses were fractured 18.7 mm from the femoral condyles in three point cantilever bending, as previously described [Engesaeter et al., 1978]. The proximal femur was fixed in a clamp, the cam of the rotating wheel engaged the femoral condyles and a fulcrum positioned anteriorly 18.7 mm from the condyles was the third point of force application (Fig. 4D). All tests were done at a loading rate of 0.095 radians/s (5.43 degrees/s) [Kaastad et al., 1997]. The load in the test apparatus, an MTS 858 Mini Bionix® Axial/Torsional Test System (MTS Systems Corporation, MN), was measured with a MTS Test Star TM Sensor Cartridge Force 250 N load cell and registered in MTS Test Star II software.

Ultimate moment, ultimate energy absorption, stiffness, and deflection were read directly or calculated from the computer recordings.

Statistical Analysis

Data shown are expressed as mean ± SEM. All data were tested for normality with Shapiro-Wilk. Normally distributed parameters were tested by means of Student's *t*-test, while parameters that were not normally distributed were tested by Mann–Whitney *U*-test. Significance was assumed at *P*-values lower than 0.05.

RESULTS

Serotonin Measurements and Clinical Signs

The serotonin injections induced clinical signs, including flushing, loose stools, and drowsiness. The flushing and drowsiness lasted 3–4 h after the injections. In dialysate collected from the femoral muscles, only one of three control rats had a detectable serotonin (2.9 nM). In dialysate sampled 1 h prior to the 10th injection, one of four serotonin receiving animals had detectable serotonin (9.2 nM) in the femoral muscles. Two hours after the 10th injection, the serotonin level reached a peak (56.8 ± 9.6 nM) (Fig. 1).

Body Fat Content and Bone Mineral Density

An interesting finding was the weight-loss induced by serotonin. At the end of the study the serotonin treated animals weighed less than the controls (305.5 ± 4.0 vs. 321.8 ± 5.3 g, $P = 0.02$). This was a result of a lower body fat content (28.08 ± 7.20 vs. 42.96 ± 8.49 g, $P = 0.0008$), as the lean body mass remained unchanged (Fig. 2A,B). Despite the lower body weight, the serotonin treated animals had higher total body BMD (0.1976 ± 0.0015 vs. 0.1913 ± 0.0012 g/cm^2, $P = 0.004$), while no difference was found in femoral BMD compared to controls (Fig. 2C,D).

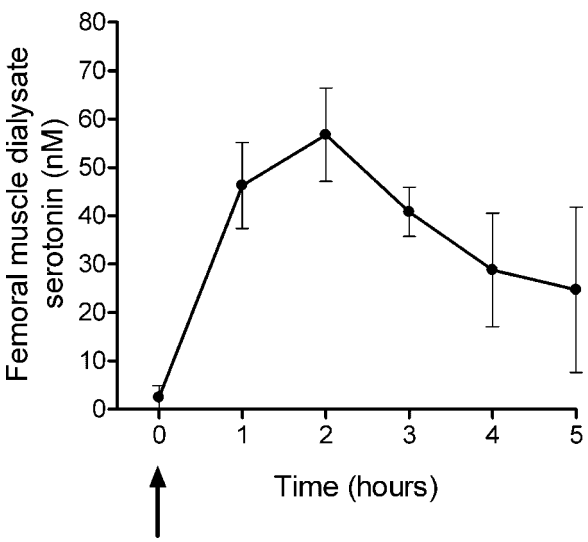

Fig. 1. Serotonin levels in dialysate from femoral muscles in animals given daily subcutaneous serotonin injections (5 mg/kg) for 10 days. At time point 0 h the serotonin concentration in dialysate, collected during 1 h prior to the 10th serotonin injection (↑), is shown. Thereafter the serotonin concentration in five consecutive, hourly collections is depicted. Values are mean ± SEM; n = 4.

The higher total body BMD in the serotonin group was a result of a lower bone area (52.99 ± 0.77 vs. 55.22 ± 0.76 cm^2, $P = 0.035$), total body BMC; however, was not different compared to controls. No difference in body weight or parameters measured by DXA was observed between the groups at baseline.

Bone Architecture

In the metaphysis, Ct.Th was significantly higher in the rats receiving serotonin versus controls (Fig. 3A). On the other hand, BV as well as EV in the metaphysis was found to be significantly lower in the serotonin group compared to the control group (Fig. 3B,C). Trabecular bone volume fraction (Fig. 3D) and Tb.Th (data not shown) remained unchanged, indicating that BV decreased due to a smaller EV. All other parameters studied were not significantly different in the metaphysis. In the femoral head no significant differences were found, although BV and EV showed the same trend as in the metaphysis.

Bone Histomorphometry

All sections studied for TRAP staining were found to be negative (data not shown). Osteoclast activity may be very low, since bone turnover may already be low in rats of this age. Furthermore, no positive staining for unmineralized matrix was found in the sections (data not shown), which may also be due to a low bone turnover state or due to the fact that mineralization is known to occur very quickly in rodents.

Femoral Stiffness is Higher in Rats Treated With Serotonin

Consistent with the phenotypes of altered BMD and architecture, femurs from rats receiving serotonin seemed to have altered mechanical properties. In comparison with controls, mean stiffness of the femur in three-point bending was 12.3% higher (Fig. 4). Bones from the serotonin rats tended to absorb more energy before breaking, but there was no statistical difference. There were no significant differences in ultimate bending moment or deflection in the femoral shaft. The lengths of the femurs were not statistically different between serotonin treated animals and controls (36.45 ± 0.27 vs. 36.45 ± 0.20 mm).

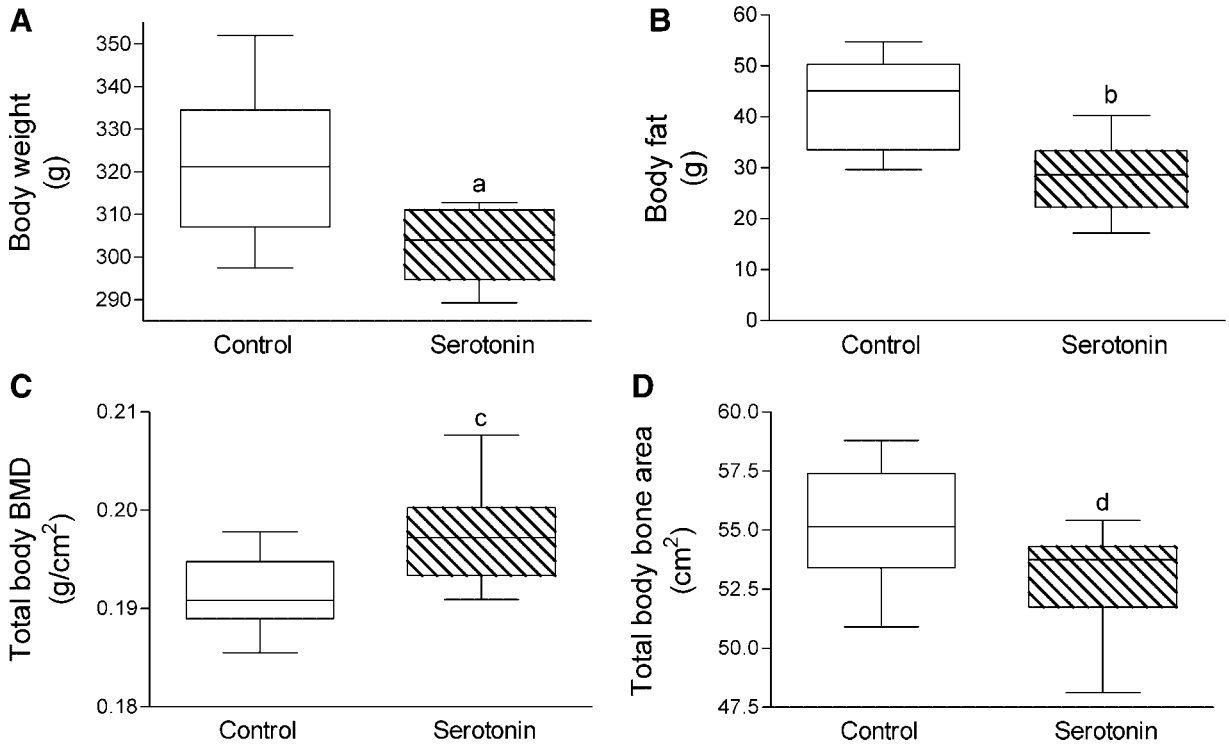

Fig. 2. Influence of long-term serotonin administration on body weight, fat content, BMD, and bone area compared to controls. Results are shown as mean ± SD; n = 10. **A**: Body weights were significantly lower in serotonin dosed animals compared to controls ($^{a}P = 0.02$). **B**: The body fat content was significantly lower ($^{b}P = 0.0008$). **C**: Total body BMD was significantly higher in the serotonin group ($^{c}P = 0.004$). **D**: Total body bone area was significantly lower in the serotonin group ($^{d}P = 0.035$).

DISCUSSION

We present here, for the first time, a study concerning long-term serotonin effects on bone in growing rats. In vitro studies have suggested that serotonin is a regulator of bone metabolism. The present work shows that serotonin seems to have important in vivo effects on bone as well.

Hormones like grehlin and leptin are involved in brain-gut regulation and recently they have been shown to have effects on bone metabolism and BMD [Fukushima et al., 2005; Martin et al., 2005]. Serotonin found in the blood circulation is mainly produced by the enterochromaffin cells of the gut. More than 99% of circulating serotonin is stored in platelet granules. As the free fraction of serotonin is believed to be biologically active, it is crucial to avoid platelet degranulation during blood sampling when serotonin measurements are to be done. We have previously demonstrated that sampling by microdialysis in femoral muscles gives a more accurate determination of the free fraction of circulating serotonin [Gustafsson et al., 2005a]. In this study we also used microdialysis combined with an improved HPLC technique to collect and analyze serotonin in femoral muscles. We found that the serotonin injections induced hyperserotoninemia, with a peak >10 times higher than controls, 2 h after the injection. The hyperserotoninemia lasted >5 h.

A disturbance in central serotonin regulation has been implicated in eating and body weight disorders, and drugs with affinity for serotonin receptors have been used in treatment of obesity [Connolly et al., 1997]. Serotonin has been shown to reduce food intake and weight gain, and a role for hypothalamic serotonergic receptor mechanisms in mediation of these effects has been suggested [Leibowitz and Alexander, 1998]. In the present work we confirm that serotonin is a potent weight reducing substance, also when injected s.c. We also found that the low-body weight was due to a reduced body fat content.

Serotonin can reach bone cells and bone cell precursors via the blood circulation, but may also affect bone tissue via serotonergic neurons. The higher total body BMD (determined by DXA) that we find in serotonin dosed animals may therefore be mediated directly through

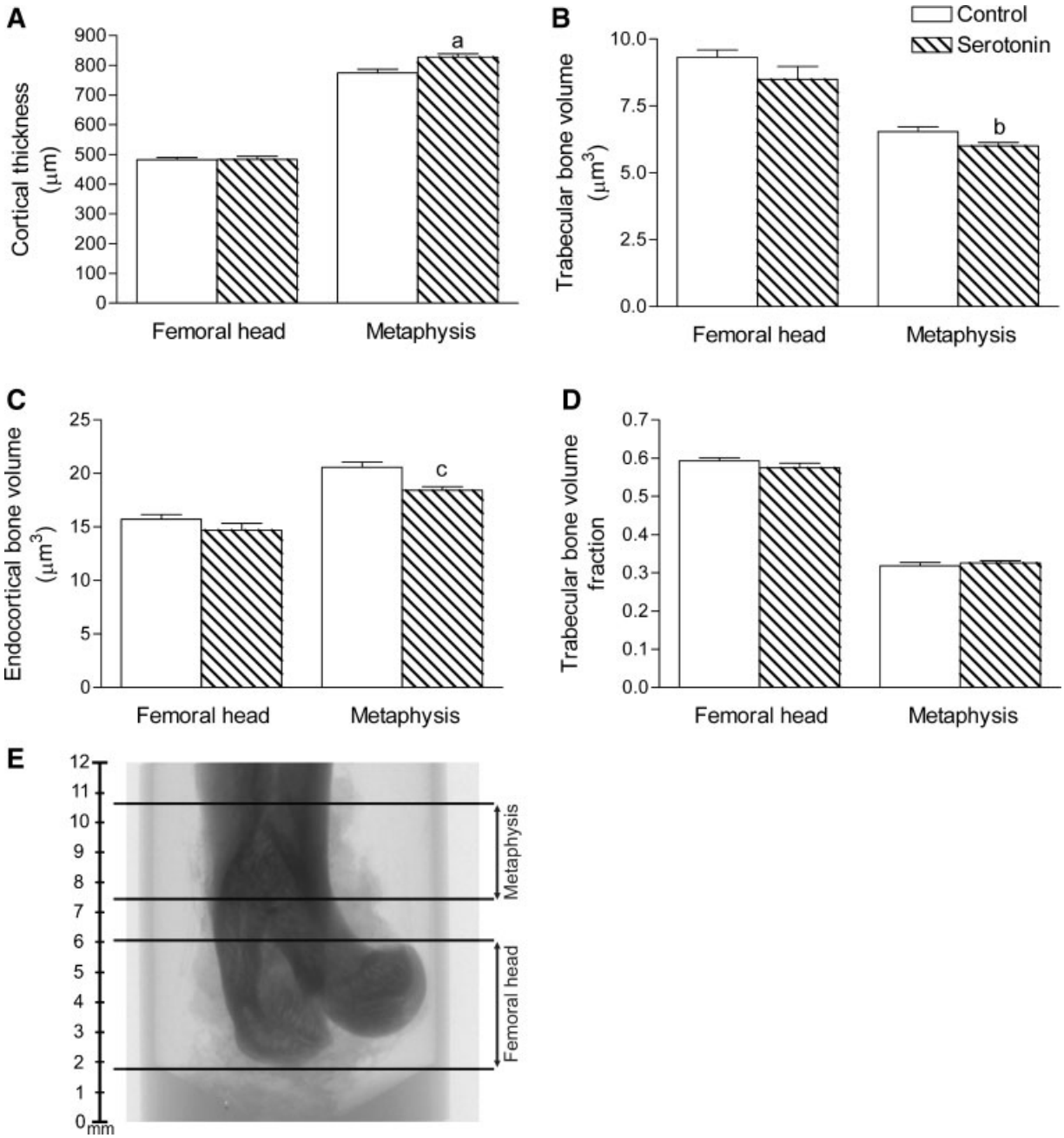

Fig. 3. Micro-computed tomography (μCT) scanning data of femoral head and metaphysis. Data are shown as mean ± SEM; n = 10. **A**: Cortical thickness (Ct.Th) is significantly higher in the metaphysis of serotonin-dosed rats versus controls ($^{a}P = 0.009$). **B**: The serotonin dosed rats had significantly lower trabecular bone volume (BV) in the metaphysis ($^{b}P = 0.035$). **C**: Endocortical bone volume (EV) was significantly lower in the metaphysis of serotonin dosed animals ($^{c}P = 0.002$). **D**: Trabecular BV fraction is similar in both groups. **E**: A typical μCT-scan image. Areas for the separate data sets of femoral head and metaphysis are depicted.

serotonin receptors on cells involved in bone metabolism [Bliziotes et al., 2001; Westbroek et al., 2001]. Indirect effects of serotonin on other neurotransmitters or hormones affecting bone metabolism; however, can not be ruled out. Although femur BMD measurements by means of DXA did not show significant changes, detailed high resolution μCT scans revealed that serotonin administration did affect bone architecture of the femur. Changes in the proximal head and the metaphysial region were similar, although they only reached significance in the metaphysis. This can be explained by the fact that normal bone turnover and adaptation of

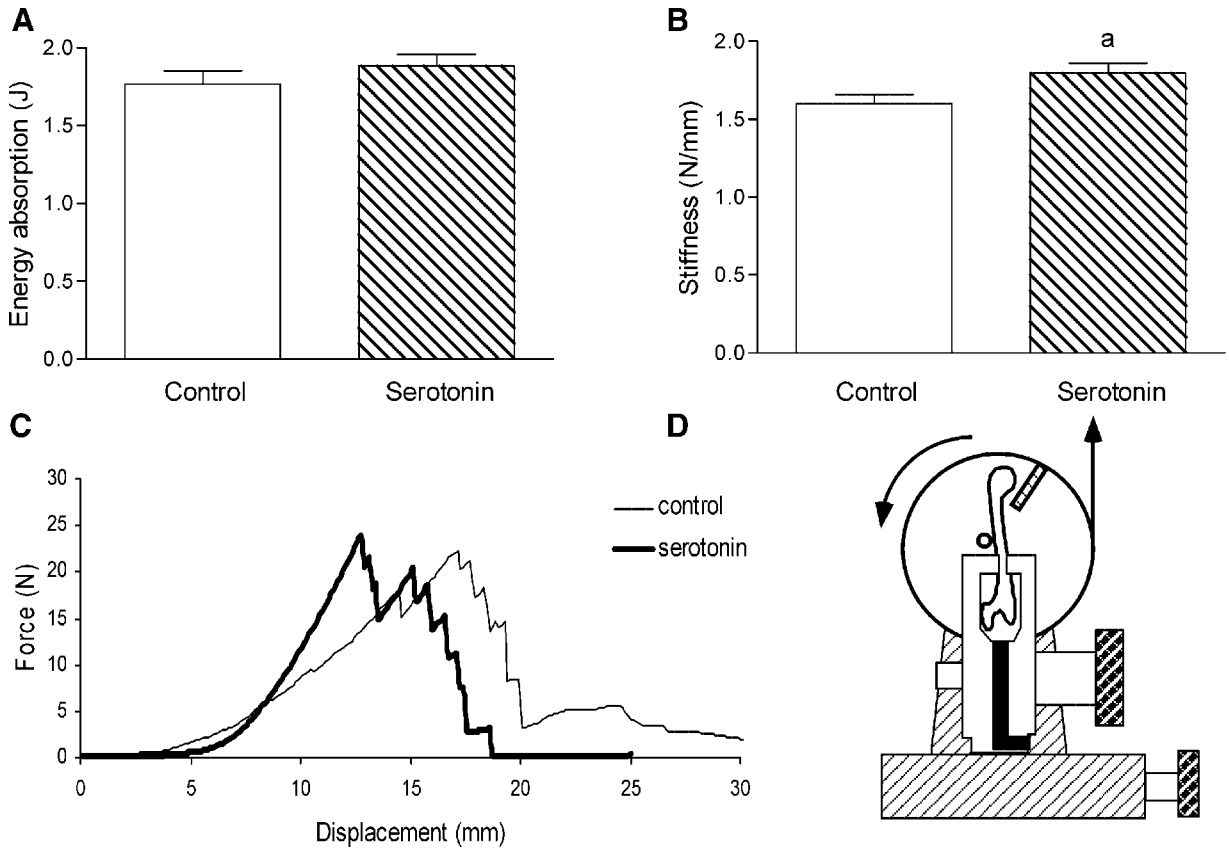

Fig. 4. Femoral midshaft mechanical properties in control and serotonin dosed rats. **A**: Energy absorption and (**B**) stiffness. [a]Statistically significantly higher compared to controls ($P < 0.05$). **C**: Load-graph showing break point. Data are shown as mean ± SEM; n = 10. **D**: Mechanical properties of the femoral midshaft were investigated using a three-point anterior bending test.

bone architecture are very high in the metaphysis of growing animals, making this region prone to react on drugs, chemicals, and hormones. We found that MOI and the perimeter of the metaphysis were unchanged, while Ct.Th was significantly higher in the serotonin group. On the other hand, we demonstrated that serotonin administration led to a lower trabecular BV, indicating that the effect of a higher Ct.Th is reversed by lower trabecular BV, resulting in similar MOI in both groups. It is also possible that there are changes in the distribution of trabecular bone in the marrow space that explains that there is no difference in MOI. For instance, the normal animals could have more trabecular bone close to the cortex, while the serotonin animals have more evenly distributed trabecular bone. The unchanged trabecular BV fraction indicates that trabecular BV was lower solely because of a smaller EV. These results indicate that bone metabolism in the metaphysis of the femurs was affected by serotonin. Our hypothesis is that endosteal resorption was decreased during growth in rats receiving serotonin leading to higher Ct.Th, lower EV, and lower trabecular BV. This is in accordance with our in vitro findings showing an increased OPG/RANKL ratio (indicating an inhibitory effect on osteoclast activity) in medium collected from osteoblasts treated with serotonin [Gustafsson et al., 2005b]. The fact that no osteoclasts could be detected in the sections of both control as well as serotonin rats, does not exclude a difference in resorption at a given time point during serotonin administration. Another possible explanation for the changes seen in bone architecture could be that serotonin induces an increased endosteal bone apposition in growing rats, which could be explained by our findings showing that serotonin induces osteoblast proliferation in vitro [Gustafsson et al., 2005b].

Furthermore, we demonstrated that the stiffness of the femurs from the serotonin

animals was increased. Bone toughness also tended to be higher, but the difference was not statistically significant. The increase in stiffness is probably due to the slightly enlarged Ct.Th, with more bone at the endosteal side. The fact that femoral stiffness was proportionally higher than the energy absorption indicates less deflection at fracture in the serotonin dosed animals.

Serotonin is a regulator of craniofacial morphogenesis, and 5-HTT is present in developing craniofacial mesenchyme in mice where it is thought to influence the morphogenetic effects of serotonin [Moiseiwitsch, 2000]. In a recent study, long-term administration of the 5-HTT inhibitor (selective serotonin reuptake inhibitor, SSRI) fluoxetine led to reduced bone accrual in growing mice [Warden et al., 2005]. Only a couple of reports on fracture risk and SSRI treatment have been published, and they suggest an increased fracture risk, even though the mechanism behind is unknown [Liu et al., 1998; Pacher and Ungvari, 2001; Hubbard et al., 2003]. Another study demonstrated decreased growth in children during therapy with SSRI [Weintrob et al., 2002]. Taken together these studies indicate that serotonin is involved in bone development and bone turnover. This is the first in vivo study showing that serotonin administration affects bone metabolism in rats. Serotonergic mechanisms are highly preserved through evolution and species differences are small. It is therefore likely that changes similar to those seen in rats receiving serotonin would develop in humans with hyperserotoninemia. Further investigation is needed to understand the physiological role for serotonin in bone metabolism.

In conclusion this study, for the first time, demonstrates that long-term serotonin administration leads to higher BMD, altered bone architecture and higher femoral bone stiffness in growing rats.

ACKNOWLEDGMENTS

This study was supported in part by, Ingrid and Torleif Hoels Legacy, grants from Nycomed Pharma AS, the Norwegian Osteoporosis Foundation and Funds for Medical Research at Trondheim University Hospital. We are grateful to the following members of our staff: Nicole Kops, Kari Slørdahl, Trine Skoglund, and Bjørn Munkvold for technical assistance in this project.

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