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GUT-DERIVED SEROTONIN CONTRIBUTES TO BONE DEFICITS IN COLITIS

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Abstract

Osteoporosis and bone fractures occur at higher frequency in patients with inflammatory bowel disease (IBD), and decreased bone mass is observed in animal models of colitis. Another consistent feature of colitis is increased serotonin (5-HT) availability in the intestinal mucosa. Since gut-derived 5-HT can decrease bone mass, via activation of 5-HT_{1B} receptors on pre-osteoblasts, we tested the hypothesis that 5-HT contributes to bone loss in colitis. Colitis was chronically induced in mice by adding dextran sodium sulfate (DSS) to their drinking water for 21 days. At day 21, circulating 5-HT levels were elevated in DSS-inflamed mice. Micro-computed tomography of femurs showed a decrease in trabecular bone volume fraction, formation, and surface area, due largely to decreased trabecular numbers in DSS-treated mice. The colitis-induced loss of trabecular bone was significantly suppressed in mice treated with the 5-HT synthesis inhibitor, p-chloro-DL-phenylalanine (PCPA; 300mg/kg/day IP daily), and in mice treated with the 5-HT_{1B} receptor antagonist GR55562 (1mg/Kg/day SC daily). The 5-HT reuptake transporter (SERT) is critical for moving 5-HT from the interstitial space into enterocytes and from serum into platelets. Mice lacking SERT exhibited significant deficits in trabecular bone mass that are similar to those observed in DSS-inflamed mice, and these deficits were not extensively worsened by DSS-induced colitis in the SERT^{-/-} mice. Taken together, findings from both the DSS and SERT^{-/-} mouse models support a contributing role for 5-HT as a significant factor in bone loss induced by colitis.

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The authors have no conflicts to disclose.

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GRAPHICAL ABSTRACT

Keywords

Peripheral 5-HT; 5-HT_{1B}; bone loss; DSS-induced colitis

1. INTRODUCTION

Bone metabolism is a highly dynamic process, involving a constant balance between the anabolic activity of the osteoblasts and the catabolic activity of the osteoclasts, that is essential for the development and maintenance of healthy bone (1). Both intrinsic and extrinsic signaling factors such as hormones, growth factors and cytokines modulate the activity of osteoblasts and osteoclasts, and therefore affect the balance between bone formation and resorption. Pathological conditions, including gut dysfunction, as well as drug treatments can lead to impaired balance of bone formation by affecting activity of the signaling factors exchanged among intramedullary bone cell populations and received from other organs and tissues (2, 3). Osteoporosis is common in patients with gastrointestinal inflammatory disorders. Inflammatory bowel disease (IBD), an umbrella term used clinically to describe the two forms of chronic inflammatory disorders of the gastrointestinal tract, Crohn's Disease and Ulcerative Colitis, affects an estimated 3 million adults (1.3% of the US population) according to the Center for Disease Control. In patients with IBD increased activity of proinflammatory cytokines has been associated with decreased bone density (4). Additionally, antiinflammatory drugs, such as corticosteroids, have a negative impact on bone formation, as well as leading to bone degradation, particularly in patients with IBD (5, 6).

In order to differentiate between the direct contribution of inflammatory cytokines in chronic IBD and the role of anti-inflammatory drugs, including steroids, on changes in bone properties, animal models of colitis have been used (7–9). In adult mice treated chronically for 2 weeks with dextran sulfate sodium (DSS), a stimulant of intestinal inflammation (7).

Colitis leads to a decrease in trabecular bone volume that results from decreased bone formation and increased bone resorption, while no changes are detected in the femur cortical zone. Specific inflammatory cytokines have been shown to contribute to decreased trabecular and cortical bone in DSS-treated juvenile mice during the inflammation phase (8). Following recovery from colitis, however, bone measurements return to control levels, as serum levels of pro-inflammatory cytokines subside to normal levels.

In addition to steroids and pro-inflammatory cytokines, another signaling factor that could contribute to colitis-associated bone loss is gut-derived serotonin (5-hydroxytryptamine; 5-HT) (10). Serotonin produced by, and released from, enterochromaffin (EC) cells in the epithelial layer of the gastrointestinal tract activates local 5-HT receptors to stimulate intrinsic and extrinsic enteric reflexes (11). Following activation of 5-HT receptors, most of the 5-HT is rapidly taken up by epithelial cells of the gut, which express the serotonin selective reuptake transporter (SERT), to terminate signaling by 5-HT from the interstitial space (11). During this process, some 5-HT enters the circulation and is sequestered by platelets, which also express SERT. Findings from a number of investigations of human tissue samples as well as animal models of colitis have demonstrated that epithelial SERT expression is decreased in inflammatory conditions (12). A decrease in expression of SERT in the epithelium likely leads to an increase in the amount of 5-HT entering the bloodstream. EC cell-derived 5-HT has been reported to be a negative regulator of bone density specifically by inhibiting osteoblast formation via activation of pre-osteoblast 5-HT_{1B} receptors (13).

The current study was undertaken to test the hypothesis that increased 5-HT available in mice treated chronically with DSS contributes to the colitis-associated bone defects. To accomplish this, we used micro-computed tomography (μ CT) imaging to evaluate changes in the properties of bone tissue in mice with chronic DSS colitis. Bone is a dynamic organ where trabecular bone in the medullary cavity is continuously being resorbed by osteoclasts to maintain serum calcium and reformed by osteoblasts, while the cortical bone has different properties to support bone strength. In trabecular bone the balance between osteoblast and osteoclast activities are critical in maintaining trabecular bone mass. The μ CT data provide quantitative information on the bone forming and resorbing parameters for both trabecular and cortical bones. Several approaches were used to assess the effects of altered 5-HT signaling on bone mass, including evaluation of femurs of SERT $-/-$ mice, mice treated with the systemic 5-HT synthesis inhibitor, p-chloro-DL-phenylalanine (PCPA) and mice treated with the 5-HT_{1B} receptor antagonist, GR55562. Previous studies have examined the relationship between inflammation and bone loss in colitis (7, 8), but the possibility that 5-HT plays a contributing role has not been previously examined. Thus, this investigation addresses the compelling question as to the specific and direct influence of 5-HT on bone loss in inflammatory colitis. The results of these experiments support a contributing role of gut-derived peripheral 5-HT acting on 5-HT_{1B} receptors, in addition to previously described action of pro-inflammatory circulating cytokines, in colitis-mediated bone deficits.

2. MATERIAL and METHODS:

2.1. Animal Preparations

Experiments conducted for this study were approved by the Institutional Animal Care and Use Committee of the University of Vermont. Mice were euthanized by isoflurane overdose and exsanguination or by carbon dioxide inhalation and cervical dislocation.

We used several mouse strains and substrains (from different sources) in order to reproduce baseline results from previous studies. Mice used in this study: C57BL/6J (Jackson Labs, Bar Harbor, ME), C57BL/6NTac (Taconic Biosciences, Albany, New York), BALB/cJ (Jackson Labs), BALB/cAnNCrI (BALB/cCrI, Charles River Labs, St-Constant, Québec, Canada). SERT^{-/-} mice (14) were transferred to the University of Vermont from Columbia University. Mice were obtained from specific pathogen free colonies and were housed in individually-ventilated caging (LabProducts™) with *ad libitum* access to food and water. Environmental parameters in the facility meet all recommendations of the Guide for the Care & Use of Laboratory Animals (NRC, 2011), with 12:12 light:dark cycle and temperatures of 72 F (+/- 2 degrees). The animal care program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALACi).

2.2. Colitis model

Colitis was induced in 7–8 week old male mice by dissolving the DSS (MW 35-50 kDa; MP Biomedicals, LLC, Solon, OH, USA) in their drinking water for 21 days. To account for the different sensitivities to DSS in the different strains and substrains of mice, its initial concentration in the drinking water ranged from 3 to 5% between the groups (15, 16). Severity of colitis was monitored daily using the disease activity index (DAI) scale that grades weight loss, stool consistency, and presence of fecal blood (17). Hemocult Single Slide testing slides (Beckman Coulter, Brea, CA) were used to detect fecal blood.

After five days, the DSS concentration was lowered to 1% and adjusted (from 0–1.5%) to maintain a moderate level of inflammation with disease activity index ranging between 4 and 10. Mice were euthanized 21 days post-DSS induction. All DSS-inflamed mice had a significantly higher disease activity index than mice in the associated control group at the time of euthanasia.

2.3. Whole Blood Serotonin

Blood was collected by cardiac puncture, mixed with 5mM EDTA and 10μM Prostaglandin E1 (Sigma-Aldrich, St-Louis MO), flash frozen and analyzed by high-performance liquid chromatography (HPLC) at the Vanderbilt Neurochemistry Core Lab (Vanderbilt University, Nashville TN). Whole blood was collected to ensure that 5-HT levels in platelets would be accounted for our analysis of bone defects.

2.4. Drug treatment

Concentration of gut-derived 5-HT was reduced in C57BL/6NTac mice by daily injection of PCPA (300mg/kg/day IP; Sigma-Aldrich, St-Louis MO), a compound that inhibits the activity of tryptophan hydroxylase (Tph), the rate-limiting enzyme for 5-HT synthesis (18).

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Injections began 5 days before DSS administration was initiated and continued throughout the 21 days of DSS treatment. Animals that were not treated with DSS, but which received saline vehicle injections served as a control group. Since 5-HT can act as a proinflammatory molecule in the colon (18–20), and decreasing 5-HT synthesis can reduce inflammation (18), mice were monitored daily and DSS concentration was adjusted to maintain comparable levels of inflammation, as measured daily by DAI scores, between all groups. Colonic 5-HT content was measured in distal colon (1cm) collected and homogenized in 0.1 M perchloric acid, buffered in 0.5 M borate buffer and analyzed using an enzyme immunoassay kit according to manufacturer's protocol (Beckman Coulter, Brea, CA). Colonic 5-HT levels were decreased by over 30% in mice receiving daily PCPA injections in both DSS-inflamed and non-DSS-inflamed mice. As reported previously (21), 5-HT levels were increased in DSS-inflamed mice when compared to controls. In studies of the role of 5-HT_{1B} receptors activation, BALB/cJ mice received the 5-HT_{1B} receptor antagonist, GR55562 (1 mg/Kg/day SC, Tocris Minneapolis, MN) or saline vehicle by SC injection daily.

2.5. Micro-computed tomography (μ CT)

Femurs were excised, fixed overnight in periodate-lysine-paraformaldehyde and scanned at a nominal resolution of 10 μ m (Scanco μ CT 40, MSK Imaging Core, UMASS Medical School, Worcester, MA) and images were reconstructed using Scanco software v5.0. Both trabecular and cortical analyses were performed. For the trabecular analysis, a 3.0 mm section of trabecular bone running proximally from the distal femoral growth plate was analyzed using a threshold range 220–1000 and a density range superior to 500mg of HA/cm³. For the cortical analysis, a 5.0 mm section of cortical bone in the center diaphysis of the femur was analyzed using a threshold range 260–1000 and a density range superior to 600mg of HA/cm³.

2.6. Dual calcein labeling

For dynamic measurements of bone formation, dual calcein labeling was carried out. Mice were given two intraperitoneal injections of 2.5 mg/ml calcein (Sigma-Aldrich, St-Louis MO) four days apart. Fixed bones were dehydrated in 70% ethanol and processed for plastic sections using JB4 embedding reagents (Polysciences, Warrington, PA). Images were captured on an Olympus AX70 fluorescence microscope using an Optronics MagnaFire digital camera and software. Distances (in microns) between 2 calcein labels were measured along the periosteal and endosteal surfaces of the cortical bone using ImageJ software (22).

2.7. Statistical analysis

Statistical analyses were performed by unpaired 2-tailed student's t-test or one-way ANOVA with Bonferroni's multiple comparisons test, as appropriate, and shown as mean $\pm$ SEM. Statistical analyses were performed using the GraphPad Prism software application (version 6.0c, GraphPad Software, La Jolla, CA).

3. RESULTS

This study was performed using DSS colitis, a well-established model of intestinal inflammation in mice (16). As previously described (15) sensitivity to DSS varied amongst the strains that were studied. Therefore, we adjusted the initial concentrations of DSS within in a range of 3–5% (see Table 1) in order to achieve a moderate level of colitis among all strains of mice used in our study. Inflammation was assessed by daily determination of their disease activity index, which was higher (4) than that of untreated mice at the time of euthanasia.

3.1. Circulating serotonin is increased in DSS colitis

The serotonin transporter, SERT is expressed by all enterocytes in the mucosa of the colon to terminate mucosal 5-HT signaling by removing it from the interstitial space (11). This rapid action is important as it prevents prolonged exposure and sensitization of the 5-HT receptors located on cells near the site of 5-HT release. Previous studies using animal models of colitis have shown a decrease in colonic SERT expression resulting in an increased availability of 5-HT in mucosa of the colon (21, 23, 24). Whole blood samples from DSS-treated BALB/cJ mice and controls were examined for levels of 5-HT. We found that the average level of 5-HT from DSS-treated mice was $24.5 \pm 1.7 \mu\text{mol/L}$ a 36% increase compared to the level observed in control mice ($15.7 \pm 4.4 \mu\text{mol/L}$) ($p < 0.03$).

3.2. Influence of colitis on bone mass

Colitis has been previously linked to decreased bone mineral density (mineral content/ mm^3) in patients (4). To measure the effect of inflammatory colitis on changes in bone structure, we performed μCT imaging of femoral bone in substrains of controls mice and in mice following 21 days of DSS treatment (Table 1). Analysis of the trabecular bone of DSS-treated mice, showed extensive reductions in the bone volume fraction (bone volume/total volume). Interestingly, compared to controls the C57BL/6NTac and BALB/cJ had the greatest DSS colitis-induced decreases in trabecular bone volume fraction at 66.6% and 49.2%, respectively, whereas the C57BL/6J and BALB/cCrl strains exhibited decreases of 38.3% and 32.2%. These strain-related differences were consistent for all other parameters measured (Table 1). For example, the C57BL/6NTac and BALB/cJ had greater decreases in connectivity density and bone surface, and increases in trabecular space, which are indicators of extensive bone loss (Fig. 1).

With respect to the cortical bone of DSS-inflamed mice, both cortical thickness and bone mineral density were reduced in all mouse strains (Table 2). However, decreased cortical bone area and bone volume were observed only in C57BL/6-inflamed mice (either substrains), while BALB/c- and C57BL/6NTac-inflamed mice additionally showed a substantial increase of bone porosity (Table 2). These effects on cortical bone are mediated by the ability of osteocytes, each cell embedded in their own lacunae, to respond to signaling molecules in the vasculature such as inflammatory cytokines (25).

3.3. Bone changes in SERT^{-/-} mice treated with DSS

The serotonin transporter, which is expressed by enterocytes and platelets, removes free 5-HT from the mucosal interstitial space and serum. Therefore, in the absence of SERT (SERT^{-/-} mice), with concomitant increased extracellular 5-HT, we could assess the influence of increased circulating 5-HT on features of bone structure in non-inflamed and DSS-inflamed mice. Consistent with the findings of Warden and colleagues (26) who compared bone features in non-inflamed SERT^{-/-} and SERT^{+/+} mice, we observed a decrease in trabecular bone in SERT^{-/-} mice as compared to control mice (Fig. 1; Table 1). Treatment of the SERT^{-/-} mice with DSS resulted in a less extensive response in bone loss as compared to the response to DSS in SERT intact mice (Fig. 1; Table 1). It is worth noting that disease activity index of the untreated SERT^{-/-} mice reached inflammatory levels that were not significantly different than the disease activity index of DSS-inflamed mice at the end of the chronic treatment period. Therefore, in the DSS-treated SERT^{-/-} mice, little or no further reductions in trabecular and cortical features of bone mass were observed (Fig. 1; Tables 1 and 2). These results support the concept that 5-HT could contribute to bone deficits related to colitis.

3.4 Effect of decreased 5-HT synthesis on bone changes in DSS-inflamed mice

Tryptophan hydroxylase is the rate-limiting enzyme for 5-HT synthesis. Our data and those of others (13, 27) support a link between higher levels of gut-derived 5-HT and bone deficits. We therefore tested the hypothesis that inhibition of 5-HT synthesis could attenuate the detrimental effects of DSS inflammation on bone formation. Mice received daily injections of PCPA (300mg/kg/day IP), a compound that decreased colonic 5-HT content by 30% (see Materials and Methods).

While no difference was noted in bone measurements in non-inflamed mice treated with PCPA (PCPA/vehicle mice; data not shown) compared to vehicle-treated mice, a protective effect by reducing 5-HT production was observed in the trabecular bone of DSS-treated mice (Fig. 2). Trabecular number and spacing for PCPA/DSS mice were statistically comparable to those of non-inflamed control mice. Additionally, while trabecular bone volume fraction, bone surface and connective density in PCPA/DSS mice were still lower than those of control mice, their levels were significantly higher than those of vehicle/DSS mice (Fig 2A). These quantitative changes are consistent with the trabecular bone images in 3 experimental groups (Fig 2B). The vehicle/DSS mice exhibit strikingly fewer trabeculae that are not connected to each other, as compared to control mice. In DSS-treated mice, the PCPA prevented the extensive bone loss and bone volume and connectivity parameters were reaching control levels (Fig 2B). Colitis-induced deficits in cortical bone were not affected by PCPA treatment (data not shown). Taken together these data support the involvement of gut 5-HT in colitis-mediated bone loss.

3.5. Contribution of the 5-HT_{1B} receptor to colitis-induced bone deficits

The negative impact of gut-derived 5-HT on bone mass involves activation of 5-HT_{1B} receptors expressed by pre-osteoblasts (13). To further test our hypothesis that 5-HT contributes to bone loss in colitis and that this process results from activation of the 5-HT_{1B} receptor, we evaluated whether administration of the 5-HT_{1B} receptor antagonist, GR55562

could ameliorate the bone loss associated with DSS colitis. Inhibition of the 5-HT_{1B} receptors appeared to have a protective effect on colitis-induced bone changes because, when compared to vehicle/DSS mice, GR55562/DSS mice had increased trabeculae number and decreased trabecular spacing (Fig. 3; $p < 0.001$). This result indicates that the resorptive activity of trabecular bone was diminished in absence of a 5-HT-mediated signal that results in decreased osteoblast formation. The finding that GR55562 did not increase trabecular bone volume and bone surface demonstrates that 5-HT_{1B} receptor inhibition could not completely rescue the bone loss induced by DSS. The 5-HT_{1B} receptor antagonist also protected cortical bone. Micro-CT imaging demonstrated that cortical thickness was significantly improved in GR55562/DSS mice as compared to vehicle/DSS mice (Fig. 4A). Furthermore, additional information provided by dynamic calcein labeling for bone formation rate on the cortical bone surfaces showed that cortical bone formation was decreased in vehicle/DSS mice, but comparable to controls in GR55562/DSS animals (Fig 4B).

Because the 5-HT_{1B} receptor antagonist treatment was effective in protecting cortical bone growth in DSS-inflamed mice, as measured by μ CT scanning, cortical bone growth was further examined by obtaining dynamic measurements of bone formation using dual injection (separated by 4 days) of the fluorescent dye, calcein. In DSS-treated mice, the decreased spacing observed between the two fluorescent bands correlated with the decreased level of bone growth activity (Fig. 4B). Moreover, daily treatment of DSS-treated mice with GR55562 supports the concept that antagonism of the 5-HT_{1B} receptor may protect bone formation in mice with colitis.

4. Discussion

Osteoporosis and bone fractures are commonly associated with IBD, particularly in Crohn's disease patients (4). In this study, we used an established chronic model of colitis in mice to investigate a potential link between increased gut-derived 5-HT and bone deficits associated with colonic inflammation. We report that DSS-inflamed mice have increased levels of circulating 5-HT and showed decreased trabecular and cortical bone parameters that were attenuated by lowering 5HT synthesis. Moreover, treatment with a 5-HT_{1B} receptor antagonist reduced bone loss in DSS-inflamed mice, indicating a possible site of action for the excess of gut-derived 5-HT in the blood. Collectively, these findings support the hypothesis that increased gut-derived 5-HT contributes to decreased bone formation in colitis, and its action involves stimulation of the 5-HT_{1B} receptor (see Fig. 5).

The possibility that an interaction exists between gut-derived 5-HT and bone metabolism was recognized when links were made between low-density lipoprotein receptor-related protein 5 (Lrp5) and gut-derived 5-HT (28, 29). Lrp5 is an anabolic mediator of Wnt signaling in bone, and gain or loss of function mutations in the Lrp5 are associated with high bone mass syndrome or osteoporosis, respectively (30, 31). Lrp5 inhibits 5-HT synthesis in EC cells, and in turn, lowers circulating 5-HT levels, by decreasing Tph1 expression (13). Deletion of Lrp5 in mice leads to a marked increase in Tph1 expression, elevated circulating 5-HT levels, and decreased bone mass. The relationship between circulating 5-HT levels and bone metabolism is further supported by the finding that bone formation is increased when

Tph1 is inhibited pharmacologically (32). However, it should be noted that other studies failed to detect evidence for a role for gut-specific Lrp5 action in bone metabolism and of a direct effect of the Lrp5 receptor on 5-HT level, and it was proposed that bone-specific Lrp5 is responsible for changes of function of the osteocyte population through the canonical Wnt pathway (33). In this model, 5-HT has a positive effect on bone mass by decreasing osteoclastogenesis. This controversy, based largely on findings in Tph1^{-/-} mice, has led to an unresolved debate about the physiological role of Lrp5's actions on EC cells and gut-derived 5-HT as it relates to bone loss (28, 34–37).

Regardless of the site(s) of Lrp's physiological actions in influencing bone metabolism, our findings support the concept that gut-derived 5-HT can have a negative impact on bone growth, at least in pathological conditions. Our study design bypasses any involvement of Lrp5 and provides evidence that bone loss in association with colitis involves, at least in part, gut-derived 5-HT. As proposed, 5-HT released from EC cells and entering the blood stream has a negative impact on bone formation by inhibiting osteoblast formation. Our data demonstrate that lowering 5-HT levels with PCPA can also attenuate the deleterious effects of colitis on bone formation in the murine DSS colitis model. Furthermore, our data also support the concept that 5-HT negatively affects osteoblast proliferation via activation of 5-HT_{1B} receptors because treatment with a 5-HT_{1B} antagonist had a protective effect on bone mass.

Further evidence supporting the idea that 5-HT can negatively impact bone mass is found in studies, including those reported here, involving deletion or inhibition of serotonin transporter SERT. Deletion or inhibition of SERT results in increased 5-HT availability whenever the indole is released, as well as in the circulation. Consistent with a previous study involving SERT^{-/-} mice (26), we found that bone mass was significantly reduced in SERT^{-/-} mice. Furthermore, increased incidence of fractures and osteoporosis in adults, as well as decreased growth in youths, have been reported in patients who are prescribed SSRIs to treat depression (38–40).

The findings reported here are clinically relevant as they demonstrate a direct link between 5-HT and bone loss in IBD, and data from SERT suppression and knockout studies are relevant to the changes in 5-HT signaling in colitis. In studies of alterations in 5-HT signaling in intestinal inflammation, a change that has been consistently reported in IBD and a variety of animal models of colitis is a decrease in SERT expression (12).

In patients with IBD many confounding factors such as diet, nutrient malabsorption and corticosteroid exposure may affect bone metabolism in addition to inflammation(4). While the current study supports the concept that gut-derived 5-HT contributes to decreased bone formation in colitis, it is most likely just one of several factors that mediate the observed bone deficits. In the current study, neither lowering gut epithelial 5-HT levels with a non-absorbable Tph inhibitor nor administration of a 5-HT_{1B} receptor antagonist completely restored bone mass in DSS-exposed mice. Moreover, there is strong evidence for a contribution of pro-inflammatory cytokines in colitis-associated bone deficits. For example, there is a direct correlation between the severity of colitis and bone loss in *Helicobacter hepaticus*-infected interleukin-10-deficient male mice (41, 42). In a chronic model of DSS

colitis, bone mass measures were reported to be negatively correlated with intestinal disease score, as well as colon and bone TNF- α levels, and this relationship was independent of weight loss (41, 42). Further, in an acute model of DSS-induced mouse colitis, increased TNF α and decreased IGF-I levels correspond to bone deficits and simultaneously return to normal level during recovery (8)

In addition to the effect of gut-derived 5-HT on bone function, this study highlights the differences between mouse strains and substrains in bone metabolism. In control non-inflamed mice, differences in bone measurements were observed between C57BL/6 and BALB/c (see Table 1). Differences between C57BL/6J and C57BL/6NTac strains were even more significant. Differences in bone characteristics and sensitivity to DSS between strains have been reported previously (15). Our data also support the recent observations regarding bone characteristics in these two substrains by Sankara and colleagues (43) and stresses the fact that substrains should be carefully selected and even the same strain obtained from two different vendors are not necessarily interchangeable. Differences in mice of the same strain between vendors could involve genetic polymorphisms and/or differences in the gut microbiota of mice raised in different facilities.

In conclusion, the findings reported here strongly support the hypothesis that changes in mucosal 5-HT signaling in inflamed regions of the intestines can contribute to the bone deficits that are associated with colitis. Bone integrity was partially protected in our model of chronic colitis by inhibition of 5-HT synthesis and by antagonism of the 5-HT_{1B} receptor. Furthermore, bone loss exhibited in SERT^{-/-} was only mildly exacerbated by colitis. The findings reported here also support the concept that gut derived 5-HT can have a negative impact on bone growth, at least in pathological conditions.

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A **B** **Figure 1:**

Effects of DSS colitis on features of trabecular (A) and cortical (B) bone in C57BL6/J and SERT-/- mice. Morphometric analysis of the femurs of DSS-inflamed C57BL6/J mice showed deficits in trabecular bone. Only trabecular thickness remained unchanged after chronic DSS treatment when compared to control. SERT-/- mice drinking water had lower bone volume fraction, bone surface and connective density as well as reduced trabecular number and increased trabecular spacing when compared to control C57BL6/J mice. Of these trabecular bone measurements, only bone volume fraction and connective density were

further decreased following DSS-induced inflammation. Of the cortical bone measurements, only cortical thickness was affected by colitis. C57BL6/J+Vehicle, n=5; C57BL6/J+DSS, n=5; SERT^{-/-} +Vehicle, n= 4; SERT^{-/-} + DSS, n=4. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$; unpaired 2-tailed student's t-test.

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Figure 2.

Deficits in trabecular bone of DSS-inflamed mice were protected in mice with attenuated 5-HT synthesis. **A.** Trabecular thickness and spacing in DSS-inflamed mice receiving PCPA (300mg/kg/day IP, daily) were similar to those observed in control mice, showing a protective effect of this treatment on bone mass. Bone volume fraction, bone surface and connective density were also significantly increased in the PCPA-treated DSS mice compared to DSS mice, but did not reach the levels measured in control mice. **B.** Representative images from μ CT scans of the femoral trabecular bone from two mice per

group. Vehicle/Vehicle, n=10; Vehicle/DSS, n=7; PCPA/DSS, n=14. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$; 1-way ANOVA with Bonferroni's correction.

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Figure 3.

Inhibition of the 5-HT_{1B} receptor has a protective effect on trabecular bone of DSS-inflamed mice. **A.** Measurement of trabecular number and trabecular spacing in DSS-inflamed mice receiving the 5-HT_{1B} receptor antagonist GR55562 (1 mg/Kg/day SC, daily) were significantly improved compared to DSS-inflamed mice. They did not, however, reach control levels. **B.** Representative images from μ CT scans of the femoral trabecular bone from two mice per group. Vehicle/Vehicle, n=5; Vehicle/DSS, n=5; GR55562/DSS, n=5. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; 1-way ANOVA with Bonferroni's correction.

Figure 4.

Changes in cortical thickness and bone growth in DSS-inflamed mice were partially protected by inhibition of the 5-HT_{1B} receptor. Cortical thickness was protected by inhibition of the 5HT_{1B} receptor with GR55562 (1 mg/Kg/day SC, daily). **A.** Parameters of cortical bone growth/homeostasis that were decreased in femurs from DSS-inflamed mice included cortical thickness, bone porosity, bone density and bone volume fraction. **B.** Dual injection of fluorescent calcein was used to measure dynamic bone growth. Decreased spacing of the bands growth in DSS mice correlates with decrease level of bone growth

activity. Bone formation in inflamed animals was protected by daily treatment with the 5-HT_{1B} antagonist. Vehicle/Vehicle, n=5; Vehicle/DSS, n=5; GR55562/DSS, n=5. ** $P < 0.01$, *** $P < 0.001$; 1-way ANOVA with Bonferroni's correction.

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Figure 5. Schematic diagrams depicting the proposed relationship between 5-HT released from EC cells under healthy conditions and in colitis. In colitis, there is a reduction in SERT expression by enterocytes, which leads to more 5-HT entering the circulation and greater activation of 5-HT_{1B} receptors in bone, which has a negative impact on bone mass.

Table 1.
Effects of DSS Colitis on Features of Trabecular Bone in Different Strains and Substrains of Mice

	Jackson				Taconic			
	Vehicle (n=5)		3% DSS (n=5)		Vehicle (n=10)		4% DSS (n=7)	
	Mean	± SEM	Mean	± SEM	Mean	± SEM	Mean	± SEM
C57BL/6								
Bone Volume Fraction (BV/TV; %)	6.44	± 0.12	3.97	± 0.14	3.56	± 0.14	1.17	± 0.07
Trabecular Thickness (µm)	40.7	± 1.8	36.3	± 1.1	41.4	± 1.5	34.6	± 1.8
Trabecular Number (1/mm)	3.76	± 0.18	3.19	± 0.08	2.19	± 0.13	1.62	± 0.14
Trabecular Space (µm)	267.7	± 13.7	313.3	± 7.6	477.5	± 28.5	656.5	± 65.2
Connective Density (1/mm ³)	51.5	± 1.5	26.7	± 1.3	26.5	± 0.7	4.6	± 0.2
Bone Density (mg HA/cm ³)	806.8	± 3.0	778.9	± 4.955	787.2	± 6.2	760.0	± 11.4
Total Volume (TV; mm ³)	4.714	± 0.127	5.084	± 0.165	6.189	± 0.175	6.719	± 0.307
Bone Volume (BV; mm ³)	0.303	± 0.011	0.201	± 0.009	0.220	± 0.010	0.080	± 0.007
SMI	2.87	± 0.09	3.05	± 0.01	2.89	± 0.07	3.30	± 0.05
Bone Surface (BS; mm ²)	19.44	± 0.46	13.95	± 0.23	13.80	± 0.70	5.68	± 0.63
BS/BV (1/mm)	6.44	± 0.19	3.97	± 0.14	69.10	± 2.70	83.30	± 3.28
	Jackson				Charles River			
	Vehicle (n=5)		5% DSS (n=5)		Vehicle (n=5)		5% DSS (n=5)	
	Mean	± SEM	Mean	± SEM	Mean	± SEM	Mean	± SEM
BALB/c								
Bone Volume Fraction (BV/TV; %)	8.50	± 0.29	4.32	± 0.19	7.44	± 0.17	5.19	± 0.30
Trabecular Thickness (µm)	41.1	± 0.8	37.9	± 0.7	41.4	± 0.32	40.48	± 1.4
Trabecular Number (1/mm)	3.66	± 0.10	2.48	± 0.07	3.30	± 0.09	2.58	± 0.10
Trabecular Space (µm)	275.1	± 7.8	409.3	± 12.1	305.7	± 9.2	392.9	± 14.5
Connective Density (1/mm ³)	114.1	± 4.4	41.0	± 2.4	90.1	± 2.6	52.3	± 3.3
Bone Density (mg HA/cm ³)	817.5	± 5.3	810.0	± 2.4	819.9	± 4.0	798.4	± 4.7
Total Volume (TV; mm ³)	3.250	± 0.070	3.440	± 0.070	3.080	± 0.060	3.39	± 0.080
Bone Volume (BV; mm ³)	0.276	± 0.009	0.149	± 0.007	0.227	± 0.004	0.175	± 0.007
SMI	2.46	± 0.02	2.79	± 0.04	2.54	± 0.05	2.72	± 0.08

Bone Surface (BS; mm ²)	17.04	± 0.49	9.67	± 0.32	****	13.80	± 0.14	10.71	± 0.19	****
BS/BV (1/mm)	66.49	± 1.32	72.45	± 1.72	*	65.58	± 0.87	67.65	± 2.97	ns
SERT/- on a C57BL/6 background	Vehicle (n=4)		3% DSS (n=4)							
	Mean	± SEM	Mean	± SEM	Diff.					
	2.48	± 0.14	2.06	± 0.02	*					
Bone Volume Fraction (BV/TV; %)										
Trabecular Thickness (µm)	36.1	± 2.2	32.7	± 1.3	ns					
Trabecular Number (1/mm)	2.39	± 0.22	2.47	± 0.15	ns					
Trabecular Space (µm)	432.3	± 38.2	409.6	± 24.5	ns					
Connective Density (1/mm ³)	13.73	± 1.07	8.78	± 0.67	**					
Bone Density (mg HA/cm ³)	790.1	± 9.2	760	± 4.69	*					
Total Volume (TV; mm ³)	4.05	± 0.17	4.93	± 0.26	*					
Bone Volume (BV; mm ³)	0.100	± 0.005	0.101	± 0.005	ns					
SMI	3.24	± 0.03	3.19	± 0.02	ns					
Bone Surface (BS; mm ²)	6.93	± 0.68	7.59	± 0.39	ns					
BS/BV (1/mm)	79.32	± 5.45	87.13	± 4.41	ns					

Trabecular bone µCT measurements of femurs of DSS-treated mice (initial concentration differed between strains) compared to controls (vehicle) with unpaired 2-tailed student's t-test

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$
**** $P < 0.0001$

ns non-significantly different

Table 2. Effects of DSS Colitis on Features of Cortical Bone in Different Strains and Substrains of Mice

C57BL/6	Jackson				Diff.	Taconic				Diff.	
	Vehicle (n=5)		3% DSS (n=5)			Vehicle (n=10)		4% DSS (n=7)			
	Mean	± SEM	Mean	± SEM		Mean	± SEM	Mean	± SEM		
	4.764	± 0.070	4.727	± 0.088		ns	4.433	± 0.087	4.377		± 0.067
Bone Surface (mm ²)	0.397	± 0.015	0.346	± 0.009	*	0.362	± 0.014	0.300	± 0.011	**	
Bone Volume (BV; mm ³)	0.164	± 0.006	0.144	± 0.001	**	0.161	± 0.004	0.135	± 0.004	***	
Cortical Thickness (mm)	11.260	± 0.830	12.900	± 0.267	ns	12.570	± 0.472	15.540	± 0.289	***	
Cortical Porosity %	0.794	± 0.029	0.692	± 0.018	*	0.724	± 0.028	0.599	± 0.023	**	
Bone Density (mg HA/cm ³)	995.7	± 5.5	976.1	± 3.0	*	980.7	± 5.4	958.5	± 1.3	**	
Bone Volume Fraction (BV/TV; %)	88.730	± 0.831	87.120	± 0.272	ns	87.430	± 0.476	84.480	± 0.290	***	
	Jackson					Charles River					
BALB/c	Vehicle (n=5)		5% DSS (n=5)		Diff.	Vehicle (n=5)		5% DSS (n=5)		Diff.	
	Mean	± SEM	Mean	± SEM		Mean	± SEM	Mean	± SEM		
	3.753	± 0.052	3.857	± 0.093		ns	3.744	± 0.058	3.673		± 0.037
	0.404	± 0.004	0.381	± 0.013		ns	0.381	± 0.008	0.353		± 0.010
Bone Surface (mm ²)	0.212	± 0.003	0.194	± 0.003	**	0.200	± 0.004	0.189	± 0.006	ns	
Bone Volume (BV; mm ³)	5.280	± 0.222	6.480	± 0.188	**	7.200	± 0.311	8.020	± 0.464	ns	
Cortical Thickness (mm)	0.808	± 0.008	0.761	± 0.025	ns	0.762	± 0.016	0.706	± 0.021	ns	
Cortical Porosity %	1140.0	± 5.1	1099.0	± 4.1	***	1080.0	± 8.6	1061.0	± 8.1	ns	
Bone Density (mg HA/cm ³)	94.720	± 0.237	93.530	± 0.190	**	92.800	± 0.313	91.980	± 0.463	ns	
Bone Volume Fraction (BV/TV; %)											
SERT ^{-/-} on a C57BL/6 background	Vehicle (n=4)		3% DSS (n=4)		Diff.	Vehicle (n=4)		3% DSS (n=4)		Diff.	
	Mean	± SEM	Mean	± SEM		Mean	± SEM	Mean	± SEM		
	4.288	± 0.245	4.396	± 0.129		ns					
	0.311	± 0.009	0.317	± 0.016		ns					
Bone Surface (mm ²)	0.144	± 0.006	0.142	± 0.004	ns						
Bone Volume (mm ³)	12.030	± 0.932	12.830	± 0.382	ns						

Cortical Bone Area (mm ²)	0.623	± 0.018	0.635	± 0.033	ns							
Bone Density (mg HA/cm ³)	991.0	± 18.5	982.9	± 1.7	ns							
Bone Volume Fraction (BV/TV; %)	87.990	± 0.931	87.200	± 0.368	ns							

Cortical bone μ CT measurements of femurs of DSS-treated mice (initial concentration differed between strains) compared to controls (vehicle) with unpaired 2-tailed student's t-test

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

**** $P < 0.0001$

ns non-significantly different.