Björn Gustafsson

The Serotonin Producing Enterochromaffin Cell, and Effects of Hyperserotoninemia on Heart and Bone

Doctoral thesis
for the degree of doktor ingeniør

Norwegian University of Science and Technology
Faculty of Medicine
Department of Cancer Research and Molecular Medicine
Section for Gastroenterology and Section for Endocrinology

NTNU
Innovation and Creativity

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List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. **Gustafsson BI, Bakke I, Tømmerås K, Waldum HL.** A new method for visualization of gut mucosal cells, describing the enterochromaffin cell in the rat gastrointestinal tract. Accepted for publication in Scandinavian Journal of Gastroenterology.

II. **Gustafsson BI, Tømmerås K, Nordrum I, Loennechen JP, Brunsvik A, Solligard E, Fossmark R, Bakke I, Syversen U and Waldum H.** Long-term serotonin administration induces heart valve disease in rats. Circulation 2005;111(12):1517-22.

III. **Gustafsson BI, Thommesen L, Stunes AK, Tømmerås K, Westbroek I, Waldum HL, Slørdahl K, Tamburstuen MV, Reseland JE and Syversen U.** Serotonin and fluoxetine modulate bone cell function *in vitro*. Accepted for publication in Journal of Cellular Biochemistry.

IV. **Gustafsson BI, Westbroek I, Waarsing JH, Waldum HL, Solligard E, Brunsvik A, Dimmen S, van Leeuwen JP, Weinans H and Syversen U.** Long-term serotonin administration increases bone mineral density and affects bone architecture as well as bone mechanical properties in rats. Accepted for publication in Journal of Cellular Biochemistry.

Summary

Neuroendocrine (NE) cells are found in a majority of the body organs. In the gastrointestinal (GI) tract, enterochromaffin cells (EC) constitute the largest NE cell population and they are distributed from the cardia to the anus. The EC cell population includes several different sub-populations, and morphological differences in shape, luminal endings and secretory granules suggest region-specific functions. The main secretory product of EC cells is serotonin and EC cells account for more than 90 % of all serotonin synthesized in the body. Serotonin is thought to be released from the EC cell by degranulation at the base of the cell as a response to luminal stimuli acting on the apical part of the cell. Serotonin functions as a key regulator of regional blood flow, motility and secretion in the gut. The embryological origin of EC cells is still under debate. Many researchers today believe that EC cells are derived from a local mucosal stem cell. In paper (I) we described a new method for visualizing morphologically intact mucosal EC cells. Some EC cells made contact with mucosal cells via axon-like, infranuclear cytoplasmatic extensions, while others had extensions that connected with underlying neurons. A third EC cell type had no or only short and blunt extensions. The serotonin released from these EC cells may reach targets such as neighboring cells or fenestrated capillaries through diffusion. EC cells were found to have striking morphological similarities with serotonergic neurons, thus indicating that they are derived from the neural crest. The finding of EC cells in mitosis, also makes the local mucosal stem cell theory less plausible.

Carcinoid tumors arising from the EC cell produce large amounts of serotonin and other hormonally active substances, giving rise to the carcinoid syndrome. The major features of the carcinoid syndrome are flushing, diarrhea, asthma and the carcinoid heart disease. Carcinoid heart disease occurs in more than 65 % of patients with the carcinoid syndrome and is characterized by fibrous thickening of cardiac valves, leading to heart failure. Whether serotonin is directly responsible for these cardiac abnormalities has so far been unknown. In order to address this issue we injected rats with high doses of serotonin once daily for three months (II). For the first time we could show that serotonin administration leads to a carcinoid heart-like

condition in rats, thus proving the relationship between serotonin and heart valve disease.

Serotonin is a well-known mitogen with proliferative effects on different cells of mesenchymal origin as well as macrophages via specific serotonin receptors. Two key cell types involved in bone metabolism are the mesenchymally derived bone forming osteoblasts and the bone-resorbing osteoclasts derived from the monocyte/macrophage lineage. It was recently shown that osteoblasts and osteoclasts have functional serotonin receptors. In paper (III) we performed *in vitro* experiments demonstrating that serotonin induces proliferation of human bone marrow stem cells, human osteoblasts and murine preosteoblasts. Serotonin also increased osteoclast differentiation and activity. This effect, however, seemed to be opposed by the finding that serotonin induced an increase in the OPG/RANKL ratio in osteoblast cell culture medium, indicating an inhibitory effect on bone resorption. A regulatory function for serotonin in bone became even more likely when we found that osteoblasts and osteoclasts expressed tryptophan hydroxylase 1 (Tph 1), the rate-limiting enzyme in serotonin synthesis, indicating that they are able to produce serotonin. We also investigated the effects of the selective serotonin reuptake inhibitor (SSRI) fluoxetine on bone metabolism *in vitro*. Fluoxetine inhibited osteoblast proliferation and reduced the OPG/RANKL ratio, indicating an overall negative effect on bone metabolism. These results may be of clinical importance as fluoxetine is the most used antidepressant drug worldwide. To evaluate possible effects of serotonin on bone formation *in vivo*, a long-term study with daily, low dose serotonin injections was performed in growing rats (IV). After three months, a significant increase in bone mineral density (BMD) developed. Micro-computed tomography (μCT) scans were performed to study bone architecture. In the serotonin group, the femoral cortex was thicker, whereas the trabecular bone volume was lower compared to controls, indicating a decrease in bone resorption or/and increased apposition of endosteal bone. These data were in accordance with the fact that the serotonin dosed animals had stiffer bones in mechanical tests. The *in vivo* findings may be explained by the serotonin-induced increase in proliferation of osteoblastic cells and elevated OPG/RANKL ratio induced by serotonin *in vitro*.

Introduction

1.1 Endocrine cells of the gut

In 1902, Bayliss and Starling were the first to introduce the concept that chemical messengers (hormones) played an important role in the control of physiologic functions (1). It is now well established that normal secretory, absorptive and motor functions of the GI tract are controlled by a complex combination of regulatory mechanisms that are chemically mediated. These so-called regulatory chemical messengers (gut hormones and neurotransmitters) are usually either biogenic amines or polypeptides, and are normally present in the nerve terminals of the myenteric plexuses and in endocrine cells dispersed within the mucosal lining of the gut. Endocrine cells within the gut epithelium from the stomach to the rectum represent the largest population of hormone producing cells in the body (2). They are scattered as individual cells throughout the mucosa, comprising approximately 1 % of the cells lining the GI lumen.

Feyrter was the first to describe the “diffuse neuroendocrine cell system” in 1938 (3). During the 1960s, gastrointestinal endocrine cells were found to express markers for neuronal differentiation, including those involved in the biosynthesis of neurotransmitters, as well as showing ultrastructural properties common to those of neurons. In 1969, Pearse described the amine precursor uptake and decarboxylation properties of NE cells and introduced the APUD concept, and he also proposed that APUD cells stemmed from the neural crest (4). The resemblance between gut endocrine cells and nerve cells gave birth to the “paraneuron” concept (5). The microvilli were regarded as the sensory part of the nerve cell, the cell body as the signal transport portion and the exocytosed secretory granules as the message. The hypothesis that NE cells are of neural crest origin has been opposed by others, and using embryonic cell tracing techniques they conclude that NE cells are derived from a common endodermal stem cell (6-8).

1.2 The enterochromaffin (EC) cell

In 1870 Heidenhain described the chromaffin (from their ability to stain a brownish color with chromic salts) cells in the gut (9). Later Kultschitzky described them as basigranular acidophil cells and as a result the cells were named Kultschitzky cells (10). The term enterochromaffin cell was first used by Ciaccio in 1907 (11). The functional significance of these cells remained obscure until Masson observed their affinity for silver salts (argentaffinity) and suggested that they probably served an endocrine function (12).

EC cells are distributed all along the gastrointestinal tract, from the cardia to the anus. They comprise the major population of gut endocrine cells and play a pivotal role in several aspects of gut function including secretion, motility and sensation (13, 14). EC cells synthesize, store and release the biogenic amine serotonin (5-hydroxytryptamine (5-HT)) as well as a variety of peptides (15-19). The biochemical pathway for serotonin synthesis initially involves the conversion of the essential amino acid tryptophan to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase (Tph). This enzyme provides the rate limiting step for serotonin synthesis. The subsequent metabolic step in the synthesis of serotonin involves the decarboxylation of 5-hydroxytryptophan into 5-hydroxytryptamine by the action of the enzyme 5-hydroxytryptophan decarboxylase. EC cells accumulate serotonin in secretory vesicles via vesicular monoamine transporter 1 (VMAT-1) (20). In the secretory vesicles, serotonin is colocalized with chromogranins, which are acidic proteins with diverse functions such as amine storage proteins and pro-hormones in enteroendocrine cells (21-24). Most EC cells are of the "open" type with apical cytoplasmatic extensions which project into the glandular lumen with short microvilli (Fig. 1). These structures represent the anatomical basis for the cell response to physical or chemical variations in luminal content (25). EC cells have been referred to as "taste buds of the gut" and are believed to function as sensory transducers that activate mucosal processes of both intrinsic and extrinsic primary afferent neurons through their release of serotonin from granule stores located at the base of the cells (14, 26). Secreted serotonin may also influence adjacent cells by paracrine actions and exert hormonal effect on distant cells via the blood circulation. EC

cells with cytoplasmatic processes sometimes projecting into adjacent glands have been shown, suggesting a role for EC cells in functional synchronization of neighboring glands (27, 28). After release, most of the serotonin is rapidly transported by the cell membrane-bound serotonin transporter (5-HTT) into a number of cell types, with platelets serving as a reservoir. It has been estimated that the adult human body has about 5 to 10 mg of serotonin, 90 % of which are in the intestine and the rest in blood platelets and the brain. The free circulating fraction of serotonin is very low due to a rapid degradation to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase in the liver and lungs.

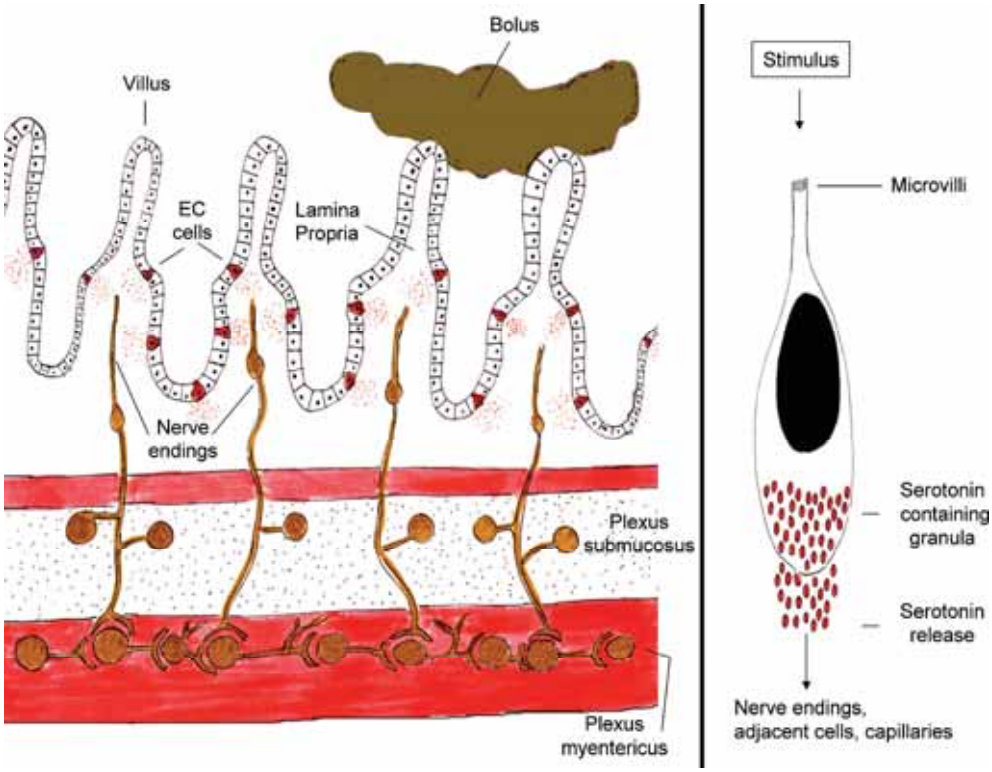

Figure 1. Left, diagram illustrating the hypothesis regarding initiation of the peristaltic reflex. A bolus in the intestinal lumen exerts pressure on the mucosa that causes serotonin to be secreted from enterochromaffin (EC) cells. The serotonin enters the *lamina propria*, where it reaches serotonin receptors on the terminals of submucosal sensory neurons. Serotonin thus participates in the initiation of the peristaltic reflex. Right, diagram showing how a luminal stimulus acts on EC cell microvilli, leading to degranulation of infra-nuclear stores of serotonin (illustration by Sarah Bååth Krantz).

From a historical perspective a number of histochemical techniques have been used to visualize EC cells (29, 30). Nowadays immunohistochemical staining techniques using antibodies specifically directed against serotonin represent a very convenient, reproducible and specific method for the visualization and identification of EC cells.

1.3 The carcinoid syndrome

Carcinoid tumors were first described by Lubarsch in 1888, when he reported the autopsy findings of two patients with multiple tumors in the distal ileum (31). The term *carcinoid* was introduced in the literature by Oberndorfer in 1907 in his description of a class of malignant tumors that behaved less aggressively than the more common adenocarcinomas of the GI tract (32). The exact nature of the tumor was not determined until 1928 when Masson described its origin as the enterochromaffin cell (33). A syndrome associated with these neoplasms was independently reported by Isler and Rosenbaum in 1953 and Thorson in 1954 (34-36). The carcinoid syndrome is caused by biologically active tumor products, such as serotonin, dopamine, cathecholamines, bradykinin and tachykinins (37-40). The primary tumor, most often located in the ileum, rarely gives any symptoms. If metastases to the liver occur, however, the tumor products drain into the caval system via the hepatic veins, thus bypassing inactivation by the liver. The result is that large amounts of tumor products enter the blood circulation and the carcinoid syndrome develops, typically consisting of episodic skin flushing, diarrhea, bronchoconstriction, sweating, abdominal cramping, and valvular heart disease.

1.4 Carcinoid heart disease

Carcinoid heart disease describes cardiac and vascular changes associated with the carcinoid syndrome. Fibrous plaques on the endocardial surface of the valvular cusps and the cardiac chambers, and on the intima of the great veins and arteries occur. The plaques contain deposits of myofibroblasts, fibroblasts, and smooth muscle cells in a

myxoid matrix (41). Carcinoid plaques are seen in areas subjected to the greatest concentrations of tumor products (42, 43). The cause of carcinoid heart disease is unknown, but serotonin was early mentioned as a possible agent involved in the pathogenesis (44). Among patients with the carcinoid syndrome, those with the highest levels of tachykinin and serotonin in serum and 5-HIAA in the urine are prone to develop heart valve changes (45-48). In addition to its role as a regulator of secretive processes, serotonin has been found to modulate cell proliferation in fibroblasts, valvular subendocardial cells and other cells of mesenchymal origin (49-53).

Serotonin produces its effects through a variety of membrane-bound receptors (54). With the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel, 5-HT receptors belong to the G-protein-coupled receptor (GPCR) super family and, with at least 14 distinct members, represent one of the most complex families of neurotransmitter receptors. Human heart valves have been shown to express mRNA for the 5-HT_{1B}, $_{1D}$, $_{2A}$ and $_{2B}$ receptors (55, 56). Fenfluramine, a serotonergic drug used as an appetite suppressant, was withdrawn from the market in 1997 because it induced a valvular heart disease similar to that seen in the carcinoid syndrome (57). This effect may be mediated via the 5-HT_{2B} receptor (56, 58). It has also been shown that ablation of the 5-HT_{2B} receptors in mice leads to abnormal cardiac development with hypoplastic ventricles (59). On the other hand, the 5-HT_{2A} receptor seems to be involved in upregulation of transforming growth factor-β and stimulation of glycosaminoglycan production in sheep aortic interstitial cells (60). In conclusion, several *in vitro* studies point out serotonin as the major pathogen in carcinoid heart disease, *in vivo* however, this has not been verified.

1.4 The skeleton

Bone is a specialized connective tissue that makes up, together with cartilage, the skeletal system. These tissues serve three main functions: 1. mechanical: support and site of muscle attachment for locomotion, 2. protective: for vital organs and bone marrow; and 3. metabolic: as a reserve of ions, especially calcium and phosphate. Anatomically, two types of bones exist in the skeleton: flat bones (skull bones, scapula,

mandible and ileum), and long bones (tibia, femur and humerus). The skeleton is composed of an outer layer of compact or cortical bone with low porosity and the inner trabecular (cancellous) with high porosity (Fig. 2 and 3). The skeleton consists of approximately 80 % cortical bone, largely in peripheral bones, and 20 % trabecular bone, mainly in the axial skeleton.

The diverse functions of bone are reflected in its composition. Bone tissue is composed of an extracellular matrix (largely mineralized), collagen and cells responsible for the formation and maintenance of the bone matrix. The cells of the bone maintain the structure of the skeleton and adapt it to mechanical demands placed upon it. Powerful systems organize and control the numbers, location, and work efficiency of cells involved in bone metabolism.

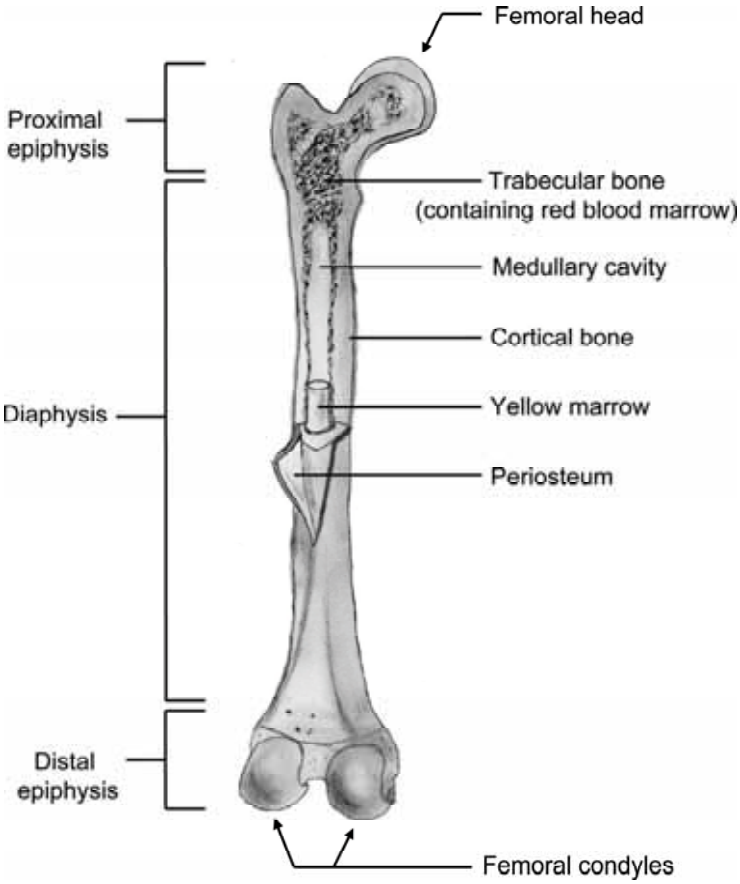

Figure 2. A human long bone (femur) (illustration by Sofie Gustafsson).

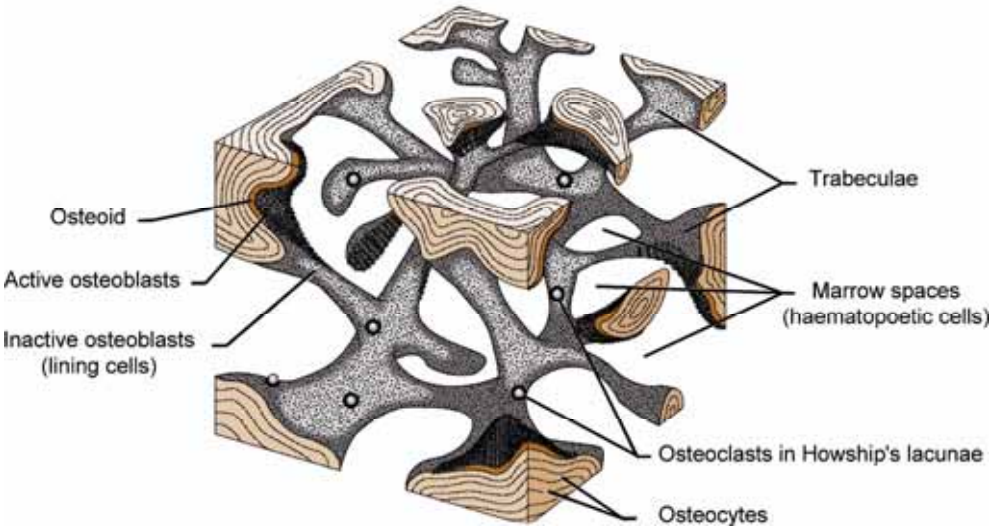

Figure 3. Structure of trabecular bone (with permission from professor Philip Sambrook, University of Sydney).

1.6 Bone cell function, modeling and remodeling

While trabecular bone accounts for the minority of total skeleton tissue, it is the site of higher bone turnover because its total surface is greater than that of cortical bone. In a growing individual bone continuously changes its shape, structure, and mass by two types of bone formation, endochondral and periosteal apposition, determining the length and width of the bones (61). During this process called modeling, the bone formation exceeds bone resorption. In adulthood the process of bone remodeling maintains the mechanical integrity of the skeleton. Remodeling is a strict coupling of bone resorption and formation. This process continues throughout life, in order to replace damaged bone with new bone (62-64). If this balance of bone formation and resorption is disturbed (un-coupled), pathological states with loss of bone like osteoporosis or with increased bone mass like osteopetrosis, may develop (65-67).

The process of bone remodeling involves three different cell types that can respond to various environmental signals. The osteoblast is situated on the bone surface at sites of active bone matrix formation (Fig. 4). It originates from a local mesenchymal

stem cell and in cell culture, osteoblasts are nearly indistinguishable from fibroblasts (68-70). Osteoblasts produce bone matrix proteins including type I collagen, the most abundant extracellular bone protein, and also takes charge of mineralization of the tissue. Other cells in the osteoblastic lineage are the osteoblast precursor cells (pre-osteoblasts), bone lining cells, and osteocytes. Pre-osteoblasts are located near bone formation sites, and can rapidly develop into mature osteoblasts. In adult bone some surfaces are not actively involved in bone formation or resorption. These surfaces are covered with a thin layer of flattened cells, the bone lining cells. Bone lining cells are formed from osteoblasts when bone formation stops, but are thought to have the ability to re-differentiate into active osteoblasts when needed (71). Osteocytes are considered to be osteoblasts that were trapped in the bone matrix that they produced. They possess several long extensions that can contact other osteocytes, osteoblasts, lining cells or/and osteoclasts and their precursors. It has been suggested that osteocytes sense mechanical load to bone, and thus participate in the modulation of bone (72, 73).

Osteoclasts are multinucleated, bone resorbing cells originating from hematopoetic mononuclear cells (74). They are usually found in contact with a calcified bone surface and within a lacuna (Howship`s lacunae) that is the result of its own resorptive activity. Bone remodeling follows an ordered sequence. In this cycle, bone resorption is initiated by recruitment of osteoclasts. Osteoclasts have a membrane called the ruffled border through which hydrochloric acid and lysosomal enzymes are released, causing bone resorption. This resorptive phase is followed by a bone formation phase where osteoblasts fill the lacunae produced by osteoclasts with osteoid, which is subsequently mineralized to form new bone matrix.

The functions of osteoblasts and osteoclasts are closely linked. Cells from the osteoblast lineage synthesize and secrete molecules that initiate and control osteoclast differentiation. It is known that two hematopoetic factors are crucial for osteoclastogenesis, the polypeptide growth factor M-CSF-1 (Macrophage-Colony Stimulating Factor-1) and Receptor Activator of NF κB Ligand (RANKL), a member of the TNF super-family. Both are expressed by osteoblasts and their immature precursors (75, 76). RANKL activates its receptor, RANK, which is expressed on osteoclasts and their precursors. Thus, RANKL promotes osteoclast formation and activation and prolongs osteoclast survival by suppressing apoptosis (77).

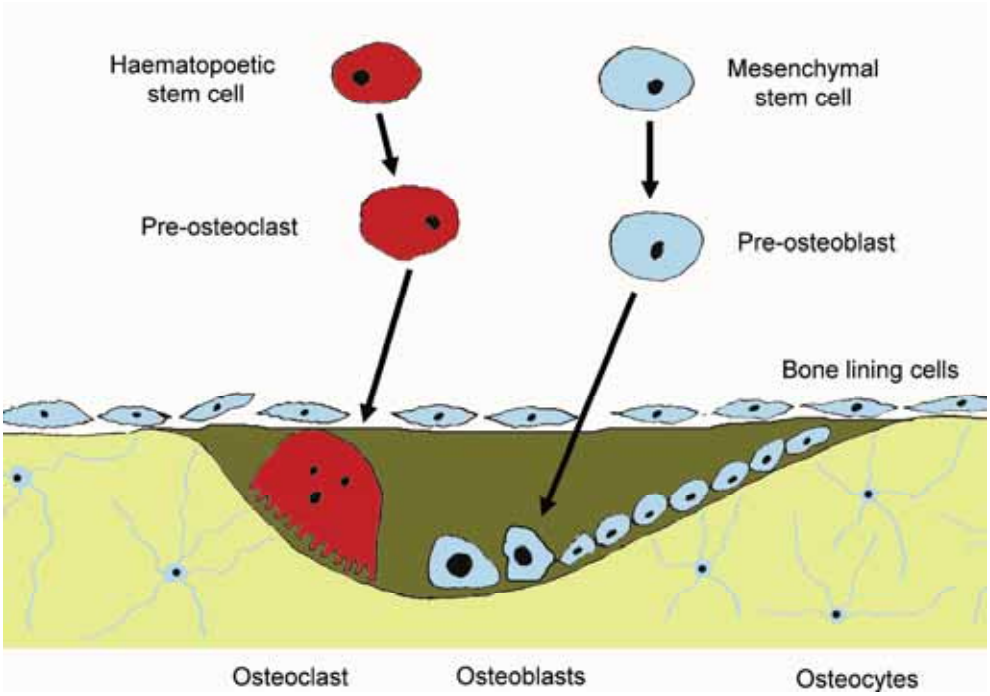

Figure 4. Diagram showing the different cell types involved in bone remodeling (illustration by Sarah Bååth Kranz).

Osteoprotegerin (OPG) is a secretory glycoprotein that is also expressed by the osteoblast and its precursors. It inhibits osteoclast differentiation and activation by binding to RANKL and preventing it from activating RANK (78). The balance between RANKL and OPG is regulated by multiple factors including cytokines, growth factors, systemic hormones and transcriptional factors, which together determines the overall osteoclast function (79-81).

Recently, signaling molecules present in the peripheral nervous system and mechanisms controlled by the central nervous system, have been shown to be involved in the regulation of bone metabolism (82-84). Studies on nerve terminals in bone have demonstrated the presence of several neuropeptides (85). In the 1990s, Rahman *et al.* showed effects of bradykinin and vasoactive intestinal peptide (VIP) on bone cell activity *in vitro* (86, 87). A few years later two groups, almost simultaneously, discovered that glutamate could act as a signaling molecule in bone, and it is now recognized that both osteoclasts and osteoblasts can be regulated by glutamate (88-92).

Receptors for the neuropeptides VIP and pituitary adenylate cyclase-activating polypeptide (PACAP) have been demonstrated on both osteoblasts and osteoclasts and activation of these receptors causes profound changes in the activity of the cells (93, 94). It has also been shown that leptin is expressed in and secreted from primary cultures of human osteoblasts and promotes bone mineralization, (95) and recently it was demonstrated that ghrelin stimulates bone formation (96). In 2000, functional serotonin receptors in both osteoblasts and osteocytes were demonstrated (97). Furthermore, 5-HTT was demonstrated in rat osteoblasts (98).

1.7 Biomechanics of bone

The strength of bone and its ability to resist fracture is dependent on its mass and geometry, but also on intrinsic (material) properties of the bone tissue itself (99). The mineral content provides strength and stiffness to the tissue, but at increasing levels of mineralization, the tissue can become more brittle, reducing the energy required for fracture (100, 101). The collagen matrix also contributes to a large extent to the mechanical properties of bone (101). It has been shown that changes in collagen structure contribute to the age-associated reduction in bone toughness and an increased fracture risk independent of BMD (102). Bone fragility can be defined as the susceptibility to fracture. The biomechanical definition of bone fragility include at least three components: strength, brittleness and work to failure. A fourth measure, stiffness, is also used to assess mechanical integrity of bones. When a force (load) in a known direction is placed on a structure, the displacement (deformation) of the structure can be measured and plotted on a force-displacement curve (Fig. 5A). Bone strength (ultimate force) is defined as the height of the curve, and describes the maximum force the bone can sustain before it breaks. The energy it can store before fracture, or work to failure, is the area under the curve.

Skeletal disease can cause fragile bones by affecting bone structure in different ways. Osteopetrosis, with an increased bone mass, causes stiff, brittle bones (Fig. 5B). They absorb very little energy before breaking (reduced work to failure) and are therefore more susceptible to fracture resulting from trauma. In osteomalacia with a

deficiency of vitamine D and calcium, the bone tissue becomes soft, which also leads to reduced work to failure. These bones, however, can deform considerably before breaking. An ideal drug to cure bone fragility would improve strength and decrease brittleness (Fig. 5C).

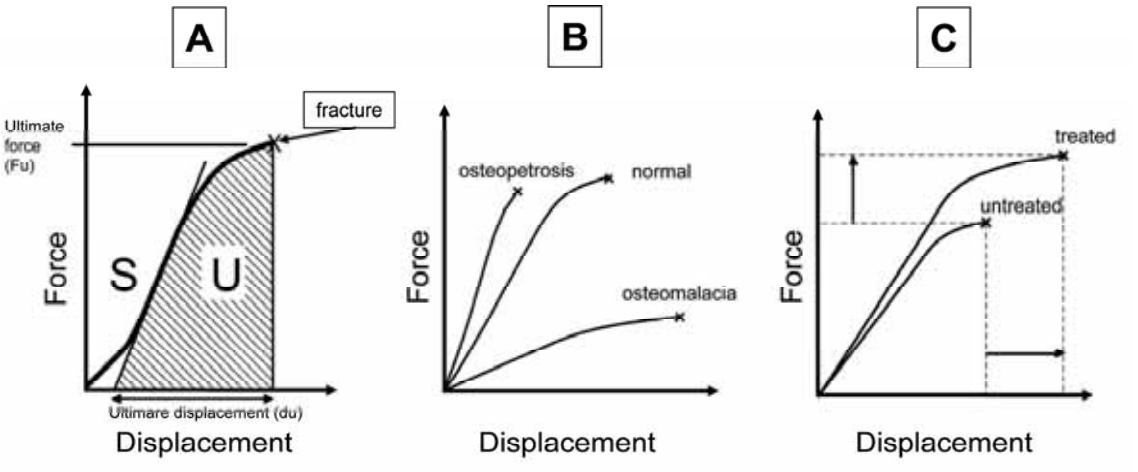

Figure 5. Diagrams showing force-displacement curves. **A;** the height of the curve represents the strength, the area under the curve is the work to failure (U), the maximum slope of the curve is the stiffness (S) and the width of the curve is the ultimate displacement (reciprocal of brittleness). **B;** osteopetrosis reduces the displacement before failure and thus increases brittleness. Osteomalacia decreases brittleness but reduces the force at failure and thus weakens bone. **C;** an ideal treatment for bone fragility.

Aims of the studies

1. To develop a new technique for visualization of morphologically intact gut mucosal cells.

2. To study the morphology of the enterochromaffin cell in different parts of the rat gastrointestinal tract.

3. To examine the expression of serotonin receptors in rat aortic valves.

4. To create a carcinoid syndrome-like condition in rats and investigate whether heart valve changes similar to those seen in the carcinoid heart disease appear.

a. By echocardiography.
b. By histological and immunohistological examinations.

5. To describe the effects of serotonin and the selective serotonin reuptake inhibitor fluoxetine on bone metabolism *in vitro*.

a. By investigating the effects of serotonin and/or fluoxetine on proliferation of human and murine osteoblastic cells *in vitro*.
b. By investigating the effects of serotonin and/or fluoxetine on proliferation, differentiation and activity of human and murine osteoclastic cells *in vitro*.
c. By examining the expression of serotonin receptors and the serotonin transporter in human osteoclasts, and the rate limiting enzyme in serotonin synthesis (Tph) in osteoblasts and osteoclasts *in vitro*.
d. By examining the release of factors modulating bone metabolism from osteoblasts treated with serotonin and/or fluoxetine *in vitro*.

6. To study the consequences on bone of long-term administration of serotonin to growing rats.

a. By measuring the bone mineral density with DXA.

b. By examining the femurs with micro-computed tomography.

c. By studying the histology of bones with histomorphometry.

e. By performing mechanical testing on femurs.

Methodological considerations

The details of all procedures have been described in each paper and only general comments concerning the different methods will be given.

1.1 Dispersion of formalin-fixed gut mucosal cells

Dispersion of tissues is done to obtain a single-cell suspension with all the different cell types composing the tissue. Traditionally, dispersion of gut mucosa cells has been done using a fresh and unfixed organ. To disperse mucosal cells from the stomach or the intestinal wall, a combination of proteolytic enzymes, acalcemia and mechanical force is needed (103-105). What is unique about the method used in paper I is that the different parts of the GI tract were fixed in formalin before dispersion, which results in morphologically intact cells in the cell suspension. To accomplish the dispersion of mucosal cells from formalin-fixed tissue we created a modified method based on the principals of enzymatic, chemical and mechanical detachment, and segregation of cells.

1.2 Immunohistochemical staining

Immunohistochemistry is a method using specific antibodies to detect molecules in their *in situ* localization on a tissue slide or in cell smears. The principle of immunohistochemistry has been known since the 1930s, but it was not until 1941 that the first immunohistochemistry study was reported (106). All stainings used in this thesis were done by the EnVision-system (DAKO, Glostrup, Denmark). The EnVision-system is based on dextran polymer technology. This chemistry permits binding of a large number of enzyme molecules (horseradish peroxidase or alkaline phosphatase) to a secondary antibody via the dextran backbone. The benefits are many, including increased sensitivity, minimized non-specific background staining and a reduction in the total number of assay steps as compared to conventional techniques. In brief, the protocol was as follows; i) Application of primary antibody; ii) Application of enzyme

labeled polymer; iii) Application of the substrate chromogen. In paper I, specific antibodies directed against serotonin were used, whereas antibodies directed against Ki-67 were used in paper II. The antigen Ki-67 is a ubiquitous nuclear protein expressed in G_1-, S-, and G_2-phases of the cell cycle, but not in the G_0-phase, and is therefore a measure of the growth fraction of cells (107-109).

1.3 Animal studies

All animals used were female Sprague Dawley rats. The reason for working only with female rats was that I had developed a rat allergy. The allergens produced by rats are well characterized, and it is believed that the urine of adult male rats is the most important source of allergen (110). In paper I, the animals were sacrificed without any prior treatment or procedures. Paper II and IV were *in vivo* studies to investigate the biological effects caused by long-term administration of serotonin (5-Hydroxytryptamine Creatinin Sulfate Complex, Sigma-Aldrich). Serotonin is well recognized as an unstable compound and decomposes quickly if treated improperly. Temperature, pH, and some metal ions are known to affect its degradation. As serotonin can not be administered orally, we first performed long-term infusion with serotonin using mini pumps. However, due to degradation of serotonin in the pumps, no detectable increase in plasma serotonin occurred (unpublished observation). The problem was overcome by daily subcutaneous injections of freshly dissolved serotonin.

1.4 Blood and microdialysis sampling

Serotonin is stored in platelet granules, and free circulating levels of serotonin are very low. Due to degranulation of platelets during blood sampling, large amounts of serotonin may leak out and cause a false, elevated serotonin concentration in plasma. Special techniques to prepare platelet-poor plasma (PPP) have therefore been developed (111, 112).

Microdialysis is based on diffusion through a semi-permeable membrane. The microdialysis catheter mimics a blood capillary. The tubular dialysis membrane is

continuously perfused by a liquid that equilibrates with the surrounding interstitial fluid (Fig. 6). After insertion of the microdialysis catheter into the tissue, dialysate samples can be continuously collected. The technique has been in use since the 1980s, and collection of serotonin from the brain is well established. It has also been used for determination of free serotonin in other tissues and in blood (113-117).

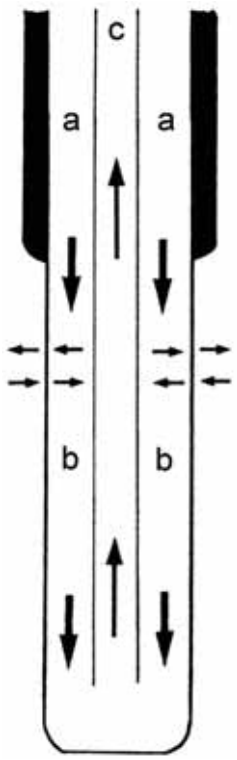

Figure 6. The tip of a microdialysis catheter. The perfusion fluid is guided through the double-lumen shaft (a). In space (b) the dialysis takes place between the inner outlet tube and the surrounding tubular outer dialysis membrane. The dialysate is collected (c).

1.5 High-performance liquid chromatography (HPLC)

Serotonin concentrations in paper II and IV were determined by HPLC. The technique was developed in the late 1960s and early 1970s (118). HPLC separates compounds that are dissolved in solution. HPLC instruments consist of a reservoir of mobile phase, a

pump, an injector, a separation column, and a detector. A small amount of liquid sample is injected into a moving stream of liquid (called the mobile phase) that passes through a column packed with particles of stationary phase. Separation of a mixture into its components depends on different degrees of retention of each component in the column. The extent to which a component is retained in the column is determined by its partitioning between the liquid mobile phase and the stationary phase.

1.6 Echocardiography

Cardiac ultrasonography (echocardiography) is a non-invasive tool for imaging the heart and surrounding structures. The evolution of ultrasonography dates back to 1880, when Pierre and Jacques Curie discovered piezoelectricity (119). The pioneers of echocardiography were Inge Edler, a cardiologist at Lund University in Sweden, and Hellmuth Hertz, a Swedish physicist (120). During the 1970s, Liv Hatle and Bjørn Angelsen in Trondheim, Norway, established the clinical use of ultrasound doppler (121). Diagnostic ultrasound employs pulsed, high frequency (>20 000 Hz) sound waves that are reflected back from body tissues and processed by the ultrasound machine to create characteristic images. In paper II, a GE Vingmed Ultrasound system Five scanner with an 8-MHz phased array probe was used to examine if leakage over heart valves existed. The examination was mainly performed as earlier described (122-125).

1.7 Reverse-transcriptase polymerase chain reaction (RT-PCR)

In 1969, the Norwegian scientist Kjell Reppe presented a new method for “repair replication” at a conference in New Hampshire, which described the principles for what we today call PCR (126). Accordingly, Kleppe was the first to describe the theoretical basis of PCR, and later Kary Mullis developed it into a technique that has became one of the most useful techniques in molecular biology (127). Kary Mullis, but unfortunately not the original inventor Kjell Reppe, was awarded the Nobel Prize in Chemistry in 1993 for this achievement (128, 129). RT-PCR is the most sensitive

technique for detection of mRNA. In papers II-IV, we have used this technique to look at expression of serotonin receptors, the serotonin transporter and Tph in total RNA extracted from tissue or cell cultures.

1.8 Cell cultures

Primary human osteoblasts (NHOst cell system, Cambrex, Walkersville, MD, USA) and human mesenchymal stem cells (MSC) isolated from the iliac crest and human osteoclasts differentiated from human peripheral blood mononuclear cells (PBMC) were used in paper III. In the same paper, two immortalized cell lines, the murine MC3T3-E1 preosteoblasts and murine RAW264.7 osteoclasts were also used. Primary cell cultures are initially established by dissociation of a tissue into single cells. Most cells will die as a result of their limited life span, which is characteristic for all somatic cells. By exposing normal, mortal cells to radiation, chemical carcinogens or certain oncogenic viruses they can be made immortalized. These cells can be dissociated by proteolytic treatment and subcultured (or passaged) into fresh cultures. In contrast to most primary cultures, immortalized cell lines are able to grow at low cell density and allow the cloning of single cells into homogeneous populations. However, they invariably display abnormal karyotypes, are in many respects already preneoplastic, and may undergo spontaneous transformation. Proliferation of cultured cells depends on numerous nutrients that are routinely supplied by a synthetic medium and on many other components including growth factors that are typically provided by supplementing the medium with serum. As serum contains large amounts of serotonin, cell culture studies looking at the effects of serotonin on proliferation, differentiation and release of mediators must be done using serotonin depleted media.

In vitro results from cell cultures must be interpreted very carefully. In fact, cell lines are poor models of their *in vivo* counterpart. Receptors and signal transduction proteins are differentially expressed during embryogenesis, growth and even under different physiological conditions. The effects of a compound may therefore vary at different cell stages. Lack of circulating hormones, neurotransmitters and paracrine signals from neighboring cells can also make results from cell culture studies difficult to

interpret and firm conclusions for *in vivo* effects should not be drawn. Yet, to define mechanisms, simplified systems are necessary. The biological relevance of the findings, however, needs to be validated in living animals or in the human organism.

1.9 Dual X-ray absorptiometry (DXA)

DXA, a technique for measuring bone mineral density (BMD) and soft tissue composition (body fat and lean tissue mass), was used in paper IV (130). A DXA scanner produces two X-ray beams, each with different energy levels. One beam is high energy while the other is low energy. The amount of x-rays that passes through the tissue is measured for each beam and it will vary depending on the thickness of the tissue. Based on the difference between the two beams, the BMD, fat content and lean mass can be measured.

1.10 Micro-computed tomography (μCT)

Measuring the BMD alone is not sufficient to understand how a compound or a disease may affect bone composition and fracture risk. In paper IV, we therefore studied the bone architecture by μCT (131-133). Based on the architecture, μCT can also be used to determine the strength and stiffness of the bone sample (134).

1.11 Mechanical testing

Strength and stiffness are important mechanical properties of bone. These properties can best be understood by examining the bone under loading. In paper IV, a three-point bending test was used to determine the mechanical properties of rat femurs. The test was performed, with a few modifiations, as earlier described (135).

Results and discussion

1.1 Cells dispersed from formalin-fixed mucosa retain their morphological characteristics

In paper I, the results of a new method for visualization of morphologically intact gut mucosal cells are shown. Due to the formalin-fixation prior to dispersion, the mucosa cells maintained their 3-dimensional features. The morphological description of GI mucosal cells has more or less been based upon histological examination of thin sections, resulting in 2-dimensional images (13, 136). Serial sectioning makes it possible to create 3-dimensional reconstructions of the histological anatomy, but the techniques are very time-consuming and the resulting 3-dimensional computer reconstructions are not telling the full truth (28).

In contrast, the current method is easy to perform and the whole procedure is done in a few hours. Using cell-specific antibodies, the different cell types of the cell-suspension smears can be visualized and their morphology thus studied.

1.2 The enterochromaffin cell has a neuron-like appearance

EC cells have traditionally been described as bottle-shaped with apical extensions towards the gut lumen. A few studies have shown that they sometimes possess basal processes directed towards the submucosa as well (27, 28, 137). We found that practically all EC cells posessed projections towards the lumen and that “closed” EC cells, without luminal contact, are very rare. This finding underlines that EC cell function is to sense and respond to changes in the luminal content (14). A large population of EC cells, especially in the colon and in the rectum, had very long extensions, projecting from the base of the cells as well. These extensions were directed towards the base of neighboring cells and in some cases also into the *lamina propria*. A few EC cells had extensions that connected with neuron-like structures. All EC cell

extensions, the luminal as well as those at the base of the cells, had serotonin containing granules throughout their whole length. The EC cells thus have the anatomic basis to sense changes in the gut content and directly pass on the information to neighboring cells, the capillary net or to neuron endings in the *lamina propria*, using serotonin as the messenger. These findings are in accordance with the paraneuron concept which points out the resemblance between neuroendocrine cells and neurons (5). It has been suggested that EC cells are lacking in vertebrates only when there is an innervation of the gut mucosa by nerve fibers containing high concentrations of serotonin (138-140). As EC cells have serotonin-containing axon-like projections that probably make synaptic contact with other cells and neurons, it is tempting to suggest that EC cells are specialized serotonergic neurons. Differentiated EC cells with the ability to divide were also found, casting further doubt on a role for a local mucosal stem cell in EC cell renewal (141). The striking resemblance between EC cells and serotonergic neurons indicates that they may be neural crest derived, as stated already in the 1960s (4). It is therefore possible that the well accepted local stemcell theory, suggesting that all gut mucosal cells derive from a common local stemcell, may be wrong (7, 142).

1.3 An animal model for the carcinoid syndrome

By injecting rats with high doses of serotonin a carcinoid syndrome-like condition was created (paper II). The carcinoid syndrome is caused by high circulating levels of vasoactive substances secreted by carcinoid tumors arising from EC cells. The major pharmacologic agent causing diarrhea in the carcinoid syndrome has been proven to be serotonin (143). Substances like histamine, kinins and tachykinins have been reported to cause the periodical flushing seen in the carcinoid syndrome, but a correlation to high levels of serotonin and catecholamines has also been shown (39, 144-146). In the current study, clinical signs as loose stools and flushing were seen as a result of serotonin injections. The flushing appeared only a few minutes after the injections, indicating a direct correlation between serotonin and flushing.

Determination of free serotonin in plasma is hard to accomplish due to degranulation of the serotonin storages in platelets. Despite that protocols to avoid

platelet degranulation were followed, serotonin levels were extremely high in PPP (10 940 ± 739.2 nM) after serotonin injections. To be sure that the high PPP serotonin levels seen reflected the free circulating serotonin levels, microdialysis in the femoral muscles was performed. The basal level of serotonin in microdialysate was below the detection limit for the HPLC method used in paper II. The improved HPLC method used in paper IV, however, showed that microdialysate from the femoral muscles in a control rat contained 2.8 nM serotonin, which was less than one 10^{th} of the serotonin level seen in PPP (51.8 nM) of control rats. Serotonin injections resulted in a peak concentration in microdialysate after 2 h (302 ± 11 nM), and thus a 100-fold increase compared to control. The serotonin concentration remained manifold increased for more than 6 hours. The serotonin injections induced a carcinoid-like syndrome both regarding clinical signs and the level of free circulating serotonin. Microdialysis seems to be the most accurate way to collect samples for determination of free circulating serotonin.

1.4 Serotonin administration induces a carcinoid heart-like condition in rats

Echocardiographic examination of rats with hyperserotoninemia demonstrated that a similar heart valve leakage as seen in the carcinoid heart disease had developed (43). The echocardiographic results correlated with the histopathological findings of thickened and retracted aortic cusps with carcinoid heart-like plaque formation. Rat aortic cusps expressed mRNA for the 5-$HT_{1A, 2A}$ and $_{2B}$ receptors, confirming that one or more of these receptors may be involved in the pathogenesis of carcinoid heart disease (55, 56, 60, 147). The 5-HTT was also expressed, indicating that SSRIs like fluoxetine have the potential to affect heart valves via a local increase in serotonin (148, 149). No clinical studies, however, have so far been able demonstrate any negative cardiac effects of these drugs (150). The fact that long-term serotonin administration induces a carcinoid heart-like disease in rats, suggests that serotonin is the agent causing the carcinoid heart disease in humans.

1.5 Serotonin affects bone metabolism

It has previously been shown that functional serotonin receptors are present in osteoblast precursors, osteoblasts and osteocytes (97). In paper III, we demonstrated that the proliferative effect of serotonin on preosteoblasts was mediated, at least partly, through binding to 5-HT_2 receptors and activation of the PKC pathway. In MSC and NHO cells, 5-HT_{2A} receptor expression was found to be up-regulated by serotonin, indicating that this receptor is involved in the proliferation induced by serotonin in these cells. The effect of serotonin was biphasic, which is also known from studies on other cell types (151-153). Serotonin also affected osteoclast proliferation *in vitro*. A direct stimulation of osteoclast proliferation as well as differentiation and activity in a resorption pit assay was seen. On the other hand, serotonin increased the OPG/RANKL ratio in osteoblast cultures. The OPG/RANKL system is known as the most potent regulator of osteoclast formation and activity (76, 154), indicating that the overall effect of serotonin on osteoclast activity *in vivo* may be inhibitory. As demonstrated in paper IV, serotonin administration leads to higher BMD and thicker cortex in the femur metaphysis in growing rats. The femur perimeter, however, was not different from control rats and the bone marrow volume was lower, indicating that the increased cortical thickness could be due to an increased apposition of endosteal bone or perhaps also result from a reduced osteoclast activity leading to less endosteal bone resorption.

The changes in bone architecture also altered mechanical properties in femurs collected from serotonin dosed rats. As a result of a thicker cortex and less trabecular bone, the bones were stiffer.

Both osteoblasts and osteoclasts expressed Tph 1, indicating that they, like fibroblasts and macrophages, may be able to produce serotonin (155, 156). It is thus possible that serotonin is a coupling factor, regulating osteoblast and osteoclast activity.

1.6 Fluoxetine affects bone metabolism *in vitro*

As shown in paper III, fluoxetine induced proliferation of human MSC and osteoblasts, as well as murine MC3T3-E1 preosteoblasts in nM concentrations. On the contrary, μM concentrations had an inhibitory effect on MSC and MC3T3-E1 cell proliferation. The proliferative effect seen at lower concentrations may be of less importance as the serum levels of fluoxetine in patients treated with this drug are 0.65–2.5 μM (fluoxetine + norfluoxetine) and the bone marrow concentration of fluoxetine may be as high as 100 μM (157). Fluoxetine had a similar direct effect on osteoclast proliferation and also on osteoclast differentiation and activity; at nM concentrations osteoclast proliferation, differentiation and activity were increased, whereas concentrations > 1 μM were inhibitory. On the other hand, fluoxetine suppressed the OPG/RANKL ratio at all concentrations, indicating increased osteoclast formation and activation. Fluoxetine has been shown to affect bone mass negatively in rats, and in humans there are indications for an increased fracture risk and reduced growth in children using the drug (158-161). The overall fluoxetine effect on bone thus seems to be negative both *in vitro* and *in vivo*.

Fluoxetine did not induce serotonin elevation in the media and addition of serotonin to the media tended to reverse the fluoxetine effects on osteoblast proliferation. In addition, antagonists of the 5-HT_2 receptors also reversed fluoxetine-induced effects. It is therefore plausible that fluoxetine exerts direct effects on bone cells via 5-HT_2 receptors and not indirectly through inhibition of serotonin reuptake by blocking 5-HTT. Fluoxetine has been demonstrated to have affinity for these receptors, which supports this theory (148, 149, 162).

Conclusions

1 Dispersion of formalin-fixed gut mucosa is a new method to investigate single gut mucosa cell morphology.

2 EC cells have a neuron-like appearance.

3 Daily subcutaneous injections with serotonin results in a carcinoid syndrome-like condition in rats.

4 Long-term hyperserotoninemia leads to a carcinoid heart-like condition in rats.

5 Serotonin directly affects proliferation of osteoblastic cells via binding to 5-HT_2 receptors and subsequent activation of the PKC pathway.

6 Serotonin increases the OPG/RANKL ratio *in vitro*.

7 Serotonin affects osteoclast proliferation, differentiation and activation in a bell-shaped manner *in vitro*.

8 Long-term hyperserotoninemia results in a higher total body BMD in rats.

9 Long-term hyperserotoninemia leads to a thicker femoral cortex and a smaller bone marrow cavity in rats.

10 Long-term hyperserotoninemia alters bone mechanical properties in rats.

11 Fluoxetine has a biphasic effect on osteoblast and osteoclast proliferation *in vitro*.

12 Fluoxetine reduces the OPG/RANKL ratio *in vitro*.

13 Fluoxetine seems to have an overall negative effect on bone metabolism *in vitro*.

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Paper I

A New Method for Visualization of Gut Mucosal Cells, Describing the Enterochromaffin Cell in the Rat Gastrointestinal Tract.

Running head: THE MORPHOLOGY OF THE ENTEROCHROMAFFIN CELL

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Abstract

Background: Enterochromaffin (EC) cells in the gastrointestinal tract have important functions as regulators of secretion, motility and sensation. The EC cell has traditionally been described as bottle-shaped with basally located stores of serotonin. Stimuli acting on the apical membrane trigger serotonin release, which in turn activates the sub-epithelial sensory nerve terminals. To better describe the EC cells appearance we developed a new method for visualization of mucosal cells.

Methods: The stomach, small intestine and large intestine were excised from Sprague Dawley rats and then fixed in formalin. The organs were everted and filled with pronase solution. Single cells and aggregates of formalin-fixed mucosal cells were collected by scraping the mucosa off the muscularis mucosa. EC cells were visualized by staining for immunoreactivity against serotonin.

Results: EC cells with luminal extensions and very long (up to 80 μM) basally located axon-like extensions, sometimes connecting to neuron-like structures were found. Other EC cells had no or only short and blunt basal extensions. Dividing, serotonin-containing EC cells were also seen.

Conclusions: These findings may be of importance to further understand EC cell function in gastrointestinal physiology. The new method described can easily be applied to better visualize the morphology of other mucosal cells as well.

Key words: Enterochromaffin cells, immunohistochemistry, morphology, neuroendocrine

Introduction

The EC cells of the gut are characterized by their high content of serotonin (5-hydroxytryptamine (5-HT)). They comprise the major population of gut endocrine cells and play a pivotal role in several aspects of gut function including secretion, motility and sensation [1]. EC cells are distributed all along the gastrointestinal tract, from the cardia to the anus [2]. Serotonin producing tumours arising from EC cells lead to the carcinoid syndrome [3] and hyperserotoninemia has also been shown to cause a carcinoid heart like disease [4]. The EC cell population seems to include several different sub-populations [5]. Morphological differences in shape, luminal endings and secretory granules suggest region-specific functions. Most EC cells are of the "open" type with apical cytoplasmatic extensions which project into the glandular lumen with short microvilli. These structures represent the anatomical basis for the cell response to physical or chemical variations in luminal content [6]. EC cells are believed to function as sensory transducers that activate mucosal processes of both intrinsic and extrinsic primary afferent neurons through their release of serotonin [1].

In biopsies from human duodenum, fibres showing serotonin immunoreactivity (IR) at the base of EC cells have been seen [7]. These fibres were proposed to be either infra-basal projections of the cells or branches of the enteric nervous system. Cytoplasmic processes sometimes projecting into adjacent glands have also been shown, suggesting a role for EC cells in functional synchronization among neighbouring glands [8,9]. Synaptic contact between EC cell extensions and underlying nerve fibres has not been demonstrated.

To further investigate the appearance of EC cells we have developed a method for visualization of formalin-fixed, single mucosal cells and described the

morphology of EC cell sub-populations in different regions of the rat gastrointestinal tract.

Materials and Methods

Buffers

Buffer A consisted of: NaCl 80 mM, KCl 5.0 mM, NaH_2PO_4 0.5 mM, Na_2HPO_4 1.0 mM, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 50 mM, glucose 11 mM, $NaHCO_3$ 20 mM, ethylenediaminetetraacetic acid (EDTA) 2.0 mM and bovine serum albumin (BSA) 10 g/L. Buffer B was analogous to buffer A, but contained $CaCl_2$ 1.0 mM and $MgCl_2$ 1.5 mM instead of EDTA and BSA 1.0 g/L. Both buffers were adjusted to pH 7.4.

Cell fixation and isolation

The animal experiments were approved by the Animal Welfare Committee of the University Hospital of Trondheim, Norway. Female Sprague-Dawley rats (Møllegaard's Breeding Center, Skensved, Denmark) (~200 g) were housed under standard conditions with free access to food and water. The rats were killed by gas (CO_2) and the different parts of the gastrointestinal-tract (rectum, colon, ileum, jejunum, duodenum and antrum) were collected and washed clean of content in 0.9 % saline. The organs were then fixed in 4% buffered formaldehyde at room temperature. After fixation the sacks were carefully everted and filled with pronase solution (7.5 mg/mL buffer A) (Merck cat no 1074330001, Darmstadt, Germany) until maximally extended and put in buffer A. The dispersion was performed at 37°C, one hour for the antrum and 30 minutes for the intestine. Cell suspensions were prepared by gently scraping the mucosa off the muscularis mucosa and dissolving it in buffer B.

Whole wall specimens from the rectum, colon, ileum, jejunum, duodenum and antrum were fixed by immersion in 4% buffered formalin. Following dehydration of the specimens and embedding in paraffin, 10 μm thick sections were cut perpendicularly to the mucosal surface.

Immunohistochemistry

Cell smears for immunohistochemistry were prepared by placing a drop of each cell suspension on glass slides, followed by air drying over night. The cells were then rehydrated and treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Rat monoclonal anti-serotonin (ab6336, Abcam Limited, Cambridgeshire, UK) was diluted (1:75) in PBS containing 0.25% Triton X-100 (Calbiochem, San Diego, CA, USA) and 0.25% BSA (Sigma, St Louis, MO, USA). The cell smears were incubated with primary antiserum for 2 hours at room temperature. An EnVision-HRP kit (K5007, Dako, Glostrup, Denmark) and an AEC peroxidase kit (SK4200; Vector, Burlingame, CA, USA) were used to visualize the immunoreaction. We also performed immunohistochemistry on 10 μM sections from paraffin embedded tissue. Except for deparaffinization the procedure described above was used for serotonin immunostaining of the sections as well. Most preparations were lightly counterstained with hematoxylin.

Results

In the rectum, the vast majority of EC cells was of the "open" type with luminal extensions. In some cases the dispersed EC cells were still attached to one or more mucosal cells. Two morphologically distinct subpopulations of EC cells were found. One of the subpopulations had a luminal extension and two or more relatively

short (10-15 μm) and blunt basal extensions containing serotonin granules (Fig. 1A). The body and nucleus of these cells appeared to lie in the glands. The other subpopulation had very long (50-80 μm) and thin basal extensions (Fig. 1B). The basal extensions had serotonin containing granules and often demonstrated a terminal button with accumulation of serotonin. The cell bodies were in some cases located outside the glands but the cells were still in contact with the lumen via slender luminal projections. The EC cells in the colon also comprised two distinct sub-populations similar to those seen in the rectum (Fig. 2). In a few cases we found EC cells with long basal extensions and terminal buttons connecting to neuron-like (dendrites?) structures without serotonin IR. In traditional histological sections from the colon, basal extensions seemed to surround the glands. Serotonin positive fibres were also seen in the *lamina propria* (Fig. 3).

In the ileum, jejunum and in the duodenum (Fig. 4A), most cells had an apical projection but no basal extensions. When present (~ 10 %), the basal extensions were shorter than in the large intestine but they had a similar appearance, with serotonin containing granules and sometimes terminal buttons.

The antral EC cells constituted a less homogeneous population (Fig. 4B). Some cells had luminal projections and short basal extensions. Others had a spider-like appearance with multiple extensions. As these cells rarely were attached to neighbour cells, the direction of the extensions could not be determined. When whole antral glands were preserved, EC cells in contact with other EC cells were demonstrated. EC cells without distinct projections were seen in less then five percent of all examined parts of the gastrointestinal tract. These could represent a mature "closed" type of EC cells or perhaps non-mature EC cells. A few serotonin IR cells undergoing mitosis were also demonstrated (Fig. 5).

Discussion

This study demonstrates a new technique for visualization of formalin-fixed gut mucosa cells. Despite its simplicity, it elicits new insights concerning three-dimensional features at the single mucosa-cell level. We have applied the method to describe the appearance of EC cells in different parts of the rat gastrointestinal tract, but it can easily be applied on other mucosal cells as well.

The dispersed formalin-fixed EC cells possessed very long and extremely thin extensions, indicating that the dispersion technique had been gentle. Serotonin negative mucosal cells did not show basally located elongations. It is not likely that this difference between serotonin positive cells and other mucosal cells was due to artefacts. The protease digestion and mechanical dispersion may affect the cell anatomy to some extent, major morphological features, however, seem to be preserved.

Prior studies using thymidine labelling have suggested that EC cells are renewed by self-proliferation [10-12]. These results were confirmed in our study by the finding of dividing EC cells. Previous descriptions of EC cells have been based on standard specimen preparation, sectioning and histological examination, which results in a 2-dimensional picture. We found EC cells with basal projections as long as 80 μM and only 0.1 μM thick. As these projections seemed to be headed in different directions, traditional techniques, including serial sectioning, may have failed to fully visualize them [2,5,9]. While EC cells have been described as open or closed regarding their contact with the gut lumen [5], the present study illustrates that most EC cells seem to have luminal contact, even when the body of the cell lies outside the gland. This is not surprising as EC cells are believed to sense and respond to variations in luminal content. In some cases however, especially in antrum, the EC

cells were not attached to neighbour cells after dispergation, making it difficult to determine whether they were of the closed or open type.

The resemblance between gut endocrine cells and nerve cells gave birth to the "paraneuron" concept [13]. The microvilli were regarded as the sensory part of the nerve cell, the cell body as the signal transport portion and the exocytosed secretory granules as the message. In recent studies, serotonergic neurons in the medulla and in the midbrain have been found to act as chemoreceptors [14] and others have shown that mouse taste buds use serotonin as a neurotransmitter [15]. From an evolutionary perspective, it has been suggested that EC cells in vertebrates are lacking only where there is an innervation of the gut mucosa by nerve fibres containing high concentrations of serotonin [16-18]. We found that EC cells have serotonin-containing axon-like projections, sometimes connecting with neuron-like structures. The idea that EC cells are of neural crest origin was first put forward by Pearse *et.al*. [19] and has later been supported by others [20]. This theory, however, has later been opposed and most researchers in the field now believe that gut neuroendocrine cells are derived from a local endodermal stem cell [21,22]. If the endodermal stem cell theory is correct, sensory serotonergic neurons in the gut mucosa have been replaced during evolution by EC cells, which have striking morphological similarities with neurons, but different embryological origin. As shown in this study, serotonin-producing EC cells like adult endocrine cells in the pancreas [23] and enterochromaffin-like cells in the stomach [24], are able to divide. The fact that differentiated EC cells can replicate cast further doubt on the idea that mucosal stem cells have a role in EC cell renewal.

As dispersed mucosal cells have lost contact with underlying structures, the targets for the basal EC cell extensions were not possible to demonstrate. In

traditional histological sections, however, serotonin IR fibres were seen in the *lamina propria* and similar fibres seemed to surround glands. It is likely that these fibres represent the projections that we found at the base of dispersed formalin-fixed cells. The EC cell may thus function as a primary neuron, sending its message to neurons in *lamina propria* [25,26]. Others have proposed that EC cells send instant paracrine messages to neighbouring cells through long cytoplasmatic extensions and thus initiate a functional synchronization among neighbouring gland cells [8,9]. A similar function has also been proposed for somatostatin producing cells in the stomach [27]. The third and fourth type of EC cells that we found had only short and thick basal extensions or no basal extensions at all. These cells had large amounts of serotonin stored in basally located granules. A possible function for these cells could be to release their hormonally active content to fenestrated capillaries or perhaps also work paracrine on cells in the *lamina propria.*

The current work gives new important insights in EC cell morphology. These findings might be helpful in order to further understand the EC cells role as a regulator of gastrointestinal physiology. The method described can also be used to isolate and visualize other morphologically intact GI mucosal cell types.

Acknowledgements

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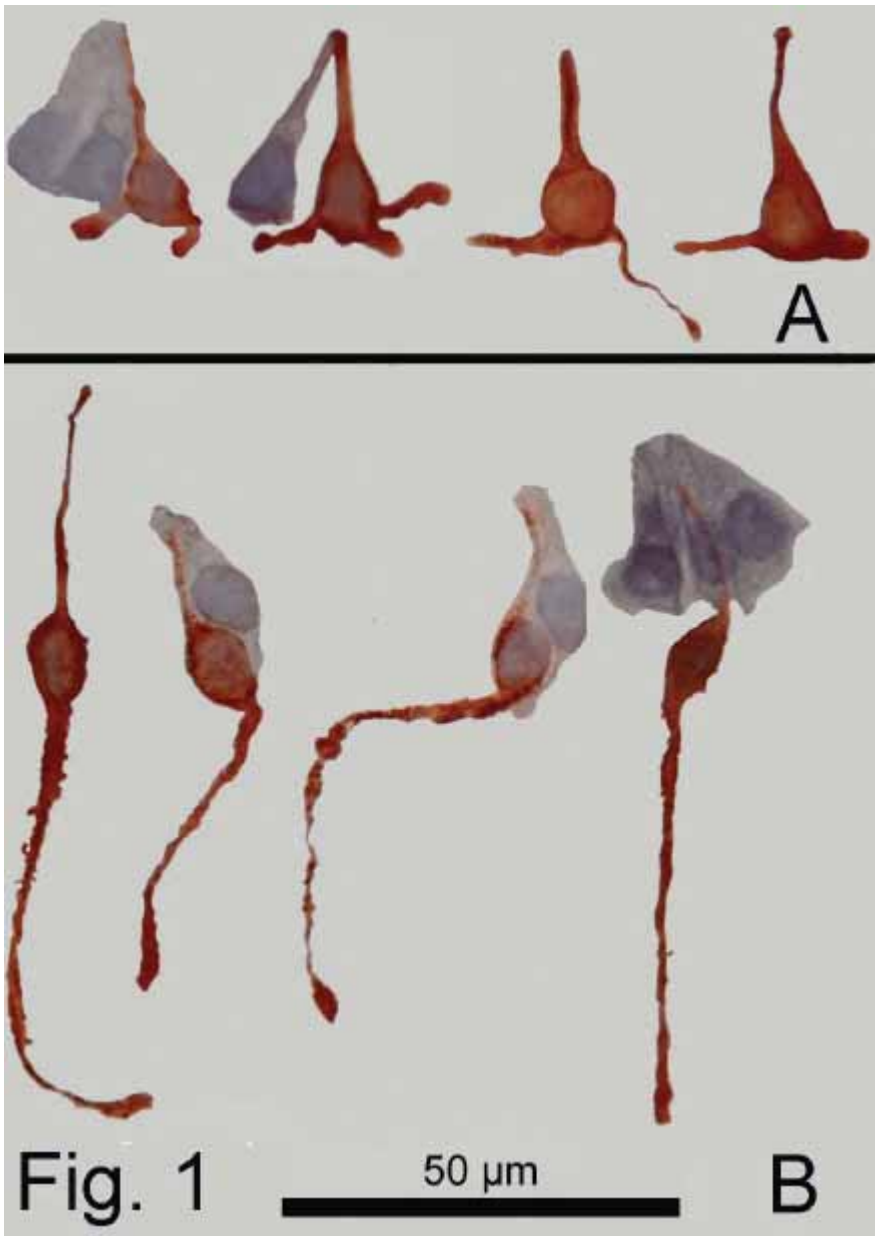

Figure 1. Photomicrographs showing serotonin immunolabelled, formalin-fixed EC cells (red) dispersed from the rectal mucosa. (A) Cells with luminal projections and thick basal projections with accumulation of serotonin containing granules. (B) Cells with long and slender, axon-like, basally located extensions with serotonin containing granules. The EC cells are in some cases attached to neighbour mucosa cells.

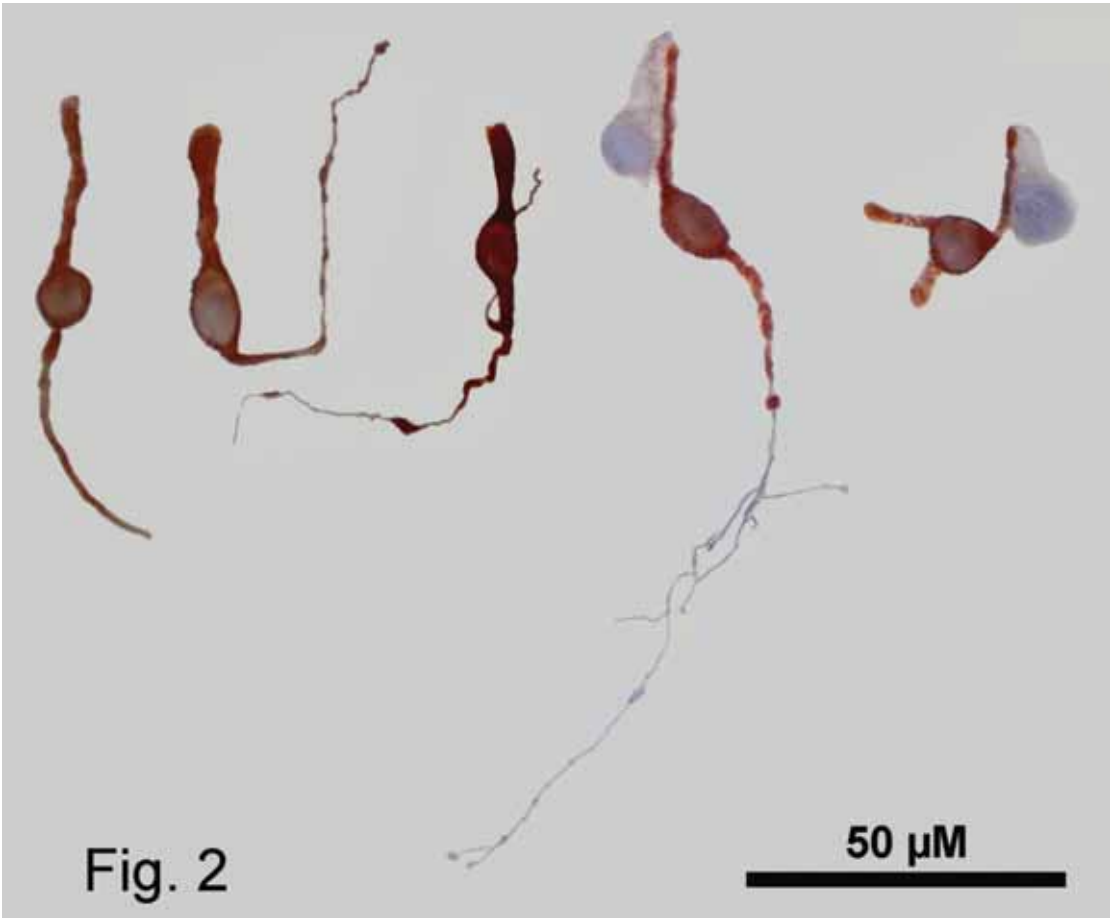

Figure 2. Photomicrographs showing serotonin immunolabelled, formalin-fixed EC cells (red) dispersed from the colonic mucosa. All cells have luminal projections. Left, four cells with one or two basally located, axon-like projections are seen. The fourth cell from the left connects with a neuron-like structure. Right, a cell with short and blunt basal projections. The EC cells are in some cases attached to neighbour mucosa cells.

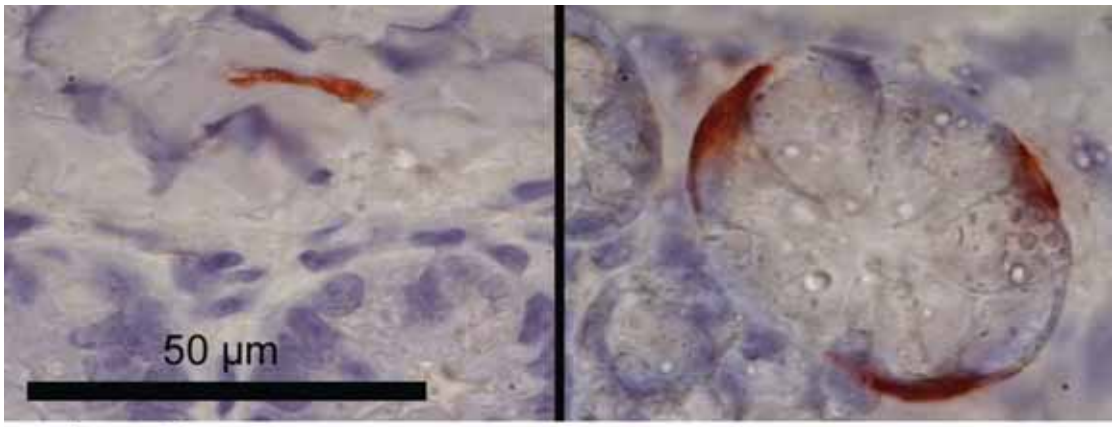

Fig. 3

Figure 3. Photomicrographs of 10 µm thick serotonin immunolabelled colon sections. Left, a serotonin containing fibre in the *lamina propria*. Right, EC cells with extensions surrounding the gland. Counterstained with haematoxylin.

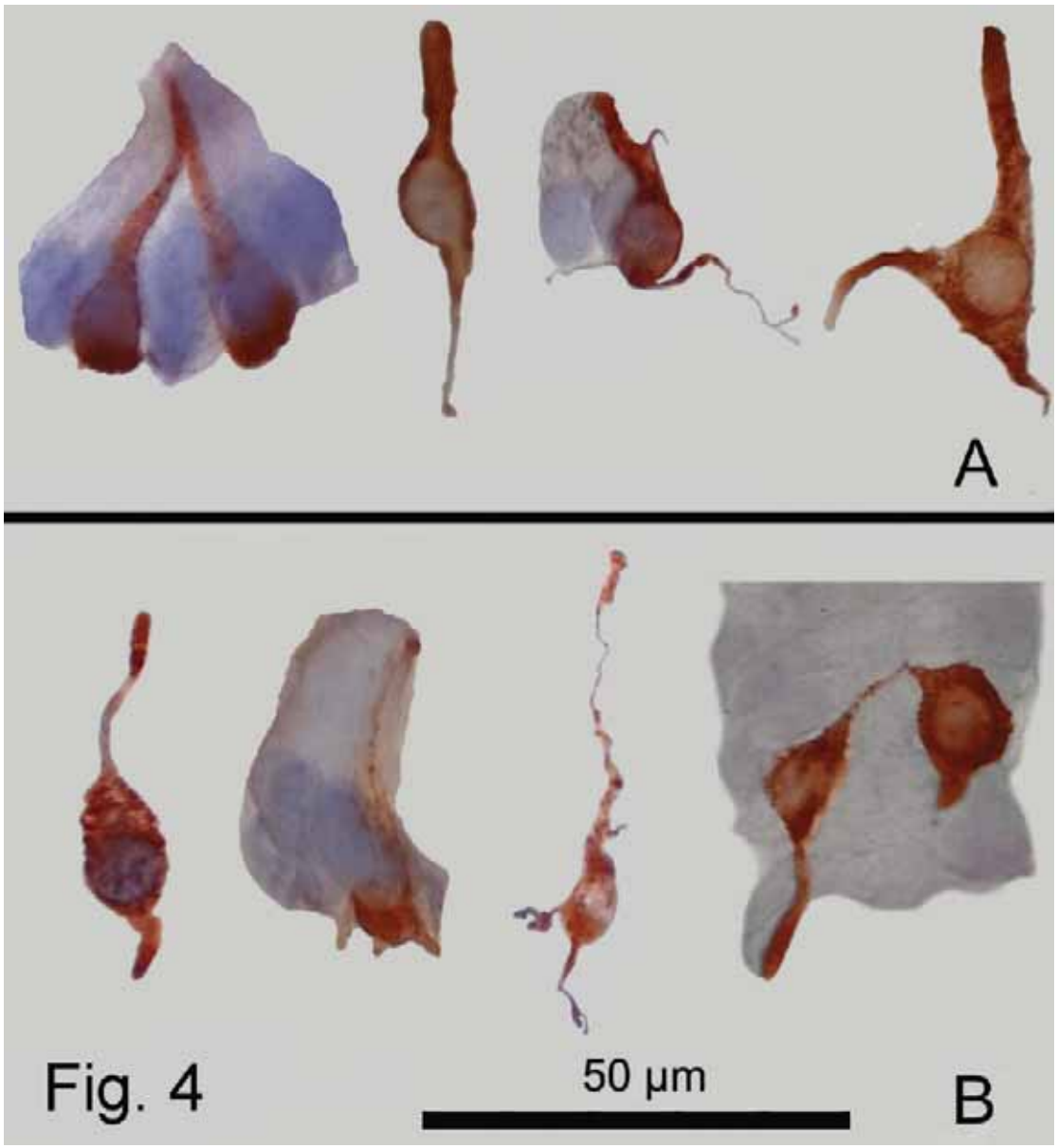

Figure 4. (A) Photomicrographs showing serotonin immunolabelled, formalin-fixed EC cells (red) dispersed from the small intestine. Left, EC cells with luminal projections, but no basal extensions. These cells have an accumulation of infra-nuclear serotonin containing granules. Right, three EC cells with both apical and basal extensions. (B) EC cells dispersed from the antral mucosa. Left, two cells with luminal extensions and short serotonin containing basal extensions. In the middle, a more spider-like cell with multiple thin extensions. Right, two EC cells located in a gastric gland. The EC cells are in some cases attached to neighbour mucosa cells.

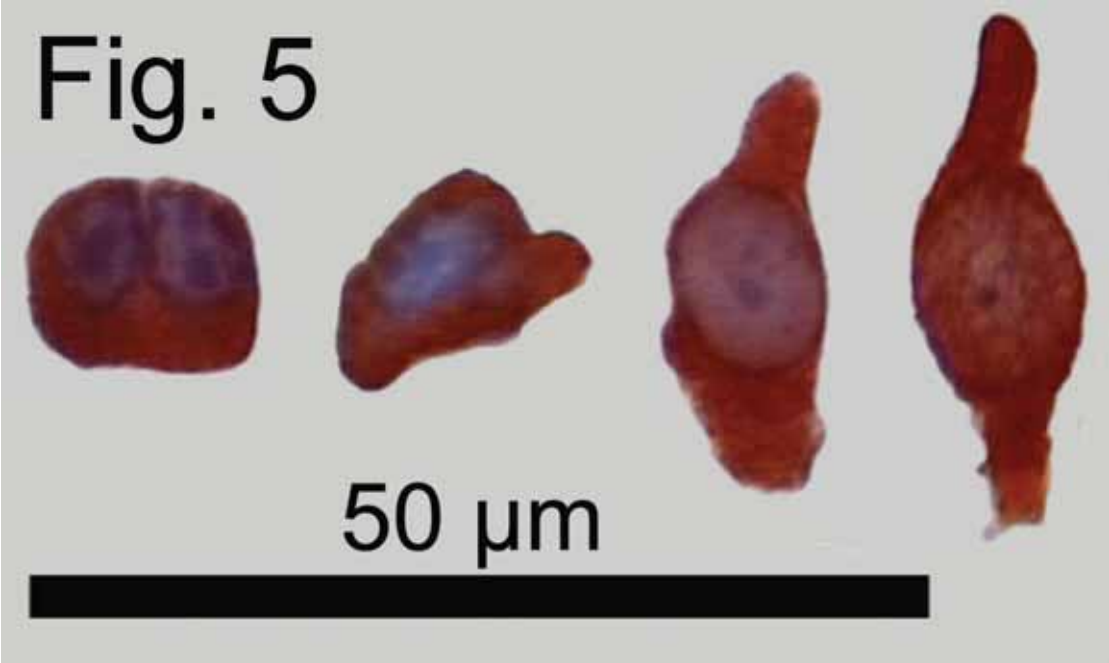

Figure 5. Photomicrographs showing serotonin immunolabelled, formalin-fixed EC cells dispersed from the jejunal mucosa. Left, a dividing cell with a non-granulated serotonin positive cytoplasm. Right, three EC cells with an immature appearance.

Paper II

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Paper III

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 preosteoclasts (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 were 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 OPG and decreased 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.

Introduction

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 (1). 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 (2) and mediates its actions by interacting with multiple serotonin receptor subtypes (3). Studies on cell cultures have shown that serotonin has mitogenic effects on fibroblasts (4), smooth muscle cells (5) and vascular endothelial cells (6) mediated through 5-HT_2 receptors. We recently demonstrated that long-term administration of toxic doses of serotonin leads to a carcinoid heart like condition with myofibroblast proliferation and plaque formation on heart valves in rats (7). 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 (8).

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 (9). It has also been suggested that platelets and macrophages are able to produce serotonin (10, 11). Our group and others have previously demonstrated functional serotonin receptors and serotonergic pathways in bone cells (12-14). An increased expression of 5-HTT in RAW264.7 cells stimulated with RANKL has been described and fluoxetine seemed to inhibit differentiation in these cells, suggesting reduced bone resorption (14). 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 (15).

Receptor activator of NFκB Ligand (RANKL) and its inhibiting decoy receptor osteoprotegerin (OPG) are central in osteoclast regulation (16, 17). RANKL, secreted mainly by osteoblastic stromal cells, is necessary for osteoclast formation from its committed precursors, which bear its receptor RANK. The cytokine IL-6 also has osteoclastic actions, acting via RANKL/OPG alterations (18). Activation of RANK leads to activation of downstream signaling pathways including NFκB, p38 kinase, and c-Jun N-terminal kinase (JNK) (19). Stimulation of JNK subsequently elicits the activation of the transcription factor c-Jun (20). c–Jun forms activator protein-1 (AP-1) complexes with cFos, an essential transcription factor for osteoclast formation (21). The AP-1 complex is also known to be involved in regulation of osteoblast proliferation and differentiation (22).

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 preosteoblasts.

Materials and methods

Cells

MC3T3-E1 (murine preosteoblasts, ATCC) cells were maintained in α-MEM (Invitrogen) supplemented with 10 % fetal calf serum (FCS) (Biological Industries), 1 mM Na- pyruvate (Gibco), 0.1 mg/ml L-glutamine (Gibco) and 10 U/ml penicillin/streptomycin (Gibco). RAW264.7 (murine preosteoclast, 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 % FCS. The IL-6 dependent mouse hybridoma cell line B9

was cultured in RPMI 1640 (Gibco) medium containing 10 % FCS, 2-mercaptoethanol (50 μM) and IL-6 (1 ng/ml)(Biosource). 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) and β-glycerophosphate (3 mM) (Sigma) to the growth medium (23-25). RAW264.7 cells were differentiated by addition of sRANKL (50 ng/ml) (Research Diagnostics, INC) and M-CSF (50 ng/ml) (Research Diagnostics, INC).

Human mesenchymal stem cells (MSC) were isolated from the iliac crest (MSC). Lymphoprep (AXIS-SHIELDPoC A/ S, 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, Grand Island, USA) with 20 % FCS, 100 U/ ml penicillin, and 0.1 mg/ ml streptomycin. The cells were maintained in humidified 95% air 5% CO_2 atmosphere at 37^{o}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, USA) were grown in Osteoblast Growth Media (OGM, Cambrex). Osteoblasts cultured to facilitate mineralization were exposed to 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). 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 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) and oligo-dT primer, according to the manufactures protocol. The cDNA was amplified using the HotMaster Taq DNA Polymerase Kit (Eppendorf).

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 DH5α -T1 cells (Invitrogen), and isolated by the SpinClean Plasmid Miniprep Kit (Mbiotech). Products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufactures protocol. Primers are listed in Table 1.

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 μl 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, USA). RT-PCR reactions were performed and monitored using iCycler iQ (Bio-Rad, Hercules, CA, USA). The 2X iQ SYBR Green Supermix was based on iTaq DNA

polymerase (Bio-Rad, Hercules, CA, USA). 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 Ct values, i.e. the cycle number at which the fluorescence signal exceeds background, versus log cDNA dilution. The Ct 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 1. 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 1).

Human osteoclast differentiation

Osteoclasts were differentiated from PBMC (26), isolated from buffycoat. Separation of PBMC was performed essentially as described by Bøyum (27). Cells were seeded into 24-well dishes, 500 000 cells/well in α-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 six and nine. 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 FCS is known to contain rather high levels of serotonin (14), we determined the amount of serotonin contamination in the media using a serotonin RIA kit (Dianova AS, Nesbru Norge). 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) 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) 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 five 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). The PKC inhibitor GF 109203x, the PKA

inhibitor H89 and the CAMK inhibitor W7 were obtained from Calbiochem, whereas the 5-$HT_{2B/C}$ receptor antagonist SB206553B was obtained from Sigma.

Human bone marrow cells and primary osteoblasts were seeded in 48 wells 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 x 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 liquid scintillation counter (Packard 1900 TR).

Plasmids and luciferase assays

pFOSLuc transfection and luciferase assay in MC3T3-E1 cells: Fifteen thousand cells/well were seeded in 96-well plates and transfected with 0.12 μg luciferase reporter plasmid per well (pcFos reporter-plasmid (nucleotides –327 to –288 of the human cFos promoter) (28), a generous gift from Dr. Ugo Moens (University of Tromsø, Norway)), using 0.35 μl Fugene transfection reagent (Roche Molecular Biochemical's, Germany). After cultivation for 24 h, cells were treated with agonist for six 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) using the Luciferase reporter Assay System (Promega Inc., USA).

pBIIXLuc and pFOSLuc transfection in RAW264.7 cells: The NFκB-driven plasmid pBIIXLuc contains two copies of a HIV-NFκB 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, RANKL and IL-6 release assays

Release of OPG, IL-6, RANKL and amount of total protein were studied in medium samples collected from MC3T3-E1 cells. Thirty thousand cells in 0.5 ml MEM-α supplied with 10 % FCS/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). The OPG standard curve was generated using recombinant mouse OPG (R&D Systems) in two-fold dilutions from 2000 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). Samples were then incubated for 20 minutes in the dark after which the reaction was stopped by addition of 1 M 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-assay 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), according to the manufacture's protocol.

Concentrations of IL-6 in the culture medium were determined by a bioassay utilizing the IL-6 dependent mouse hybridoma cell-line B9. Recombinant human IL-6 (Biosource) was used as a standard. Fifty µl of standard or sample were added as triplicate to a 96-well plate. After that mouse hybridoma B9 cells were seeded (5000 cells/well) and incubated for 3 days. The growth of cells was assessed by a (3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) / phenazine methosulfate (PMS) reduction assay essentially as described by Buttke (29). Briefly, medium was removed, cells were washed with PBS and 20 µl/well of MTS/PMS solution, (2 ml MTS (2 mg/ml in PBS) (Promega) and 100 µl PMS (0.92 mg/ml in PBS) (Acros Organics)), were added, and plates were incubated for 4 h. The optical density of each well was measured at 490 nm. Minimum detectable concentration of IL-6 was typically 5 pg/ml, and intra-assay and inter-assay variability were both less than 15 % for all measured samples.

Total protein in medium was determined using Sigma Microprotein PR assay kit with a Protein Standard Solution Calibrator (Sigma Diagnostics, Dorset, UK). Analyses were performed using Cobas Mira chemistry analyzer. Thirty µl of medium were mixed with 200 µl substrate. Intra-assay and inter-assay variability were less than 2.4 % and 3.2 % respectively. The assay detection range was 10-2000 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 x g for 5 min at 4ºC, 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, Manheim, Germany), 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 means ± 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} 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 preosteoclasts 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 (30, 31). 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 FCS.

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 preosteoclasts 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 preosteoclasts

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.

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 and 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-$HT2_{B/C}$ 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 that the effect of high-dose fluoxetine was inhibitory and not cytotoxic (data not shown). Both serotonin and fluoxetine induced an acute (24h) decrease (to 58-85 % of control) in the expression of 5-TH_{2A} receptor mRNA followed by an enhanced expression (to 130-186 % 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} receptor mRNA 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).

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 three fold- increase in RANKL release at the most and decrease to 43 % of control in OPG release (Fig. 8-D).

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 and differentiation of MC3T3 E1 preosteoblasts. Previously we have shown that functional serotonin receptors are present in osteoblast precursors, osteoblasts and osteocytes (12). Serotonin induces proliferation in cells originating from mesenchymal stem cells (smooth muscle cells, fibroblasts etc) via the 5-HT_2 receptors (32, 33), with subsequent activation of different signaling pathways in a cell specific manner (34). 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 mRNA expression was found to be up-regulated 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 (35-37). 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 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 (38). The same study also showed that traces of fluoxetine could be detected in bone marrow three months after termination of medication. We found that fluoxetine induced proliferation of human MSC, osteoblasts and murine MC3T3-E1 preosteoblasts 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 preosteoblast 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 (39-41). 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 preosteoblasts, 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* (14). 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* (15). 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 (15). 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 (15).

We demonstrate that both fluoxetine and serotonin activated NFκB activity in RAW264.7 cells even in the absence of RANKL. Battaglino *et al*. have previously shown that serotonin stimulated, whereas fluoxetine had an inhibitory effect on NFκB activity in RAW264.7 cells (14). 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 cFos 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 (8, 15). 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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TABLE I

Primer sets used in RT-PCR.

Primers	mRNA position	Sequence	Product length	T_{ann}
mTph1	1370-1389	5'-AGTTGCGGTATGACCTTGAT-3'		
	1561-1543	5'-AGGCGAGAGACATTGCTAA-3'	192 bp	60°C
h5-HT1A	1067-1086	5'-GCTGGCTGCCCTTCTT-3'		
	1232-1213	5'-TTAAACGCGTTTTGAAAGTC-3'	166 bp	54°C
h5-HT2A	1520-1538	5'-GACAATAGCGACGGAGTGA-3'		
	1943-1923	5'-GGCAATAGGTAACCAACTCAA-3'	424 bp	54°C
h5-HT2A	917-936	5'-TCTTTCAGCTTCCTCCCTCA-3'		
	1139-1120	5'-TGCAGGACTCTTTGCAGATG-3'	223 bp	58°C
h5-HT2B	1317-1336	5'-TGGCAGAGAACTCTAAGTTT-3'		
	1513-1493	5'-CTGCCAGTTCTGCTATACATA-3'	197 bp	52°C
h5-HT2C	3638-3658	5'-ACGCTTGACAGTTACTTACAC-3'		
	3906-3890	5'-GGCAAGGCAGGTAGACT-3'	269 bp	52°C
h5-HTT	2023-2038	5'-CTTGGGTTACTGCATAGGAAC-3'		
	2181-2166	5'-GCATTCAAGCGGATGT-3'	159 bp	52°C
hTph1	1057-1076	5'-CCCTTTGATCCCAAGATTAC-3'		
	1267-1248	5'-CATTCATGGCACTGGTTATG-3'	211 bp	50°C
hCalcitonin	2535-2554	5'-CTTGTGGTTGACCGCTTGTT-3'		
receptor	2733-2714	5'-ATTTCAGGTGCCAGTAACGA-3'	199 b	58°C
M13Reverse		5'-CAGGAAACAGCTATGAC-3'		55°C
GAPDH		5'-TGCACCACCAACTGCTTAGC-3'		
		5'-GGCATGGACTGTGGTCATGAG-3'		60°C

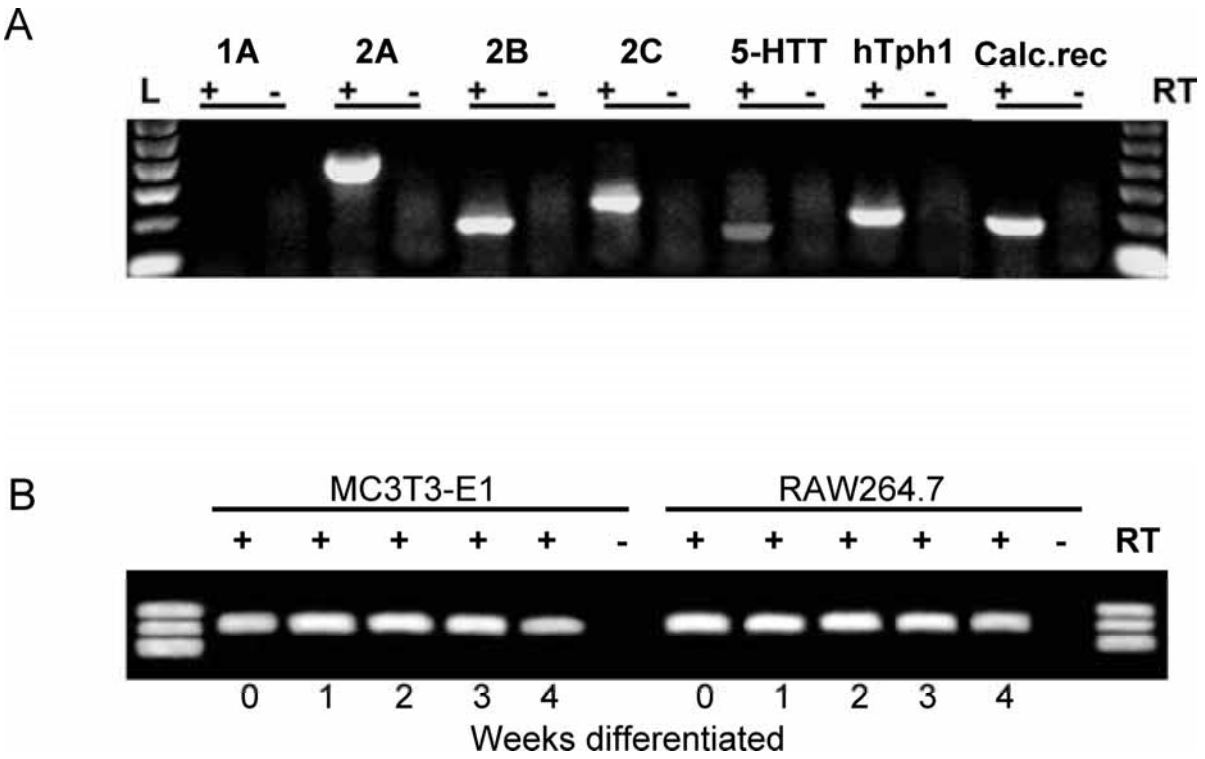

Figure 1. Gel electrophoresis of representative PCR products from one-step RT-PCR for serotonin receptors, 5-HTT and Tph1. (A) RT-PCR analysis of 5-HT_{1A} (166 bp), 5-HT_{2A} (424 bp), 5-HT_{2B} (197 bp), 5-HT_{2C} (269 bp), 5-HTT (159 bp), Tph1 (211 bp) and the calcitonin receptor (180 bp) expression 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.

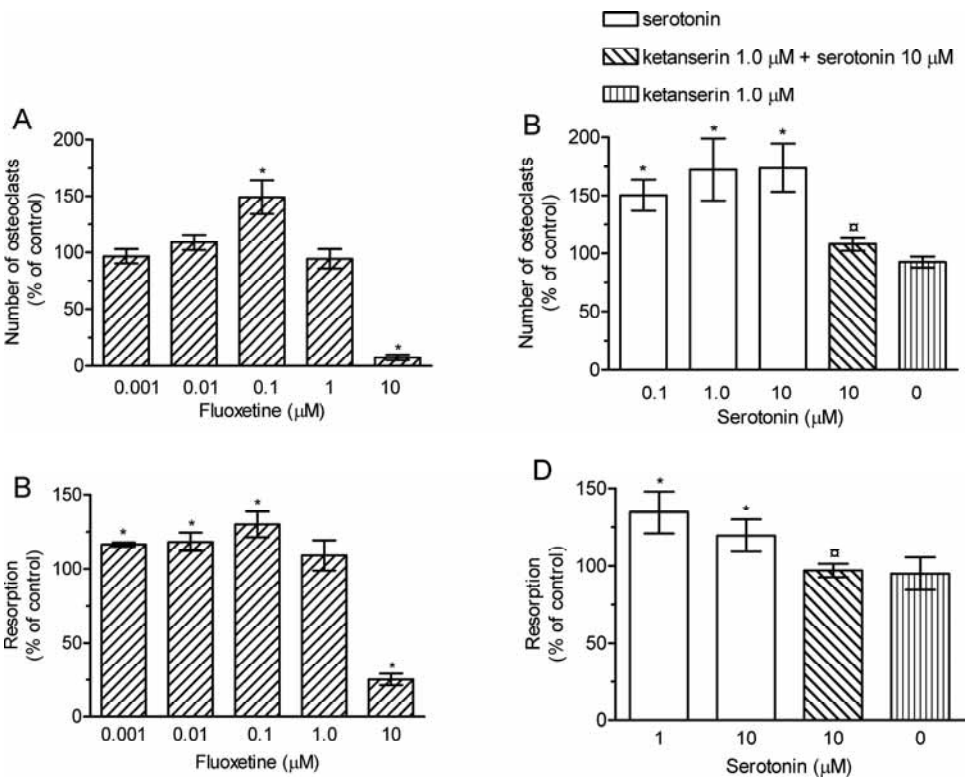

Figure 2. Serotonin and fluoxetine affect osteoclast differentiation and activation. (A) Human PBMC were cultured in medium with M-CSF and RANKL for 12 days in the presence of fluoxetine, (B) corresponding resorption pit assay. (C) An equivalent experiment with different serotonin concentrations and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

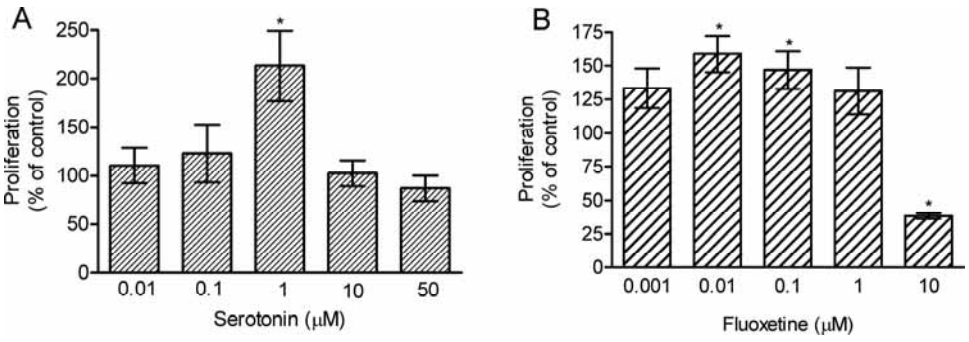

Figure 3. Serotonin and fluoxetine enhance proliferation of murine preosteoclasts. (A) RAW264.7 cells treated with serotonin in different concentrations. (B) RAW264.7 cells treated with fluoxetine in different concentrations. Data are presented as % of control (no addition) values. *$p < 0.05$.

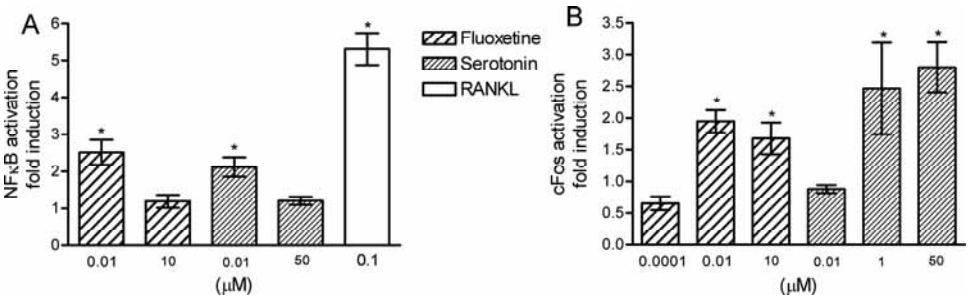

Figure 4. Fluoxetine and serotonin activate NFκB and cFos in RAW264.7 cells. (A) Fluoxetine (0.01 μM) and serotonin (0.01 μM) activate NFκB, at 10 μM however none of the compounds were effective. (B) Fluoxetine and serotonin induce cFos activation. *$p < 0.05$.

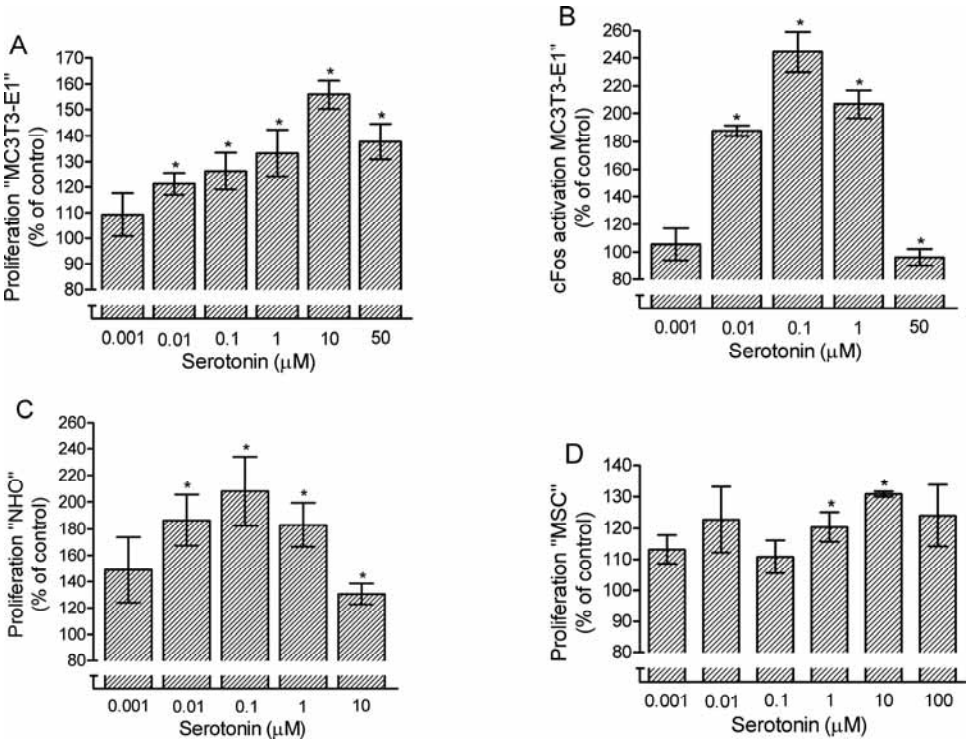

Figure 5. Serotonin stimulates osteoblast proliferation. (A) Proliferation assay on murine MC3T3-E1 cells treated with serotonin. (B) Assay on pFOSLuc transfected MC3T3-E1 cells given serotonin. (C) Proliferation assay on differentiated, human NHO cells and (D) human bone marrow stem cells (MSC). Data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

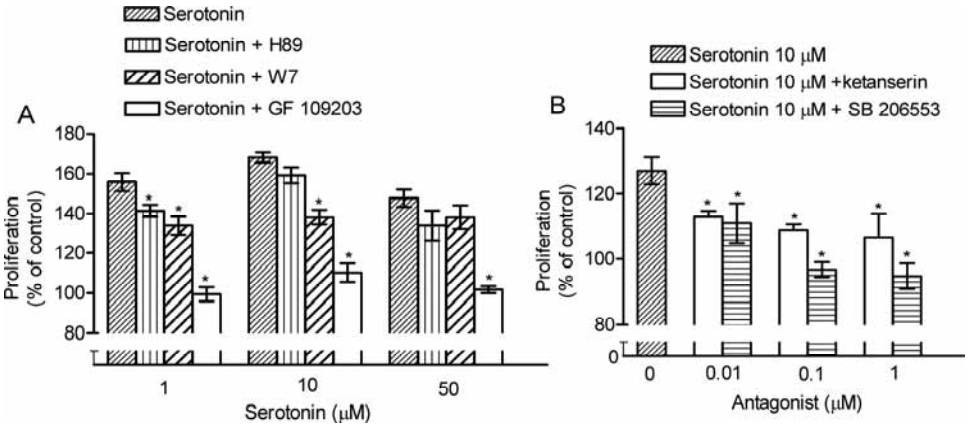

Figure 6. Effects of serotonin receptor and intracellular signaling pathway inhibition on serotonin induced proliferation of MC3T3-E1, preosteoblasts. (A) The PKC inhibitor GF 109203 and to a lesser extent the PKA inhibitor H89 as well as the CAMK inhibitor W7 inhibit serotonin induced proliferation. (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 % of control (no addition) values. *$p < 0.05$.

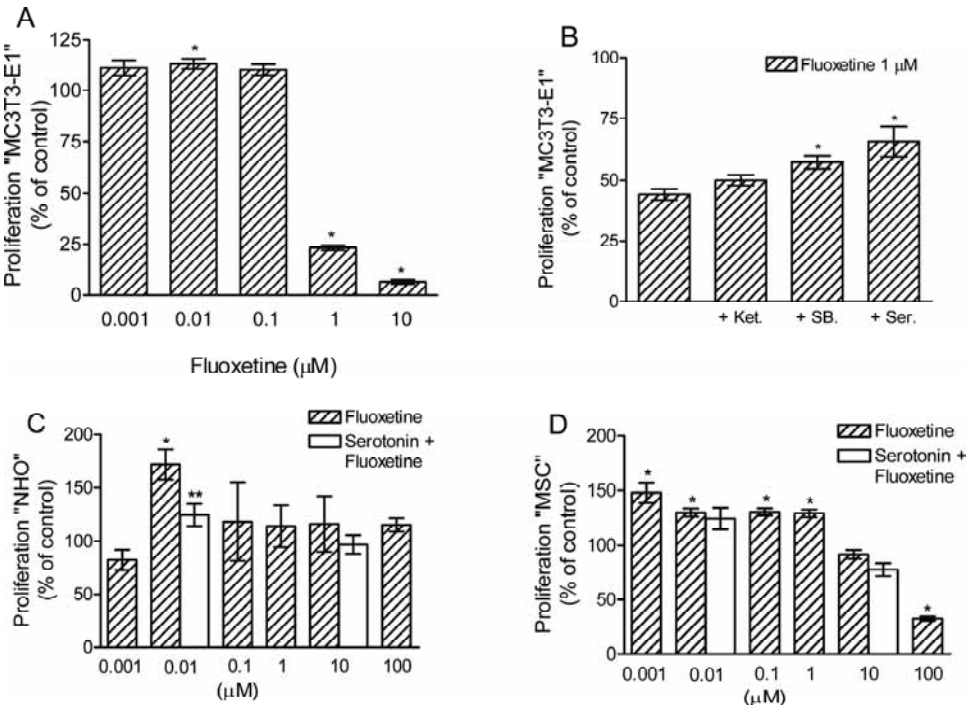

Figure 7. Effects of fluoxetine on proliferation in MC3T3-E1, NHO and MSC cells. (A) At lower concentrations fluoxetine seems to have a slight stimulating effect, whereas higher doses inhibit proliferation of MC3T3-E1 cells. (B) The effect of ketanserin (KET), SB 206553 (SB) and serotonin (Ser) on fluoxetine (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 in combination with serotonin on MSC proliferation. Data are presented as % of control (no addition) values. *$p < 0.05$.

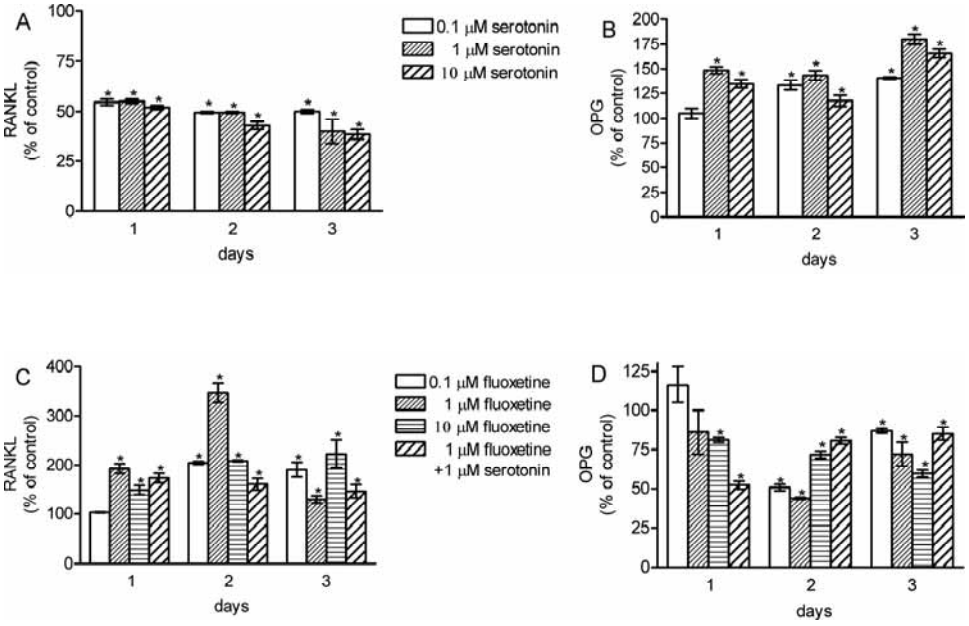

Figure 8. Serotonin and fluoxetine effects on RANKL and OPG release in MC3T3-E1 cells. (A) Inhibition of RANKL and (B) stimulation of OPG release by serotonin. (C) Stimulation of RANKL and (D) inhibition of OPG release by fluoxetine. Data are presented as % of control (no addition) values. *$p < 0.05$.

Paper IV

Long-term serotonin administration leads to higher bone mineral density and affects bone architecture as well as bone mechanical properties in rats

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Running title: **Serotonin affects bone metabolism *in vivo***

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Microabstract

To investigate possible effects of serotonin on bone, rats were injected with serotonin s.c. for 3 months. BMD, femoral cortical thickness and stiffness were significantly higher in serotonin treated animals compared to controls. These findings show that serotonin affects bone metabolism, architecture and mechanical properties *in vivo*.

Abstract

Introduction: New evidence suggests a control of bone mass by the central nervous system. Many studies indicate a role for the nervous system in embryonic skeletal development, during fracture healing, and during remodeling after insertion of implants. 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*.

Methods: Ten 2-month old female Sprague-Dawley rats were injected with serotonin subcutaneously (5mg/kg) once daily for 3 months. Controls received saline. Using microdialysis and HPLC, free circulating serotonin levels were measured. DXA scans were made after three months of serotonin administration . Bone architecture and mechanical properties were investigated by μCT, histomorphometry and mechanical testing.

Results: 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 measured by μCT analysis was also higher, whereas trabecular bone volume 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 increases apposition of endosteal bone. Mechanical testings showed that femoral stiffness was higher in serotonin dosed animals, while the energy absorption remained unchanged, indicating less deformation at fracture. As a result of a reduced body fat content (28.08 ± 7.20 vs. 42.96 ± 8.49 g), the body weight was lower in serotonin rats (305.5 ± 4.0 vs. 321.8 ± 5.3 g).

Conclusion: Hyperserotoninemia led to a higher BMD, altered bone architecture and changes in bone mechanical properties in growing rats, demonstrating that serotonin may have important effects on bone *in vivo.*

Introduction

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.[1] Functional receptors for leptin and VIP have been demonstrated in osteoblasts and in osteoblastic cell lines.[2] 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.[3] 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.[4] Until now seven serotonin receptor families ($5\text{-}HT_{1\text{-}7}$) have been characterised, and each is further divided into several subtypes. Studies on cell cultures have shown that serotonin has mitogenic effects on fibroblasts,[5] smooth muscle cells[6] and vascular endothelial cells[7] 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.[8] In 2001 we were the first to demonstrate functional serotonin receptors in both osteoblasts and osteocytes.[9] Furthermore, the expression of the serotonin transporter (5-HTT) has been demonstrated in rat osteoblasts and in osteoclasts.[10,11] Later we showed that

serotonin induced proliferation of osteoblasts and osteoclasts *in vitro* as well as increased BMD in rats *in vivo*.[12]

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, 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 ± 1°C with a relative humidity of 40 % to 50 % and a 12-hour light/dark cycle. The Rat and Mouse Diet of B&K and tap water were provided *ad libitum*. Before all procedures (except for serotonin 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); 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-gauge BD syringe. At euthanization, the animals were weighed and decapitated.

In order to perform microdialysis, an additional experiment on 7 rats was carried out.

Microdialysis

To describe the pharmacokinetics of the serotonin administration protocol, a short-term study on 7 animals was performed. Four animals were given daily serotonin injections (5 mg/kg s.c) for 10 days, 3 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 10^{th} 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 $\mu l\ min^{-1}$ using a microinfusion pump (CMA 107, CMA Microdialysis AB, Stockholm, Sweden). After a 30-minute equilibration period, baseline samples were collected for 60-minutes. Then serotonin was injected s.c followed by microdialysate sampling in 60-minute fractions for 5 hours. The samples were protected from light during the whole procedure and immediately frozen at -80°C until further analysis. *In vitro* recovery of serotonin was 59.3 ± 3.2 % (mean ± 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 µl of sample were injected on a 50 mm x 2.1mm 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.

Double 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 micro-computed tomography (µCT) scanning. Femoral head and part of the metaphysis (fig. 1) 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. In the metaphysis, cortical volume (Ct.V), and cortical thickness (Ct.Th) were measured. In the femoral head,

trabecular bone volume (BV), total bone marrow volume, including trabeculae (TV), trabecular bone volume fraction (BV/TV), trabecular thickness (Tb.Th),[13] connectivity density (CD),[14] and structure model index (SMI) were determined.[15] 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.[16] 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.[17] 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. 2) All tests were done at a loading rate of 0.095 radians/second (5.43 degrees/second).[18] The load in the test apparatus, an MTS 858 Mini Bionix® Axial/Torsional Test System (MTS Systems Corporation, Minnesota, USA), 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 means ± 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 hours after the injections. In dialysate collected from the femoral muscles, only one out of three control rats had a detectable serotonin (2.9 nM). In dialysate sampled 1 hour prior to the 10^{th} injection, one out of four serotonin receiving animals had detectable serotonin (9.2 nM) in the femoral muscles. Two hours after the 10^{th} injection, the serotonin level reached a peak (56.8 ± 9.6 nM) (Fig. 3).

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. 4A-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. 4C-D). 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, cortical thickness (Ct.Th) was significantly higher in the rats receiving serotonin versus controls (fig. 5A). On the other hand, trabecular bone volume (BV) as well as total bone marrow volume (TV) in the metaphysis were found to be significantly lower in the serotonin group compared to the control group (fig. 5B-C). Trabecular bone volume fraction (BV/TV) (fig. 5D) and trabecular thickness (Tb.Th) remained unchanged, indicating that BV decreased due to a smaller TV. All other parameters studied were not significantly different in the metaphysis. In the femoral head no significant differences were found, although BV and TV 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.

Serotonin alters rat bone mechanical properties

Consistent with the phenotypes of altered BMD and architecture, femurs from rats receiving serotonin had altered mechanical properties. In comparison with controls, mean stiffness of the femur in three-point bending was 12.3 % higher (Fig. 6). 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 similar in the two groups.

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 has 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.[19,20] 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.[8] 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 hours after the injection. The hyperserotoninemia lasted > 5 hours.

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.[21]. 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.[22] 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 serotonin receptors on cells involved in bone metabolism.[9,11] 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 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 moment of inertia and the perimeter of the metaphysis were unchanged, while cortical thickness was significantly higher in the serotonin group. On the other hand, we demonstrated that serotonin administration led to a lower trabecular bone volume, indicating that the effect of a higher cortical thickness is reversed by lower trabecular bone volume, resulting in similar moment of inertia 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 bone volume fraction indicates that trabecular bone volume was lower solely because of a smaller total bone marrow volume. 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 cortical thickness, lower total bone marrow volume, and lower trabecular bone volume. 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 (in press). 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 previous findings showing that serotonin induces osteoblast proliferation *in vitro*.[12]

Furthermore, we demonstrated that the stiffness of the femurs from the serotonin animals was increased while bone toughness remained unchanged. The increase in stiffness is probably due to the slightly enlarged cortical thickness, with more bone at the endosteal side. These results indicate less deflection and a slightly more brittle bone 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.[23] 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.[24] 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.[25-27] Another study demonstrated decreased growth in children during therapy with SSRI.[28] 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 increased BMD, altered bone architecture and changes in mechanichal properties in growing rats.

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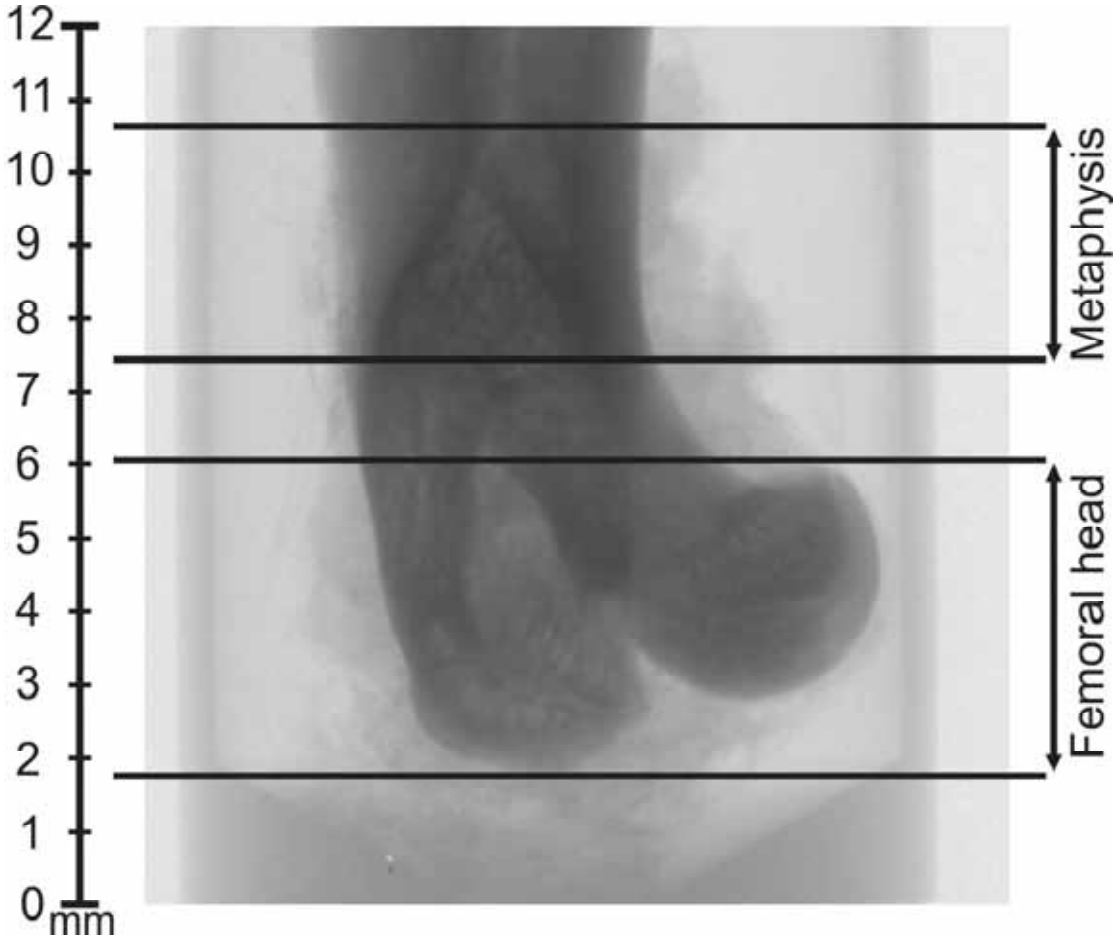

FIG. 1. A typical μCT-scan image. Areas for the separate data-sets of femoral head and metaphysis are depicted.

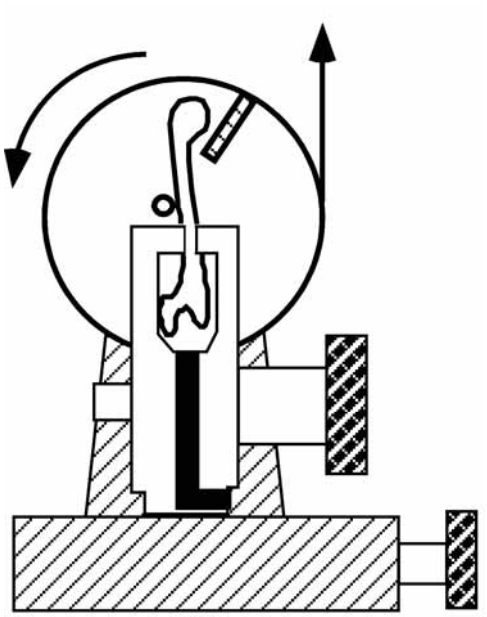

FIG. 2. Mechanical properties of the femoral midshaft were investigated using a three-point anterior bending test.

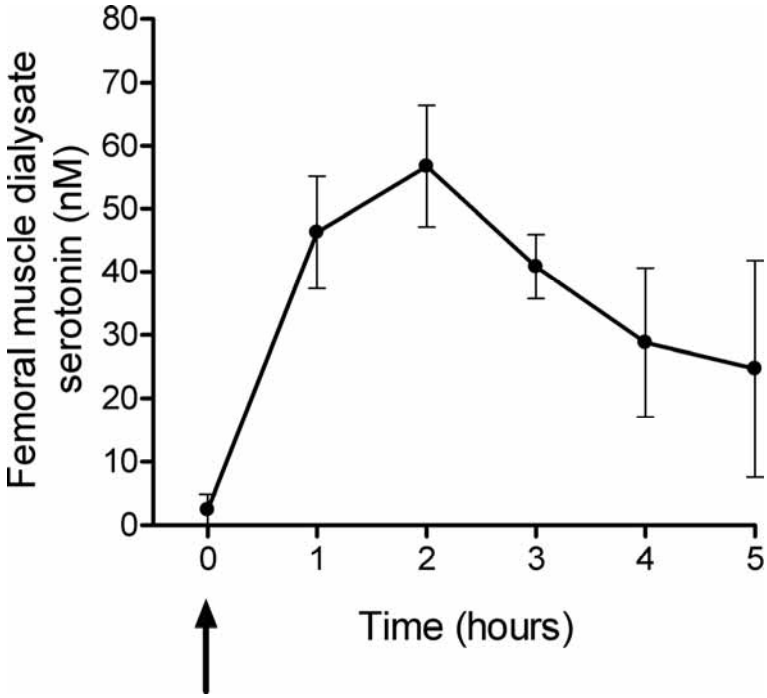

FIG. 3. Serotonin levels in dialysate from femoral muscles in animals given daily subcutaneous serotonin injections (5 mg/kg) for 10 days. Dialysate was collected 1 hour before and 5 hours after the 10th serotonin injection (↑). Values are mean ± SEM; $n = 4$.

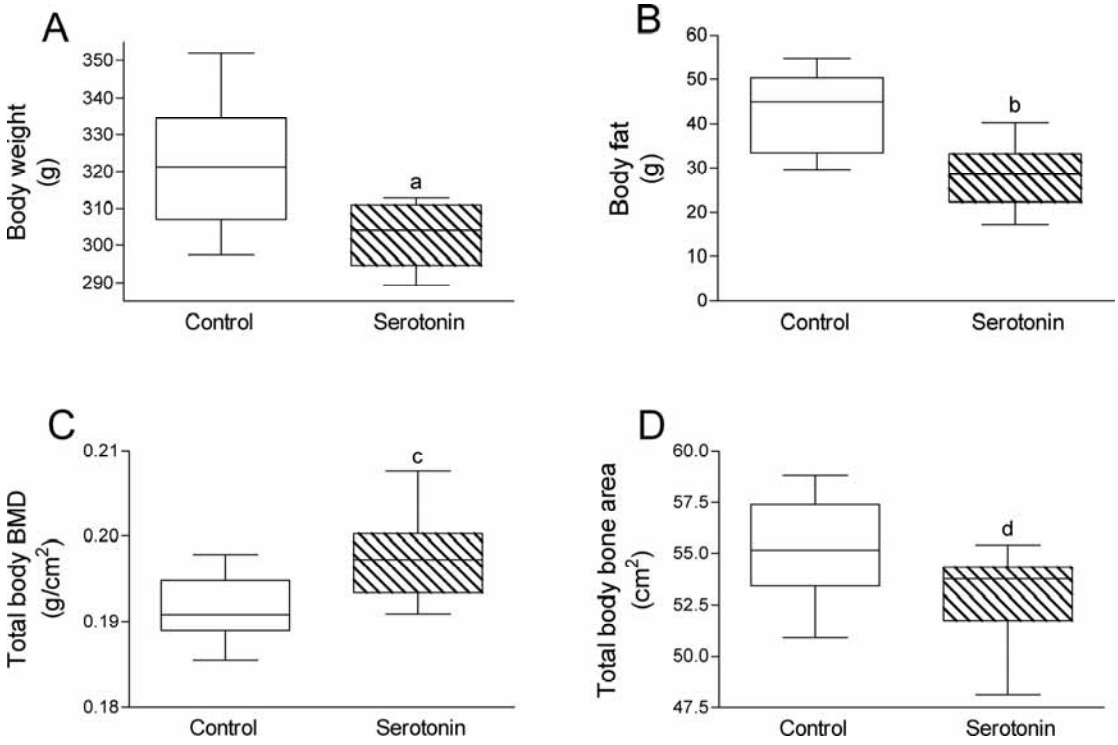

FIG. 4. Influence of long-term serotonin administration on body weight, fat content, BMD and bone area compared to controls. Results are shown as mean ± SD; $n = 10$. (A) Body weights were significantly lower in serotonin dosed animals compared to controls ($^{a}p = 0.02$). (B) The body fat content was 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 ($^{d}p = 0.035$).

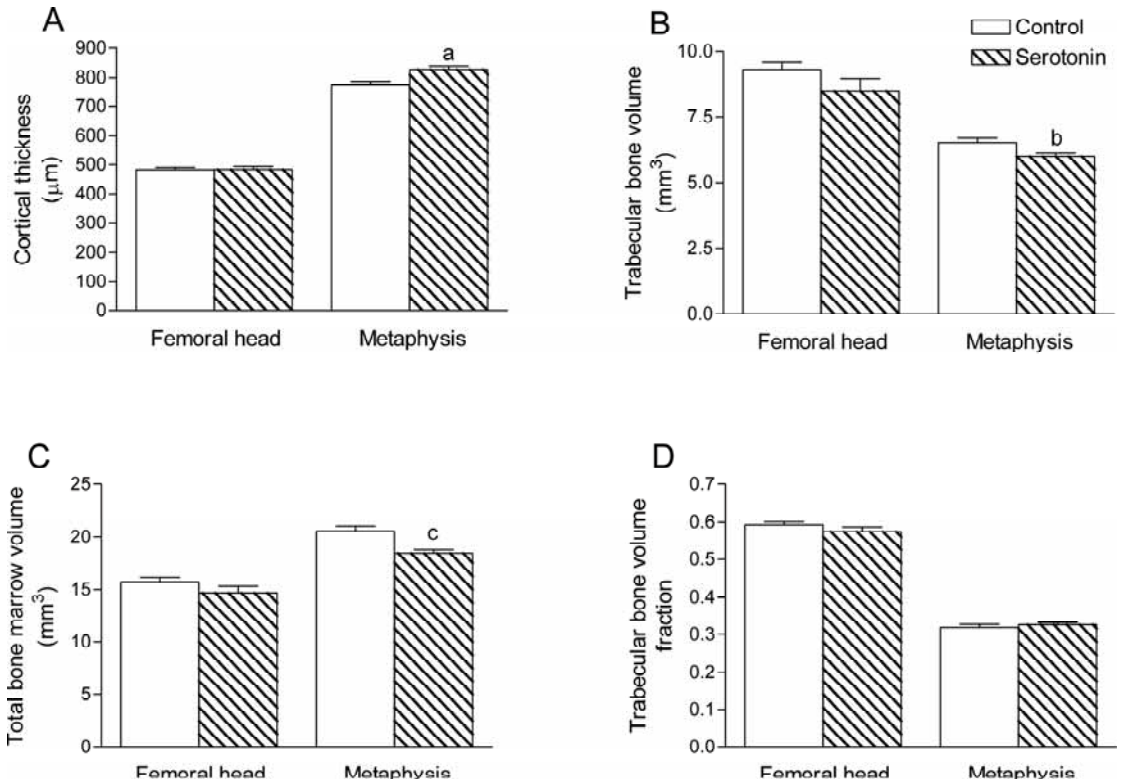

FIG. 5. μCT scanning data of femoral head and metaphysis. Data are shown as means ± SEM; $n = 10$. (A) Cortical thickness [Ct.Th] is significantly higher in the metaphysis of serotonin dosed rats versus controls ($^{a}p = 0.009$). (B) The serotonin dosed rats had significantly lower trabecular bone volume [BV] in the metaphysis ($^{b}p = 0.035$). (C) Total bone marrow volume [TV] was significantly lower in the metaphysis of serotonin dosed animals ($^{c}p = 0.002$). (D) Trabecular bone volume fraction [BV/ TV] is similar in both groups.

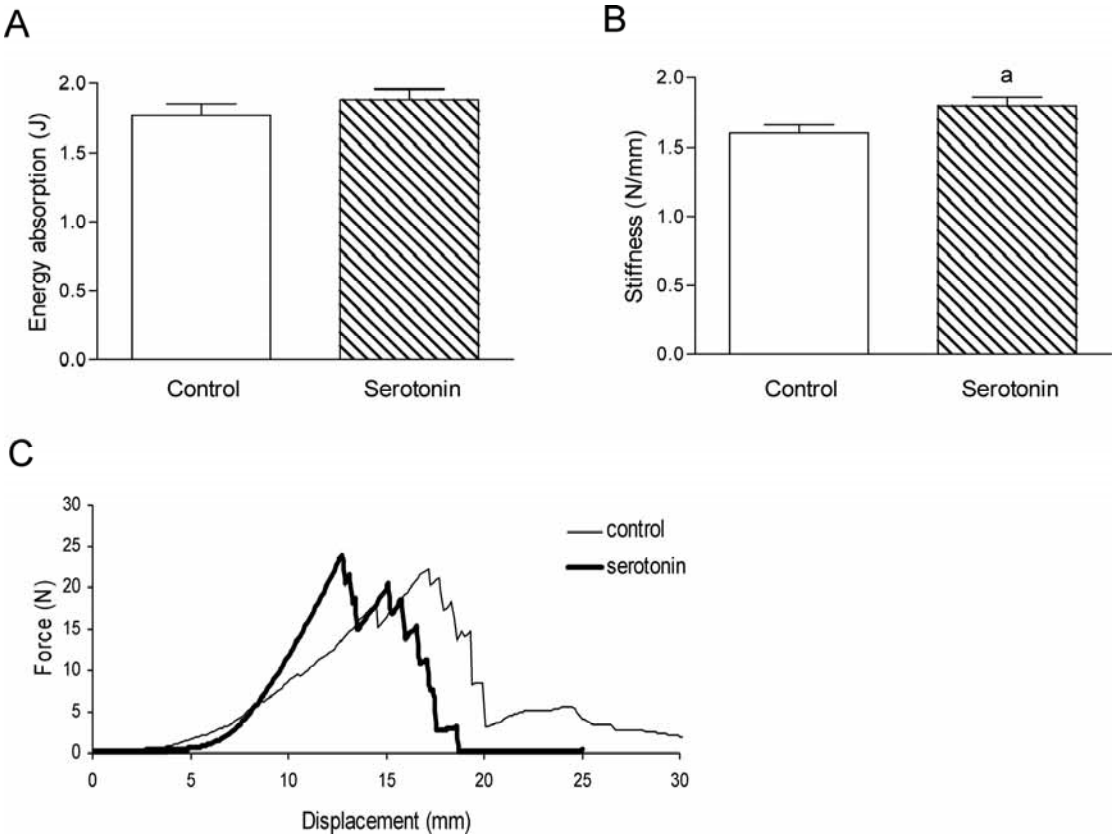

Fig. 6. Femoral midshaft mechanical properties in control and serotonin dosed rats. (A) Energy absorption and (B) stiffness. [a]Statistically significantly higher compared to controls ($p < 0.05$). (C) Load-graph showing break point. Data are shown as means ± SEM; $n = 10$.

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1978

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