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Serotonin and Fluoxetine Modulate Bone Cell Function In Vitro

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Abstract Recent studies have proposed a role for serotonin and its transporter in regulation of bone cell function. In the present study, we examined the in vitro effects of serotonin and the serotonin transporter inhibitor fluoxetine ''Prozac'' on osteoblasts and osteoclasts. Human mononuclear cells were differentiated into osteoclasts in the presence of serotonin or fluoxetine. Both compounds affected the total number of differentiated osteoclasts as well as bone resorption in a bell-shaped manner. RT-PCR on the human osteoclasts demonstrated several serotonin receptors, the serotonin transporter, and the rate-limiting enzyme in serotonin synthesis, tryptophan hydroxylase 1 (Tph1). Tph1 expression was also found in murine osteoblasts and osteoclasts, indicating an ability to produce serotonin. In murine pre-osteoclasts (RAW264.7), serotonin as well as fluoxetine affected proliferation and NFκB activity in a biphasic manner. Proliferation of human mesenchymal stem cells (MSC) and primary osteoblasts (NHO), and 5-HT_{2A} receptor expression was enhanced by serotonin. Fluoxetine stimulated proliferation of MSC and murine preosteoblasts (MC3T3-E1) in nM concentrations, μM concentrations were inhibitory. The effect of fluoxetine seemed direct, probably through 5-HT_2 receptors. Serotonin-induced proliferation of MC3T3-E1 cells was inhibited by the PKC inhibitor (GF109203) and was also markedly reduced when antagonists of the serotonin receptors 5-$HT_{2B/C}$ or 5-$HT_{2A/C}$ were added. Serotonin increased osteoprotegerin (OPG) and decreased receptor activator of NF-κB ligand (RANKL) secretion from osteoblasts, suggesting a role in osteoblast-induced inhibition of osteoclast differentiation, whereas fluoxetine had the opposite effect. This study further describes possible mechanisms by which serotonin and the serotonin transporter can affect bone cell function. J. Cell. Biochem. 98: 139–151, 2006.

Key words: serotonin; molecular biology; bone metabolism

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 taken up by an active transport mechanism into a number of cell types, with platelets serving as the major reservoir. Serotonin is a vasoactive substance with an important role in systemic blood pressure regulation [Frishman et al., 1995] and mediates its actions by interacting with multiple serotonin receptor subtypes [Hoyer et al., 2002]. 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-HT_2 receptors. We recently demonstrated that long-term administration of toxic doses of serotonin leads to a carcinoid heart like

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condition with myofibroblast proliferation and plaque formation on heart valves in rats [Gustafsson et al., 2005a]. Serotonin administration also induced a significant increase in bone mineral density compared to control rats, indicating that serotonin has a positive effect on bone formation [Gustafsson et al., 2005b].

The 5-HT_{1A} and 5-HT_2 receptors have been demonstrated in both monocytes and macrophages, and serotonin is known to exert direct effects on the immune system [Mössner and Lesch, 1998]. It has also been suggested that platelets and macrophages are able to produce serotonin [Champier et al., 1997; Martins et al., 2004].

Our group and others have previously demonstrated functional serotonin receptors and serotonergic pathways in bone cells [Bliziotes et al., 2001; Westbroek et al., 2001; Battaglino et al., 2004]. An increased expression of 5-HTT in RAW264.7 cells stimulated with receptor activator of NF-κB ligand (RANKL) has been described and fluoxetine seemed to inhibit differentiation in these cells, suggesting reduced bone resorption [Battaglino et al., 2004]. In a recent study however, long-term treatment with the 5-HTT inhibitor (selective serotonin reuptake inhibitor, SSRI) fluoxetine led to reduced bone accrual in growing mice [Warden et al., 2005].

RANKL and its inhibiting decoy receptor osteoprotegerin (OPG) are central in osteoclast regulation [Simonet et al., 1997; Lacey et al., 1998]. RANKL, secreted mainly by osteoblastic stromal cells, is necessary for osteoclast formation from its committed precursors, which bear its receptor RANK. Activation of RANK leads to activation of downstream signaling pathways including NF-κB, p38 kinase, and c-Jun N-terminal kinase (JNK) [Teitelbaum, 2000]. Stimulation of JNK subsequently elicits the activation of the transcription factor c-Jun [Kobayashi et al., 2001]. c-Jun forms activator protein-1 (AP-1) complexes with c-Fos, an essential transcription factor for osteoclast formation [Grigoriadis et al., 1994]. The AP-1 complex is also known to be involved in regulation of osteoblast proliferation and differentiation [Wagner, 2002].

The aim of the present study was to investigate the effects of serotonin and fluoxetine on human and murine osteoblast as well as osteoclast proliferation and differentiation. We also wanted to examine which intra-cellular pathways might be involved. In addition, we have studied OPG, RANKL release from serotonin and fluoxetine-treated murine pre-osteoblasts.

MATERIALS AND METHODS

Cells

MC3T3-E1 (murine preosteoblasts, ATCC) cells were maintained in α-MEM (Invitrogen life technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Bat Haemek, Israel), 1 mM Na-pyruvate (Gibco, Life Technologies, Paisley, UK), 0.1 mg/ml L-glutamine (Gibco), and 10 U/ml penicillin/streptomycin (Gibco). RAW264.7 (murine pre-osteoclast, ATCC) cells were maintained in DMEM with 4.5 g/L glucose (Gibco), supplemented with 1 mM Na-pyruvate, 0.1 mg/ml L-glutamine, 10 U/ml penicillin/streptomycin, and 10% FBS. All cell studies were performed with a cell passage less then 25.

MC3T3-E1 cells were differentiated by addition of ascorbic acid (25 μg/ml) (Sigma, St. Louis, MO) and β-glycerophosphate (3 mM) (Sigma) to the growth medium [Quarles et al., 1992; Franceschi et al., 1994; Reseland et al., 2001]. RAW264.7 cells were differentiated by addition of sRANKL (50 ng/ml) (Research Diagnostics, Inc., Flanders, NJ) and M-CSF (50 ng/ml) (Research Diagnostics).

Human mesenchymal stem cells (MSC) were isolated from the iliac crest (MSC). Lymphoprep (AXIS-SHIELDPoC AS, Oslo, Norway) was used to isolate the mononuclear cells from the bone marrow. The mononuclear cells were pelleted and cultured in MEM Alpha medium (Invitrogen life technologies) with 20% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The cells were maintained in humidified 95% air 5% CO_2 atmosphere at 37°C. Half of the medium was changed twice weekly and the cells were subcultured using 0.05% trypsin with 0.01% EDTA prior to experiments. Donor recruitment and acquisition of human bone marrow were performed in accordance with a protocol approved by the local ethical committee.

Commercially available primary human osteoblasts from both femur and tibia of different donors (NHOst cell system, Cambrex, Walkersville, MD) were grown in Osteoblast Growth Media (Cambrex). Osteoblasts cultured to facilitate mineralization were exposed to

TABLE I. Primer Sets Used in RT-PCR

Primers	mRNA position	Sequence	Product length (bp)	T_{ann} (°C)
mTph1	1370–1389	5′-AGTTGCGGTATGACCTTGAT-3′		
	1561–1543	5′-AGGCGAGAGACATTGCTAA-3′	192	60
h5-HT1A	1067–1086	5′-GCTGGCTGCCCTTCTT-3′		
	1232–1213	5′-TTAAACGCGTTTTGAAAGTC-3′	166	54
h5-HT2A	1520–1538	5′-GACAATAGCGACGGAGTGA-3′		
	1943–1923	5′-GGCAATAGGTAACCAACTCAA-3′	424	54
h5-HT2A	917–936	5′-TCTTTCAGCTTCCTCCCTCA-3′		
	1139–1120	5′-TGCAGGACTCTTTGCAGATG-3′	223	58
h5-HT2B	1317–1336	5′-TGGCAGAGAACTCTAAGTTT-3′		
	1513–1493	5′-CTGCCAGTTCTGCTATACATA-3′	197	52
h5-HT2C	3638–3658	5′-ACGCTTGACAGTTACTTACAC-3′		
	3906–3890	5′-GGCAAGGCAGGTAGACT-3′	269	52
h5-HTT	2023–2038	5′-CTTGGGTTACTGCATAGGAAC-3′		
	2181–2166	5′-GCATTCAAGCGGATGT-3′	159	52
hTph1	1057–1076	5′-CCCTTTGATCCCAAGATTAC-3′		
		5′-CATTCATGGCACTGGTTATG-3′	211	50
hCalcitonin receptor	2535–2554	5′-CTTGTGGTTGACCGCTTGTT-3′		
	2733–2714	5′-ATTTCAGGTGCCAGTAACGA-3′	199	58
M13Reverse		5′-CAGGAAACAGCTATGAC-3′		55
GAPDH		5′-TGCACCACCAACTGCTTAGC-3′		
		5′-GGCATGGACTGTGGTCATGAG-3′		60

hydrocortisone hemisuccinate (200 nM) and β-glycerophosphate (10 mM) (Cambrex) in the ambient medium. The phenotype of the cells was characterized based on the expression levels of alkaline phosphatase (ALP), collagen type 1, osteocalcin and CD44, and formation of mineralization nodules.

RT-PCR

Total RNA was isolated from human osteoclasts differentiated from human peripheral blood mononuclear cells (PBMC) using RNeasy Midi Kits (Qiagen, Valencia, CA). RT-PCR was performed on total RNA from human osteoclasts according to standard procedures using the one-step RT-PCR Kit (Qiagen), and 40 cycles of amplification. For analysis of tryptophan hydroxylase 1 (Tph1) mRNA from MC3T3-E1 and RAW264.7, 1 μg of total RNA was subjected to cDNA synthesis using M-MuLV Reverse Transcriptase (Applied Biosystems, Foster City, CA) and oligo-dT primer, according to the manufactures protocol. The cDNA was amplified using the HotMaster Taq DNA Polymerase Kit (Eppendorf AG, Hamburg, Germany).

PCR-products were cloned into the pCRII-TOPO vector using the TOPO TA Cloning Kit (Invitrogen) for sequencing, according to the standard protocol. Vectors containing PCR products were transformed by heat shock into One Shot DH5-T1 cells (Invitrogen), and isolated by the SpinClean Plasmid Miniprep Kit (Mbiotech, Seoul, Korea). Products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufactures protocol. Primers are listed in Table I.

MSC and NHO cells were lysed in lysis/binding buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, pH 8.0, 0.5 mM dithiothreitol [DTT], and 1% sodium dodecyl sulfate [SDS]). mRNA was isolated using magnetic beads [oligo $(dT)_{25}$] as described by the manufacturer (Dynal AS, Oslo, Norway). Beads containing mRNA were re-suspended in 10 mM Tris-HCl, pH 8.0, and stored at −70°C until use. One microliter of the mRNA-containing solution was applied directly to obtain a first-strand complementary DNA (cDNA) using the iScript cDNA Synthesis Kit which contains both oligo(dT) and random hexamer primers (Bio-Rad Hercules, CA). RT-PCR reactions were performed and monitored using iCycler iQ (Bio-Rad). The 2X iQ SYBR Green Supermix was based on iTaq DNA polymerase (Bio-Rad). cDNA samples were analyzed for the genes of interest and the reference genes GAPDH. The amplification program consisted of a pre-incubation step for denaturation of the template cDNA (3 min, 95°C), followed by 50 cycles consisting of a denaturation step (15 s, 95°C), an annealing step (30 s 60°C) and an extension step (30 s, 72°C). After each cycle, fluorescence was measured at 72°C. A negative control without cDNA template was run in each assay. Samples were run in duplicate. To allow relative quantification after PCR, standard curves were constructed from the standard reactions for each target and housekeeping genes by plotting C_t values, that is, the cycle number at which the

fluorescence signal exceeds background versus log cDNA dilution. The C_t readings for each of the unknown samples were then used to calculate the amount of either the target or housekeeping relative to the standard. mRNA levels were calculated as the ratio of relative concentration for the target genes relative to that for the mean between housekeeping genes. Oligonucleotide primer sequences used for the real-time RT-PCR and the specific parameters are shown in Table I. Real-time efficiencies were calculated from the given slopes in the iCycler software using serial dilutions, showing all the investigated transcripts high real-time PCR efficiency rates, and high linearity ($r > 0.99$) when different concentrations were used. PCR products were subjected to a melting curve analysis on the iCycler and subsequently 2% agarose/TAE gel electrophoresis to confirm amplification specificity, T_m, and amplicon size, respectively (see Table I).

Human Osteoclast Differentiation

Osteoclasts were differentiated from PBMC [Matsuzaki et al., 1998], isolated from buffy-coat. Separation of PBMC was performed essentially as described by Bøyum [1964]. Cells were seeded into 24-well dishes, 500,000 cells/well in MEM including M-CSF and RANKL (50 ng/ml), and dexamethasone (0.01 μM) (Sigma). Assays were performed in triplicate. The medium was replaced at day 6 and 9. After 12 days, the cells were stained for tartrate resistant acid phosphatase (TRAP) using the Sigma diagnostics acid phosphatase kit (Sigma), as described by the manufacturer. TRAP positive, multinuclear (three or more nuclei) cells were regarded as genuine osteoclasts. The 5-$HT2_{A/C}$ receptor antagonist ketanserin and fluoxetine were purchased from Sigma. To develop into osteoclasts, PBMC cells need serum in addition to differentiation factors in the medium. Since FBS is known to contain rather high levels of serotonin [Battaglino et al., 2004], we determined the amount of serotonin contamination in the media using a serotonin RIA kit (Dianova, Hamburg, Germany). The sensitivity for the kit was 4 ng/ml.

In order to investigate direct osteoclast activity a pit resorption assay was performed. The PBMC cells were seeded on BioCoat Osteologic Discs (BD Biosciences, San Jose, CA) and cultured as described above. Bone resorption was determined with the BD Biocoat Osteologic Bone Cell Culture System (BD Biosciences) according to the manufacturer.

Proliferation Assays

Cell proliferation ELISA, BrdU (chemiluminiscence) kit (Roche Molecular Biochemical's, Mannheim, Germany) was used for the proliferation assays on murine bone cells. Two thousand cells/well were seeded in 96-well plates, and cultured for 24 h. Then the cells were washed once with 180 μl serum-free medium, before addition of new serum-free medium containing test substances. After 5 h, BrdU was added, and the cells were cultured for additional 18 h before incorporation of BrdU was measured as described by the manufacturer. Light emission of the samples, expressed as relative luminescence units (RLU), was measured in a micro-plate luminometer (Fluoroskan Ascent FL, LabSystems, Inc., Helsinki, Finland). The PKC inhibitor GF 109203x, the PKA inhibitor H89 and the CAMK inhibitor W7 were obtained from Calbiochem (La Jolla, CA), whereas the 5-$HT_{2B/C}$ receptor antagonist SB206553B was obtained from Sigma.

Human bone marrow cells and primary osteoblasts were seeded in 48-well plates and cultured to 50% confluency. The cells were incubated for 12 and 48 h with serum-free medium containing serotonin and fluoxetine and pulsed with 1 mCi ^{3}H-thymidine per well 12 h prior to harvest. The medium was removed and the cells washed twice with ice-cold 1× PBS and twice with ice-cold 5% TCA to remove unincorporated ^{3}H-thymidine. The cells were solubilized in 500 ml NaOH (1M), and 400 ml of the solubilized cell solution was transferred to 8 ml of Insta-gel II Plus liquid scintillation fluid (Perkin Elmer, Applied Biosystems, Foster City, CA) and measured for 3 min in a Packard 1900 TR, liquid scintillation counter (Packard Instruments, Meriden, CT).

Plasmids and Luciferase Assays

pFOSLuc transfection and luciferase assay in MC3T3-E1 cells: 15,000 cells/well were seeded in 96-well plates and transfected with 0.12 μg luciferase reporter plasmid per well (pc-Fos reporter-plasmid (nucleotides −327 to −288 of the human c-fos promoter) [Eldredge et al., 1994], a generous gift from Dr. Ugo Moens (University of Tromsø, Norway)), using 0.35 μl Fugene transfection reagent (Roche). After cultivation for 24 h, cells were treated with

agonist for 6 h. Then the cells were washed twice in PBS before addition of 20 μl lysis buffer. Luciferase activity was measured by the Turner Luminometer, model TD-20/20 (Turner Designs, Sunnyvale, CA) using the Luciferase reporter Assay System (Promega, Inc., Madison, WI).

pBIIXLuc and pFOSLuc transfection in RAW264.7 cells: The NF-κB-driven plasmid pBIIXLuc contains two copies of a HIV-NF-κB sequence cloned upstream of the mouse *fos* promoter and *Photinus pyralis* luciferase coding sequence, and was kindly provided by Dr. M. Jättelä (Danish Cancer Society, Copenhagen, Denmark). One day before the transfection, RAW264.7 cells were seeded in 96-well plates (50,000 cells per well). Cells were transfected using 0.1 μg luciferase reporter plasmid and 0.3 μl Fugene Transfection Reagent per well. After cultivation for 24 h, cells were treated with agonist for 6 h in serum-free medium. The cells were then washed with PBS followed by lysis in 15 μl lysis buffer. Luciferase activity was measured.

OPG and RANKL Release Assays

Release of OPG, RANKL, and amount of total protein were studied in medium samples collected from MC3T3-E1 cells. Thirty thousand cells in 0.5 ml MEM supplied with 10% FBS/well were seeded in 24-well plates. After 24 h, cells were washed and cultured in 0.5 ml serum-free medium for an additional 24 h. Then fresh serum-free medium containing test substances was added. Cells were cultured up to 72 h, medium samples were harvested and frozen (−20°C) until release assays were performed.

The concentration of OPG in culture media was determined by ELISA. Briefly, 96-well plates were coated with 2 μg/ml anti-mouse-OPG-antibody (R&D Systems, Inc., Minneapolis, MN). The OPG standard curve was generated using recombinant mouse OPG (R&D Systems) in twofold dilutions from 2,000 to 31.25 pg/ml. The secondary antibody was biotinylated anti-mouse OPG (200 ng/ml) (R&D Systems), and detection was carried out using streptavidin-horseradish peroxidase (R&D Systems) in combination with OPD-substrate tablets (Dako, Glostrup, Denmark). Samples were then incubated for 20 min in the dark after which the reaction was stopped by addition of 1M H_2SO_4, and absorbance was measured at 490 nm. Minimum detectable concentration of mouse OPG was 10 pg/ml. According to the manufacturer no significant cross-reactivity or interference has been observed. Intra- and inter-assay variability were less than 15% and 9%, respectively.

RANKL concentrations in culture media were determined by an immunoassay kit for quantitative determination of free sRANKL (mouse and rat; Biomedica, Vienna, Austria), according to the manufacture's protocol.

Total protein in medium was determined using Sigma Microprotein PR assay kit with a Protein Standard Solution Calibrator (Sigma). Analyses were performed using a Cobas Mira chemistry analyzer (Roche Diagnostics, Basel, Switzerland). Thirty microliter of medium were mixed with 200 μl substrate. Intra- and inter-assay variability were less than 2.4% and 3.2% respectively. The assay detection range was 10–2,000 mg/L.

Lactate Dehydrogenase (LDH) Activity

LDH activity in the culture media after incubation was used as an index of cytotoxicity. After incubation with serotonin or fluoxetine, the culture media were collected, centrifuged at 500*g* for 5 min at 4°C, and the supernatant was stored at 4°C. LDH activity was determined spectrophotometrically according to the manufacturer's kit instructions (Cytotoxicity Detection kit, Roche Diagnostics), and presented relative to the activity in the medium of untreated cells.

Statistics

All experiments were repeated three times and the data are presented 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 with Mann–Whitney *U*-test. Significance was assumed at P values lower than 0.05.

RESULTS

Serotonin Receptors Are Expressed in Human Osteoclasts and Tryptophan Hydroxylase Is Expressed in Both Osteoclasts and Osteoblasts

In human PBMC differentiated into osteoclasts, the expression of 5-$HT_{2A,\ B,\ and\ C}$ receptors, 5-HTT, and Tph1 was shown; the 5-HT_{1A}

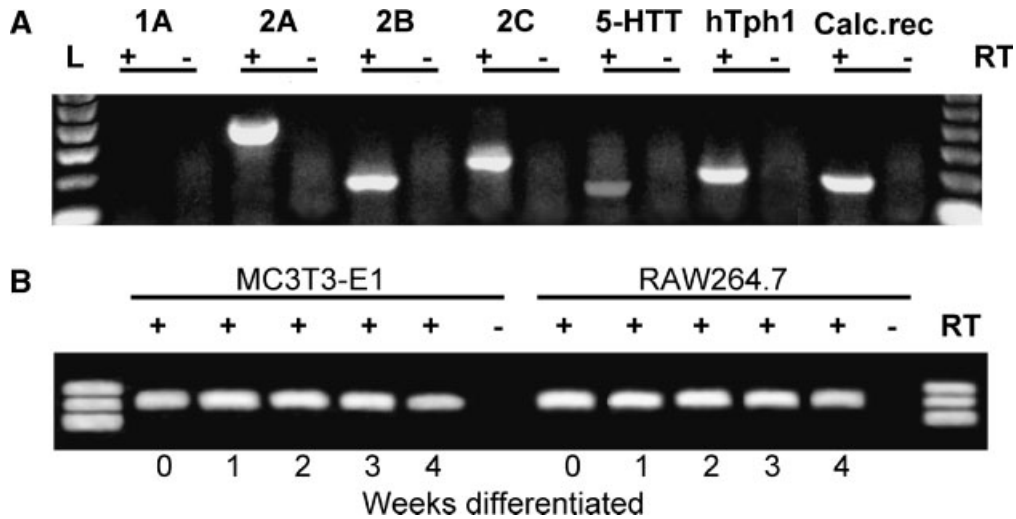

Fig. 1. Gel electrophoresis of representative PCR products from one-step RT-PCR for serotonin receptors, 5-HTT, and Tph1. **A**: RT-PCR analysis of 5-HT_{1A} (166 bp), 5-HT_{2A} (424 bp), 5-HT_{2B} (197 bp), 5-HT_{2C} (269 bp), 5-HTT (159 bp), Tph1 (211 bp), and the calcitonin receptor (180 bp) expression in human PBMC cultured with RANKL and MCSF for 12 days. PCR reactions without reverse transcriptase (RT) were used as negative controls. **B**: RT-PCR analysis for mouse Tph1 (192 bp) expression in MC3T3-E1 cells and RAW264.7 cells at different differentiation stages. RT was performed on a pool of RNA samples from MC3T3-E1 or RAW264.7 cells respectively. The DNA ladder (L) was 100 bp.

receptor however was not expressed. The calcitonin receptor was expressed, confirming that osteoclasts had developed (Fig. 1A). Undifferentiated as well as differentiated (1, 2, 3, and 4 weeks) murine pre-osteoclasts and preosteoblasts also expressed Tph1, indicating that they may be able to produce serotonin (Fig. 1B). Human MSC expressed 5-TH_{1A} and 5-TH_{2A}; and NHO cells, the 5-HT_{2A} receptor. The 5-$HT_{2B\ and\ C}$ receptors, however, were not found to be expressed in these cells (data not shown).

Serotonin Stimulates Osteoclast Differentiation and Proliferation

Previous studies have demonstrated that monocytes express serotonin receptors [Sternberg et al., 1986; Frank et al., 2001]. We therefore examined if serotonin and its transporter could influence differentiation of osteoclast precursors into osteoclasts. PBMC cells were incubated with serotonin (0.01–50 μM) or fluoxetine (0.001–10 μM) (Sigma) in addition to osteoclast differentiation factors. Furthermore, the effect of ketanserin (0.1–1 μM) (Sigma) on serotonin (10 μM)-induced osteoclast differentiation was examined. We found that serotonin as well as fluoxetine increased the total number of differentiated human osteoclasts as well as osteoclast activity (Fig. 2). At higher concentrations however, the effect of fluoxetine was inhibitory. Ketanserin inhibited the serotonin-induced osteoclast differentiation, demonstrating involvement of receptors 5-HT_{2A} and/or 5-HT_{2C}. The control media contained serotonin (0.08 μM) due to contamination from FBS.

To confirm the data on human osteoclasts, we performed a proliferation assay on RAW264.7 cells. When serotonin (0.01–50 μM) or fluoxetine (0.001–10 μM) was added to the RAW264.7 medium, the number of pre-osteoclasts also increased or decreased in a bell-shaped manner (Fig. 3). Serotonin was not detectable in media from untreated RAW264.7-cells or in media from cells treated with fluoxetine.

Serotonin and Fluoxetine Activate cFOS and NFκB in Murine Pre-Osteoclasts

To investigate which intracellular pathways are activated by serotonin and fluoxetine in osteoclasts, RAW264.7 cells were transfected with pBIIXLuc and pFOS. RANKL was used as positive control for NFκB activation. Both cFOS and NFκB were activated by serotonin and fluoxetine in RAW264.7 cells indicating a role in osteoclast formation for these transcription factors (Fig. 4). The NFκB activation was most pronounced at 0.01 μM for both substances (251% for fluoxetine and 212% for serotonin compared to control). At higher serotonin and fluoxetine concentrations, NFκB activation was absent but cFos remained activated.

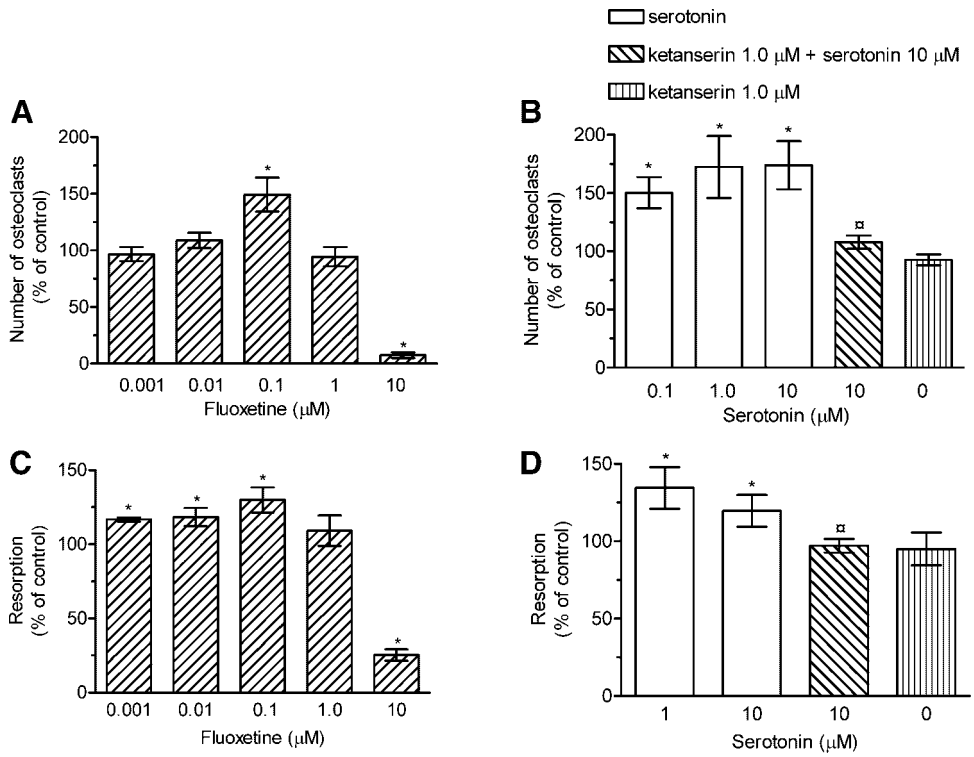

Fig. 2. Serotonin and fluoxetine affect osteoclast differentiation and activation. **A**: Human PBMC were cultured in medium with M-CSF and RANKL for 12 days in the presence of fluoxetine, (**C**) corresponding resorption pit assay. **B**: An equivalent experiment with different serotonin concentrations and the 5-$HT_{2A/C}$ receptor antagonist ketanserin, (**D**) corresponding resorption pit assay. Data are presented as percentage of control (no addition) values. *$P < 0.05$. ¤ Decrease in serotonin-induced differentiation, $P < 0.05$.

Serotonin and Fluoxetine Have a Dose-Dependent Effect on Osteoblast and Bone Marrow Stem Cell (MSC) Proliferation

Serotonin enhances the proliferation of both NHO and MC3T3-E1 cells in a bell-shaped dose-dependent manner (Fig. 5A,C). Serotonin also induced a similar dose-dependent activation of pFOSLuc in MC3T3-E1 cells (Fig. 5B), and to a lesser degree serotonin induced proliferation of MSC cells (Fig. 5D).

The PKC inhibitor GF 109203x (3.5 μM) reduced serotonin-induced proliferation, indicating involvement of this signaling pathway, the PKA inhibitor H89 (10 μM), and the CAMK inhibitor W7 (10 μM), however, had only marginal effects (Fig. 6A). The 5-HT2A/C receptor antagonist ketanserin and the 5-$HT2_{B/C}$

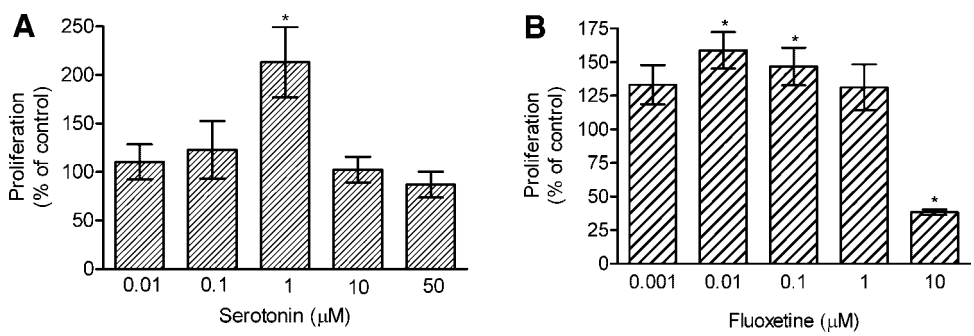

Fig. 3. Serotonin and fluoxetine enhance proliferation of murine pre-osteoclasts. **A**: RAW264.7 cells treated with serotonin in different concentrations. **B**: RAW264.7 cells treated with fluoxetine in different concentrations. Data are presented as percentage of control (no addition) values. *$P < 0.05$.

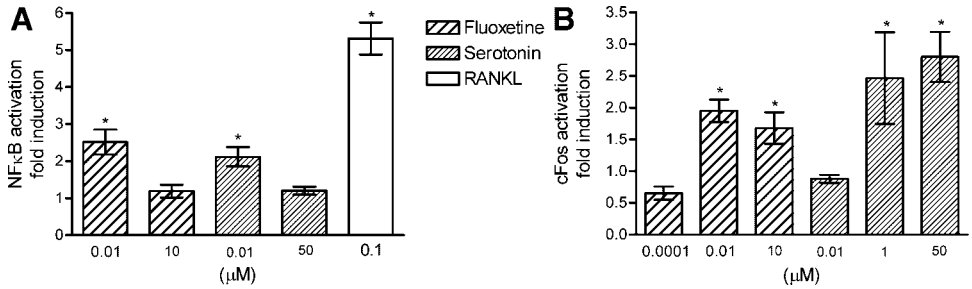

Fig. 4. Fluoxetine and serotonin activate NFκB and c-Fos in RAW264.7 cells. **A**: Fluoxetine (0.01 μM) and serotonin (0.01 μM) activate NFκB, at 10 μM however none of the compounds were effective. **B**: Fluoxetine and serotonin induce c-Fos activation. $^*P < 0.05$.

receptor antagonist SB206553B inhibited serotonin-induced osteoblast proliferation in a concentration-dependent manner (Fig. 6B). The different inhibitors and antagonists used had no influence on proliferation of MC3T3-E1 cells in the absence of serotonin.

As 5-HTT expression is found in osteoblasts, we examined the effects of fluoxetine on MSC and osteoblast proliferation. Fluoxetine seemed to stimulate proliferation of MSC and MC3T3-E1 cells at low concentrations, at higher concentrations, however, the effect was inhibitory (Fig. 7). This inhibitory effect could partly be reversed by ketanserin and SB206553B. In NHO cells, fluoxetine (0.01 μM) stimulated proliferation, higher concentrations, however, did not affect the proliferation rate. Addition of serotonin to the media reversed fluoxetine-induced inhibition of proliferation of MC3T3-E1 cells and the enhanced proliferation by fluoxetine of NHO was also reversed by serotonin. Serotonin was not detectable in cells cultured with fluoxetine or in control media. Measurements of LDH in the media confirmed

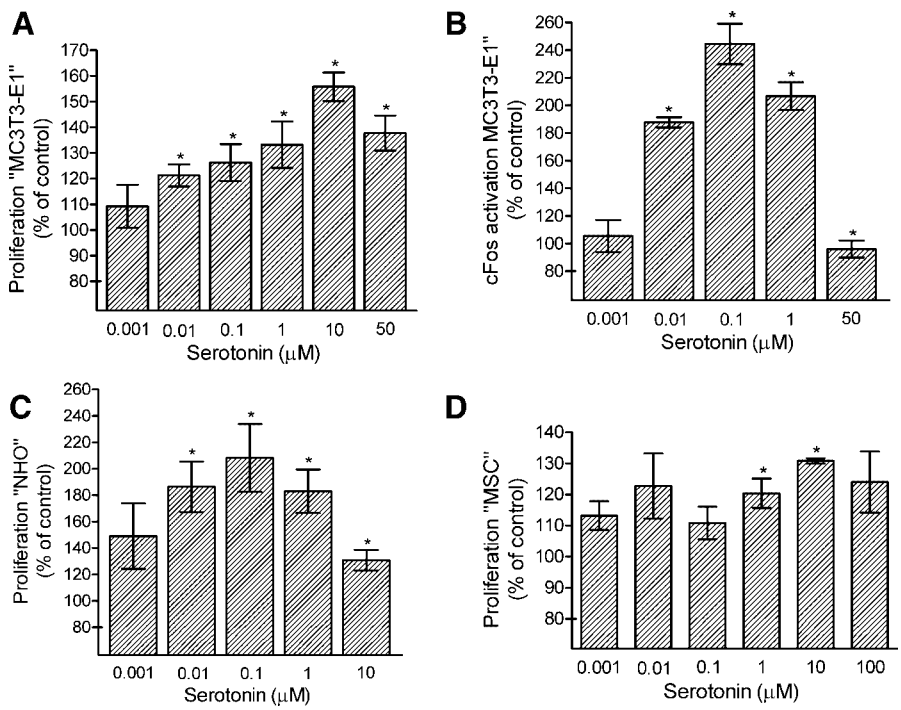

Fig. 5. Serotonin stimulates osteoblast proliferation. **A**: Proliferation assay on murine MC3T3-E1 cells treated with serotonin. **B**: Assay on pFOSLuc transfected MC3T3-E1 cells given serotonin. **C**: Proliferation assay on differentiated, human NHO cells and (**D**) human bone marrow stem cells (MSC). Data are presented as percentage of control (no addition) values. $^*P < 0.05$.

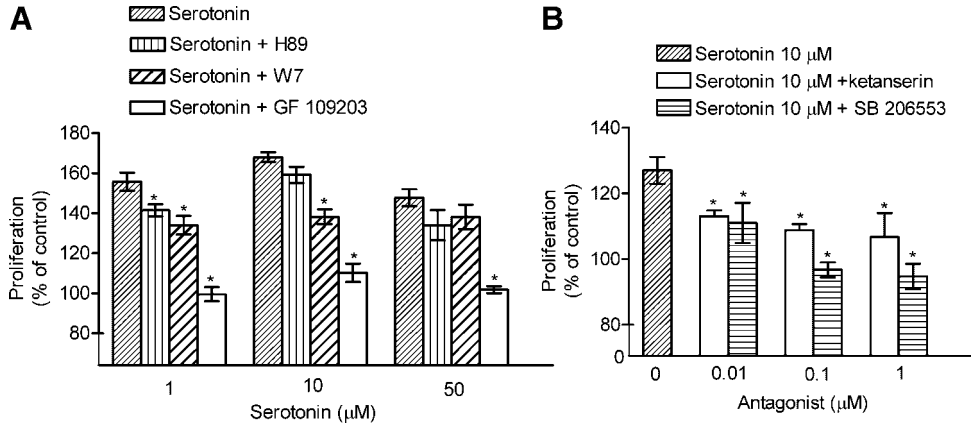

Fig. 6. Effects of serotonin receptor and intracellular signaling pathway inhibition on serotonin induced proliferation of MC3T3-E1, preosteoblasts. **A**: The PKC inhibitor GF 109203 and to a lesser extent the PKA inhibitor H89 as well as the CAMK inhibitor W7 inhibit serotonin induced proliferation. **B**: The 5-$HT_{2B/C}$ receptor antagonist SB206553 and the 5-$HT_{2A/C}$ receptor antagonist ketanserin inhibit serotonin induced proliferation. Data are presented as percentage of control (no addition) values. $^*P < 0.05$.

that the effect of high-dose fluoxetine was inhibitory and not cytotoxic (data not shown). Both serotonin and fluoxetine induced an acute (24 h) decrease (serotonin to 58% and fluoxetine 85% of control) in the expression of 5-TH_{2A} followed by an enhanced expression (to 130% and 186% of control) after 48 h in MSC, however, due to few donors and large variation in response between donors the effects failed to be significant. In NHO cells, 5-TH_{2A} expression was enhanced significantly by serotonin. The maximum effect was seen with serotonin, 1 μM, (264% of control) after 24 h and (429% of control) after 7 days (results not shown).

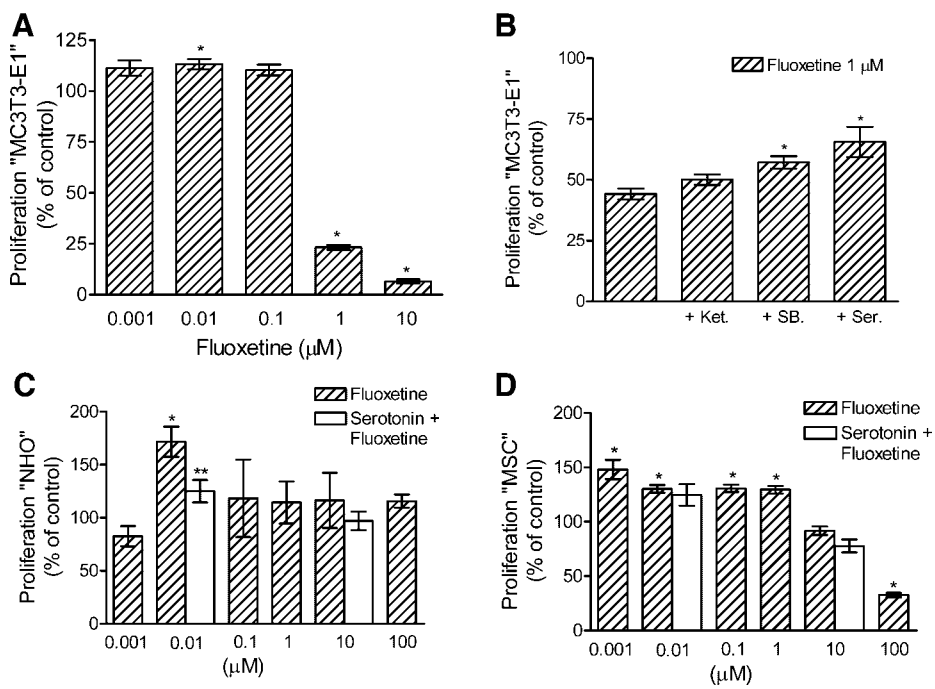

Fig. 7. Effects of fluoxetine on proliferation in MC3T3-E1, NHO, and MSC cells. **A**: At lower concentrations, fluoxetine seems to have a slight stimulating effect, whereas higher doses inhibit proliferation of MC3T3-E1 cells. **B**: The effect of ketanserin (KET), SB 206553 (SB), and serotonin (1 μM) (Ser) on fluoxetine (1 μM) induced inhibition of proliferation in MC3T3-E1 cells. **C**: The effect of fluoxetine alone and in combination with serotonin on NHO cell proliferation. ** indicates a significant reduction in fluoxetine induced proliferation, $P < 0.05$. **D**: The effect of fluoxetine alone and in combination with serotonin on MSC proliferation. Data are presented as percentage of control (no addition) values. $^*P < 0.05$.

Opposite Effects of Serotonin and Fluoxetine on OPG and RANKL Release From Osteoblasts

Serotonin reduced the RANKL release from MC3T3-E1 (Fig. 8A). The most pronounced effect (to 38% of control) was detected after 72 h incubation. Serotonin (1 μM) increased the OPG release (to 180% of control) after 72 h (Fig. 8B). Fluoxetine had a divergent effect with a more than threefold increase in RANKL release at the most and decrease to 43% of control in OPG release (Fig. 8D).

DISCUSSION

In this study, we demonstrate that serotonin induced proliferation of human primary osteoblasts (NHO) and to a lesser extent also of human MSC cells. We also find that serotonin induced proliferation of MC3T3 E1 preosteoblasts. Previously we have shown that functional serotonin receptors are present in osteoblast precursors, osteoblasts, and osteocytes [Westbroek et al., 2001]. Serotonin induces proliferation in cells originating from MSC (smooth muscle cells, fibroblasts, etc.) via the 5-HT_2 receptors [Saucier et al., 1998; Banes et al., 2005], with subsequent activation of different signaling pathways in a cell specific manner [Raymond et al., 2001]. The proliferative effect of serotonin on preosteoblasts seemed to be mediated, at least partly through binding to 5-HT_2 receptors and via activation of the PKC pathway. In MSC and NHO cells, 5-HT_{2A} receptor expression was found to be upregulated by serotonin, indicating that this receptor is involved in the proliferation induced by serotonin in these cells. In all experiments, we found a biphasic increase in proliferation, with a maximum at about 1 μM, while higher concentrations led to inhibition. This tendency is known from studies on other cell types [Pakala et al., 1997; Sharma et al., 1999; Sari and Zhou, 2003]. Serotonin activated the cFos promotor in the MC3T3 E1 cells indicating that this transcription factor is involved in serotonin-induced proliferation of osteoblasts.

Serotonin might exert its effects on bone via the blood circulation, as platelets are the main site of storage. However a direct effect via serotonergic neurons, innervating bone tissue is also possible. We showed that osteoblasts and osteoclasts expressed mRNA for Tph1, the rate-limiting enzyme in serotonin synthesis. We therefore propose that osteoblasts and osteoclasts are capable to produce serotonin, and that serotonin may act via autocrine and paracrine mechanisms. We could not find detectable levels of serotonin in serum-free osteoblast and osteoclast cell cultures, and also not after addition of fluoxetine. In vivo, bone cell serotonin production may be regulated by hormonally active

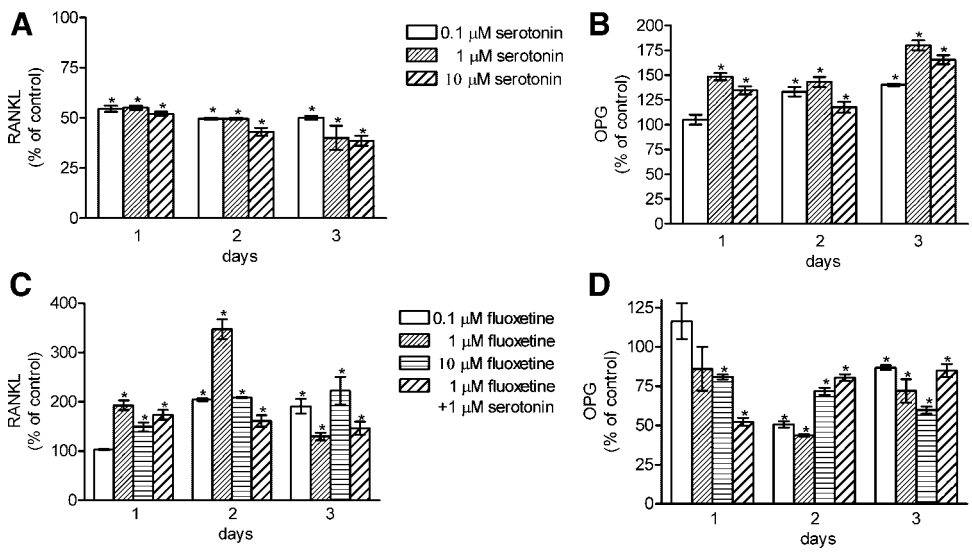

Fig. 8. Serotonin and fluoxetine effects on RANKL and OPG release in MC3T3-E1 cells. **A**: Inhibition of RANKL and (**B**) stimulation of OPG release by serotonin. **C**: Stimulation of RANKL and (**D**) inhibition of OPG release by fluoxetine. Data are presented as percentage of control (no addition) values. $^*P < 0.05$.

substances, not present in vitro, or may be under neuronal control.

The therapeutic range for fluoxetine in serum is 0.65–2.5 μM (fluoxetine + norfluoxetine). A recent study indicated that the bone marrow concentration of fluoxetine can be as high as 100 μM in patients taking the drug [Bolo et al., 2004]. The same study also showed that traces of fluoxetine could be detected in bone marrow 3 months after termination of medication. We found that fluoxetine induced proliferation of human MSC, osteoblasts, and murine MC3T3-E1 pre-osteoblasts in nM concentrations. Except for in human osteoblasts, however, μM concentrations had an inhibitory effect. If the fluoxetine concentration in bone marrow is as high as 100 μM, our findings indicate that MSC cells and pre-osteoblast proliferation will be reduced in patients taking the drug. From earlier studies it is known that fluoxetine in addition to be a serotonin reuptake inhibitor also has affinity to 5-$HT_{2A\ and\ C}$ receptors [Palvimaki et al., 1996; Ni and Miledi, 1997; Koch et al., 2002]. The fact that addition of serotonin together with fluoxetine did not enhance the effect, but slightly inhibited fluoxetine effects suggests that the fluoxetine effect is direct and not indirect through inhibition of serotonin reuptake. As the fluoxetine effects could be, at least partly, blocked by the 5-$HT_{2B/C}$ receptor antagonist SB 206553 in MC3T3-E1 cells, we believe that the fluoxetine effect could be direct on 5-HT_2 receptors. The 5-HT_{2A} receptor was expressed in MSC and NHO cells, and the expression seemed to be modulated by fluoxetine. Taken together, these data suggest that fluoxetine exerts its effects on proliferation of murine pre-osteoblasts, human MSC and primary osteoblasts via 5-HT_2 receptors, however, species differences seem to exist.

We show that serotonin stimulated differentiation of human monocytes into osteoclasts. Addition of the 5-$HT_{2A/2C}$ receptor antagonist ketanserin inhibited serotonin induced differentiation in human osteoclasts suggesting that differentiation of monocytes into osteoclasts is partly mediated through the activation of the 5-$HT_{2A/2C}$ receptor.

Fluoxetine (1–3 μM) has been shown to reduce osteoclast differentiation in vitro [Battaglino et al., 2004]. Our study confirmed that fluoxetine leads to a reduction in osteoclast differentiation and activity in μM concentrations, in the nM-ranges, however, osteoclast differentiation and activation seemed increased. Most pro- and anti-osteoclastogenic cytokines act primarily through the osteoblast to alter the levels of RANKL and OPG, the balance that determines overall osteoclast formation. The serotonin-induced augmentation of OPG and decrease of RANKL release found in the present study suggest a role in osteoblast-mediated inhibition of osteoclast generation. Fluoxetine reduced the OPG/RANKL ratio at all concentrations, indicating an osteoclastic mechanism in μM concentrations as well. No evidence of differences in resorptive indices in 5-HTT null mice was shown in vivo [Warden et al., 2005]. If the fluoxetine effect on bone is direct through activation of serotonin receptors, the comparison with 5-HTT null mice is less relevant. Mice receiving fluoxetine, however, also had reduced bone mass but increased bone resorption at the distal femur was not shown [Warden et al., 2005]. We found that fluoxetine in μM concentrations inhibited proliferation of MSCs and preosteoblasts, which may explain why fluoxetine treated mice, had a deficit in bone formation [Warden et al., 2005].

We demonstrate that both fluoxetine and serotonin activated NFκB activity in RAW264.7 cells even in the absence of RANKL. Battaglino et al. [2004] have previously shown that serotonin stimulated, whereas fluoxetine had an inhibitory effect on NFκB activity in RAW264.7 cells. We found the effect to be dose-dependent and to vanish at higher concentrations for both compounds. The dose-dependent effect of fluoxetine and serotonin on osteoclast generation can thus be regulated via NFκB activation. We also found that the transcription factor c-Fos was positively involved in serotonin- and fluoxetine-induced effects on osteoclast formation.

Taken together, serotonin and fluoxetine may affect osteoblast and osteoclast formation both positively and negatively in vitro, via different mechanisms. Their effects are markedly concentration-dependent. In vivo serotonin and fluoxetine seem to have opposite effects on BMD [Gustafsson et al., 2003; Warden et al., 2005]. Despite the limitations of in vitro approaches (immortalized phenotype, clonal nature of cultures, and lack of circulating hormones), the current study presents possible mechanisms for the serotonin- and fluoxetine-induced bone alterations seen in vivo. Studies to determine the levels of serotonin and fluoxetine

in bone marrow will be important to draw further conclusions from in vitro results. In vivo, under normal conditions, serotonergic mechanisms probably balance each other. Disruption of such a balance with serotonin interacting medications might interfere with normal bone metabolism. Medications interacting with the serotonergic system are becoming more and more common in clinical practice. SSRIs like fluoxetine are used in the treatment of depression and anxiety syndromes, 5-HT_{1A} receptor agonists in the treatment of migraine, while 5-HT_3 receptor antagonists are used for chemotherapy-induced emesis. Recently, serotonin receptor interacting medications for treating irritable bowel syndrome also became available. These drugs are often prescribed for long periods of time. Little is known about the long-term effects on the skeleton using these medications and further investigation in this field is very important.

During the last years, increasing interest has been directed against the bone modulating role of serotonin. Our data further underline that serotonin and its transporter exert important functions in bone remodeling.

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