

Skeletal effects of serotonin (5-hydroxytryptamine) transporter inhibition: Evidence from *in vitro* and animal-based studies

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Abstract

The regulation of bone metabolism continues to be an area of intense investigation, with recent evidence indicating a potential contribution from the neural system. In particular, the neurotransmitter serotonin (5-hydroxytryptamine [5-HT]) has been hypothesized to play a role in skeletal metabolism via its transporter (5-HTT). The 5-HTT is a plasma membrane transporter that is highly specific for the uptake of extracellular 5-HT, thereby facilitating the intracellular storage and/or degradation of 5-HT. The 5-HTT is clinically important as it is the key target of pharmaceutical agents aimed at treating affective disorders, such as major depressive disorder. By antagonizing the 5-HTT, selective serotonin reuptake inhibitors (SSRIs) potentiate 5-HT activity and effectively relieve the symptoms of depression. However, questions have been raised regarding the potential skeletal effects of SSRIs given the recent identification of a functional 5-HTT and functional 5-HT receptors in bone cells. This paper discusses the preclinical evidence for the skeletal effects of 5-HT and the inhibition of the 5-HTT. In particular, it discusses the: (1) role of 5-HT and the function of the 5-HTT; (2) presence of functional 5-HTTs in bone; (3) potential sources and response mechanisms for 5-HT in bone, and; (4) *in vitro* and *in vivo* skeletal effects of 5-HT and 5-HTT inhibition.

Keywords: Antidepressants, Fluoxetine, Neurotransmitter, Osteoporosis, Prozac

The regulation of bone metabolism continues to be an area of intense investigation given the ongoing clinical problem of osteoporosis. Although numerous novel skeletal regulatory pathways have recently been identified, understanding of bone biology is far from complete with the list of factors known to influence bone development and its maintenance being regularly updated. Hormonal factors have traditionally been identified as the major influence on bone; however, there is increasing evidence for the contribution of alternative systems and pathways. In particular, there is a growing body of evidence demonstrating the potential for neural regulation of bone^{1,2}.

An intimate relationship exists between the nervous system and bone tissue, with bone being richly innervated by both sympathetic and sensory neurons³. These nerves serve sensory and vascular functions, but may also influence bone cell activities. The most convincing evidence for this has been provided by studies into the central anti-osteogenic effects of the cytokine-like hormone leptin. The central effects of leptin have been shown to be mediated through hypothalamic neural circuits^{4,6} with signals being relayed to the skeleton via the sympathetic nervous system^{7,8}. Other evidence for the neural regulation of bone has been provided by studies into the skeletal effects of neuropeptide Y receptors^{4,5,9,10} and unmyelinated sensory neurons¹¹. Hypothalamic neuropeptide Y2 receptors are involved in the tonic inhibition of bone formation⁵, and hypothalamic Y4 receptors work in synergy with Y2 receptors to influence bone mass¹⁰. Meanwhile, selective lesioning of unmyelinated sensory neurons negatively influences bone metabolism¹¹. These findings suggest a functional link between the nervous system and bone.

Further evidence for a link between the nervous system and bone has been provided by studies into neurotransmit-

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ters and their transporters. Various neurotransmitters and transporters have been associated with alterations in bone metabolism. For example, osteoblasts possess functional receptors for the neurotransmitter glutamate¹², and the glutamate/aspartate transporter in osteocytes is influenced by osteogenic mechanical stimuli¹³. Meanwhile, mice with altered dopamine transporter function have a skeletal phenotype of reduced cancellous bone mass, cortical thickness and mechanical strength¹⁴.

The latest neural pathway that has been hypothesized to play a role in skeletal metabolism involves 5-hydroxytryptamine (5-HT) and the 5-HT transporter (5-HTT)^{2,15}. Interest into the skeletal effects of 5-HT has been stimulated by clinical studies demonstrating altered bone mass and fracture risk in individuals treated with pharmaceutical agents that selectively inhibit the 5-HTT—agents collectively known as selective serotonin reuptake inhibitors (SSRIs)^{16–18}. These studies are discussed in an accompanying paper¹⁹. The current paper discusses the preclinical evidence for the skeletal effects of 5-HT and the inhibition of the 5-HTT. In particular, it addresses the: (1) role of 5-HT and the function of the 5-HTT; (2) presence of functional 5-HTTs in bone; (3) potential sources and response mechanisms for 5-HT in bone, and; (4) *in vitro* and *in vivo* skeletal effects of 5-HT and 5-HTT inhibition.

The role of 5-HT and the function of the 5-HTT

5-HT is a monoamine neurotransmitter that was initially named ‘serotonin’ due to early observations identifying it as a serum agent (*sero-*) affecting vascular tone (*-tonin*)^{20,21}. However, this name is somewhat of a misnomer as it reflects the circumstances of the compound’s discovery rather than its chemical structure or the diversity of its physiological effects. With the chemical identification of the compound as 5-hydroxytryptamine, 5-HT has become the preferred name.

5-HT has recognized roles in the central nervous system (CNS), gastrointestinal (GI) tract and cardiovascular (CV) system. In the CNS, it is manufactured by pre-synaptic neurons and released into the synaptic cleft to activate pre- and post-synaptic 5-HT receptors to influence a range of behavioral, physiological and cognitive functions (Figure 1A)^{22–23}. In the GI tract, 5-HT is produced and secreted by enterochromaffin cells in response to mucosal stimulation before diffusing to enteric nerve endings to stimulate peristalsis^{24–26}. In the CV system, 5-HT is stored in platelets in dense granules²⁷ and is released following platelet activation to cause a diverse array of physiological effects^{28,29}, including blood vessel constriction or dilation³⁰, and smooth muscle cell hypertrophy and hyperplasia³¹.

The role of 5-HT in the CNS, GI tract and CV system is tightly regulated by the 5-HTT. The 5-HTT (also known as SERT) is a plasma membrane transporter that is highly specific for the uptake of extracellular 5-HT. It is a member of the neurotransmitter:sodium symporter family, which also includes the norepinephrine and dopamine transporters³².

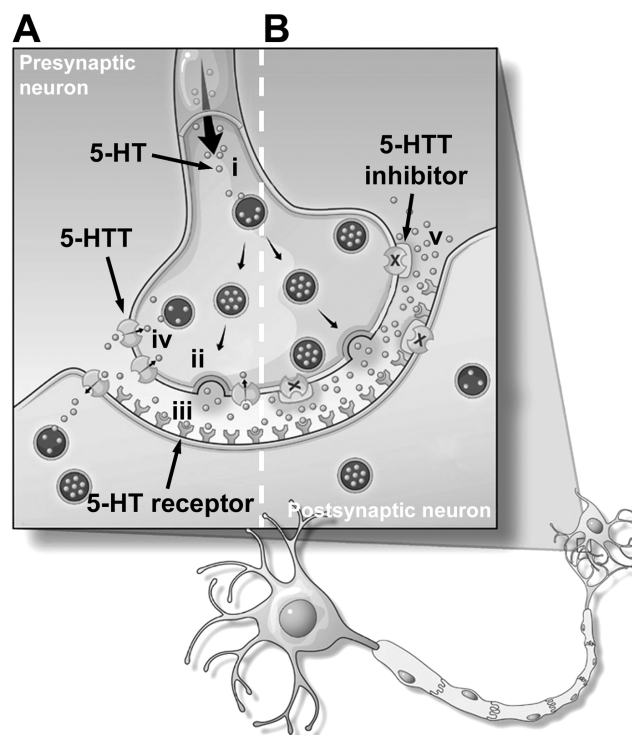


Figure 1. (A) 5-hydroxytryptamine (5-HT) transmission within the central nervous system and (B) the effects of 5-HT transporter (5-HTT) inhibition. (i) 5-HT is manufactured by presynaptic neurons and stored in vesicles. (ii) Vesicles bind with the cell membrane following an appropriate stimulus to release 5-HT into the synaptic cleft via exocytosis. (iii) Released 5-HT activates post-synaptic receptors to stimulate the post-synaptic neuron. (iv) Membrane-bound serotonin transporters (5-HTT) uptake released 5-HT to control the duration of 5-HT effects and recycle or degrade 5-HT. (v) Inhibition of the 5-HTT (i.e., by the administration of a selective serotonin reuptake inhibitor) prevents uptake of 5-HT from resulting in its accumulation within the synaptic cleft and prolonging of its effects.

The structure of these transporters remains putative; however, it is believed to consist of 12 transmembrane domains with both the amino- and carboxy-termini being intracellular, and a large extracellular loop connecting transmembrane domains 3 and 4 containing canonical glycosylation sites. Each transporter uses transmembrane ion gradients of Na⁺, Cl⁻ and K⁺, and an internal negative membrane potential for coupled transport of their substrate neurotransmitter. This active transport is important as 5-HT penetrates poorly through the lipid bilayers of plasma membranes because it is a base and positively charged at a physiologic pH²⁴.

The 5-HTT has important physiological functions and has become a key target of pharmacological agents, including SSRIs³³. In the CNS, the 5-HTT is mostly located at outer cell membranes either presynaptically or along axons³⁴ where it uptakes extracellular 5-HT released during neurotransmission (Figure 1A). This recycles the neurotransmitter for

future use and modulates 5-HT homeostasis. 5-HT homeostasis is clinically important because altered 5-HT transmission is believed to play a key role in major depressive disorder and other affective disorders³⁵⁻³⁷. By antagonizing the 5-HTT, 5-HT activity can be potentiated (Figure 1B) and the symptoms of depression effectively relieved^{38,39}. In the GI tract, the 5-HTT is located in epithelial cells of the intestinal mucosa⁴⁰ where it appears responsible for inactivating the 5-HT used in enterochromaffin cell-to-primary afferent neuron signaling⁴¹. Disruption of this 5-HT reuptake is associated with GI tract side-effects, such as nausea and diarrhea, which have been reported in individuals who take 5-HTT antagonists⁴². In the CV system, the 5-HTT has a critical role in enabling platelets to uptake 5-HT. As platelets lack the rate-limiting enzyme for the biosynthesis of 5-HT, they obtain 5-HT from the extracellular environment. They achieve this using the 5-HTT, which enables virtually all blood-borne 5-HT to be safely stored in platelets and distributed throughout the circulatory system.

Presence of functional 5-HTTs in bone

The most recognized roles of the 5-HTT are in the CNS, GI tract and CV system; however, the 5-HTT has recently been identified in bone raising questions regarding its presence and role in the skeleton. The 5-HTT has been located in the three major bone cell types – osteoblasts, osteocytes and osteoclasts⁴³⁻⁴⁶. 5-HTT mRNA expression has been identified in numerous osteoblastic cell types, including the MC3T3-E1, ROS 17/2.8, UMR 106-H5 osteosarcoma and immortalized clonal Py1a osteoblastic cell lines, and primary cultures of osteoblasts from neonatal rat calvariae^{44,45}. Similar observations have been made in both osteocytic- and osteoclastic-type cells, with 5-HTT mRNA expression being demonstrated in the immortalized murine osteocytic MLOY4 cell line⁴⁴ and osteoclasts differentiated from human peripheral blood mononuclear (hPBM) cells using receptor activator NF- κ B ligand (RANKL)⁴⁶. Likewise, 5-HTT was found to be differentially expressed in murine RAW264.7 macrophage-like cells which were stimulated with RANKL to differentiate into osteoclast-like cells⁴³.

The detection of 5-HTT mRNA in bone cells does not mean that the transporter is actually present as transcript levels are not always directly proportional to protein production. To address this issue investigators have used immunohistochemistry to demonstrate 5-HTT protein expression in the three major bone cell types^{43,44}. Bliziotis et al.⁴⁴ used this approach to demonstrate the *in situ* expression of 5-HTT protein in osteoblasts and osteocytes in whole rat bone sections. While Bliziotis et al.⁴⁴ did not identify similar *in situ* expression in osteoclasts, Battaglino et al.⁴³ demonstrated *in vitro* expression of 5-HTT protein in both RANKL-induced osteoclast-like cells and bone marrow cell-derived osteoclasts. Thus, identification of mRNA transcripts for 5-HTT in bone cells is not an aberrant finding, and the presence of mRNA appears to result in 5-HTT pro-

tein production.

These findings demonstrate the presence of the 5-HTT in bone, but do not prove the functionality of the transporter. It is possible that the 5-HTT in bone cells is vestigial having served a purpose only in embryogenesis. Transcripts for the 5-HTT have been localized in the developing craniofacial mesenchyme of the mouse⁴⁷. There it has been proposed that the 5-HTT influences morphogenesis by transporting the neurotransmitter toward epithelial uptake sites⁴⁸. Supporting this is a suggestion that inhibition of the 5-HTT during morphogenesis contributes to the development of craniofacial deformations⁴⁹, although this has been debated⁵⁰. Given its role in embryonic morphogenesis, the localization of the 5-HTT in bone may be a consequence of an earlier developmental role, with this role being lost and becoming less important with maturation. However, this theory would not explain the presence of the 5-HTT if it maintained its functionality in differentiated bone cells.

Binding and uptake studies have demonstrated the 5-HTT in osteoblastic, osteocytic and osteoclastic cells to be functional and highly specific for 5-HT uptake⁴³⁻⁴⁵. Functional capacity was determined by performing uptake studies with [³H]5-HT⁴³⁻⁴⁵, while antagonist and substrate affinities for the 5-HTT were evaluated using [¹²⁵I]RTI-55 binding to membrane preparations^{44,45}. In each of the three major bone cell types [³H]5-HT was readily taken up and this uptake was inhibited by the addition of 5-HTT antagonists (imipramine and/or fluoxetine). The combined results of these studies indicate that osteoblasts, osteocytes and osteoclasts all possess a functional 5-HTT for the uptake of 5-HT.

Potential sources of 5-HT in bone

For the skeletal 5-HTT to be biologically relevant bone cells need to have access to 5-HT. While the source of skeletal 5-HT has not been rigorously investigated, skeletal 5-HT has the potential to be derived from either indirect or direct sources (Figure 2). Indirect sources may come from distant 5-HT synthesis sites, which require subsequent transport to skeletal sites. More than 90% of the body's 5-HT is synthesized and contained within enterochromaffin cells within the GI tract⁵¹. From here, a large proportion is released into the blood where platelets rapidly bind and store the amine so that little exists freely in the plasma under normal conditions⁵². As platelets store 5-HT in dense granules and release it only following activation²⁷, and any 5-HT that is not taken up by platelets is metabolized by the liver and lungs, the effects of 5-HT derived in the GI tract and circulating in the CV system are effectively localized³⁰. Thus, this 5-HT is unlikely to represent a useful source of 5-HT for bone cells.

5-HT within the serotonergic neurons of the CNS also does not appear a likely source of 5-HT for bone cells. Serotonergic neurons within the CNS are primarily located in the midline raphe nucleus, residing in the brain stem from the midbrain to the medulla⁵³. Serotonergic neurons have yet to be identified in bone. As the blood-brain barrier is imper-

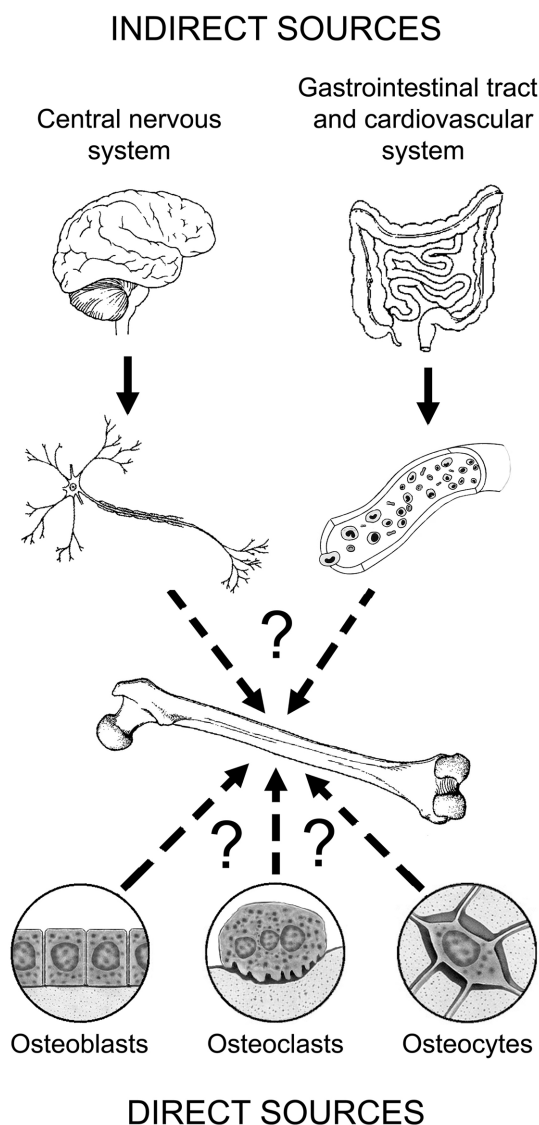


Figure 2. Potential sources of 5-HT in the skeleton. Indirect sources include 5-HT derived from the central nervous system, gastrointestinal tract and cardiovascular system. Direct sources include the possible synthesis of 5-HT by bone cells themselves, in which case skeletal 5-HT effects may be autocrine/paracrine in nature (Reprinted from Warden SJ, et al. Neural regulation of bone and the skeletal effects of serotonin (5-hydroxytryptamine). *Mol Cell Endocrinol* 2005;242:1-9, with permission from Elsevier).

meable to 5-HT, it is unlikely that 5-HT within the CNS can influence bone cells located in the periphery. It is possible that enhancing 5-HT signaling in the CNS could indirectly regulate bone development and its maintenance by affecting serum levels of skeletally relevant hormones; however, this does not explain how 5-HT could reach and directly influence bone cells.

As indirect sources appear unlikely sources of 5-HT to bone cells, the question has been raised regarding the possi-

ble production of 5-HT by bone cells themselves. 5-HT is synthesized from the amino acid tryptophan by a short metabolic pathway consisting of two enzymatic reactions involving tryptophan hydroxylase (TPH) and aromatic amino acid decarboxylase (Figure 3). TPH catalyzes the synthesis of 5-hydroxytryptophan by hydroxylating tryptophan⁵⁴. 5-hydroxytryptophan is subsequently decarboxylated by aromatic amino acid decarboxylase to produce 5-HT. As tryptophan hydroxylation is the first and rate-limiting step in 5-HT biosynthesis, TPH has become a marker for 5-HT synthesis⁵⁵. Osteoblasts (MC3T3-E1 cells), osteocytes (MLO-Y4 cells) and osteoclasts (RAW264.7 and hPBM cells stimulated with RANKL) all express mRNA for TPH1^{44,46}—the peripheral form of TPH⁵⁶. Thus, each of the major bone cell types is potentially capable of synthesizing 5-HT. Confirmation of intracellular synthesis of 5-HT would indicate that any 5-HT effects within the skeleton may be autocrine/paracrine in nature.

Response mechanisms to 5-HT in bone

In addition to having access to 5-HT, bone cells need to possess a means of responding to it in order for 5-HT and the 5-HTT to have physiological effects. 5-HT effects throughout the body are mediated via membrane-bound 5-HT receptors. To date, there are 14 genetically, pharmacologically, and functionally distinct 5-HT receptors belonging to seven families termed 5-HT₁ through 5-HT₇^{57,58}. With the exception of the 5-HT₃ receptor, which is a ligand-gated ion channel, 5-HT receptors belong to the G-protein-coupled receptor superfamily⁵⁷. A number of these receptors have been located in bone cells. For instance, the 5-HT_{1A}, 1D, 2A, 2B and 2C receptors have been identified in osteoblasts^{44-46,59,60}, 5-HT_{1A}, 2A and 2B receptors identified in osteocytes^{44,60}, and 5-HT_{1B}, 2A, 2B, 2C and 4 receptors identified in osteoclasts^{43,46}. The presence of these receptors suggests that bone cells possess a potential means of responding to 5-HT.

In vitro effects of 5-HT and 5-HTT inhibition in bone cells

In vitro studies have been undertaken to confirm the functionality of 5-HT receptors located on bone cell membranes, and to investigate the potential cellular effects of 5-HT and 5-HTT inhibition. However, as there have been few published studies on this topic, the data are currently incomplete, and diffuse in terms of the cell lines and cellular effects investigated.

In terms of the potential effects of 5-HT on bone formation pathways, 5-HT augmented the parathyroid hormone-induced increase in activator protein-1 activity in the UMR 106-H5 osteosarcoma cell line⁴⁵ and increased the release of prostaglandin-E₂ from osteocyte-like (MLO-Y4) cells⁴⁴. Similarly, the addition of 5-HT to cell cultures of avian periosteal fibroblasts (a population containing osteoblast

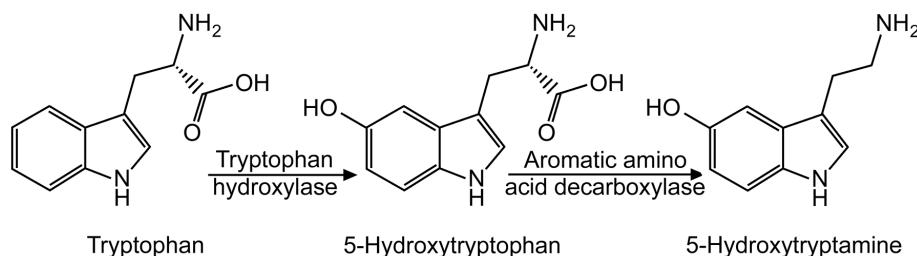


Figure 3. Biosynthesis of 5-HT from tryptophan. Tryptophan hydroxylation is the first and rate-limiting step. The enzyme responsible for this step (tryptophan hydroxylase) has been identified in all of the major bone cell types (osteoblasts, osteocytes and osteoclasts) suggesting that each may be capable of synthesizing 5-HT.

precursor cells), MC3T3-E1 cells and primary human osteoblasts enhanced proliferation^{46,60}. The stimulation of proliferation in MC3T3-E1 cells and primary human osteoblasts was inhibited by the antagonism of the 5-HT_{2A}, _{2B}, and _{2C} receptors, indicating the functionality of these osteoblastic 5-HT receptors. In addition, proliferation in MC3T3-E1 cells and primary human osteoblasts was inhibited by antagonism of protein kinase C (PKC) signaling, indicating the potential involvement of the PKC pathway in skeletal 5-HT signal transduction⁴⁶. 5-HT (0.1-10 μ M) also reduced the release of RANKL from MC3T3-E1 cells and increased the release of osteoprotegerin⁴⁶, suggesting 5-HT reduced osteoblastic signaling for the differentiation and activation of osteoclasts.

Further evidence for the involvement of the 5-HT_{2B} receptor in osteoblastic responses to 5-HT was provided by the finding that this receptor in differentiated osteoblasts from the mesoblastic (C1) cell line is functionally coupled with cyclooxygenase (COX) via the phospholipase-2 mediated release of arachidonic acid⁵⁹. In addition, the 5-HT_{2B} receptor in C1 cells regulated the production of nitric oxide (NO)⁵⁹. As inhibition of either the COX or NO pathways resulted in a decrease in *in vitro* mineralization⁵⁹, these cumulative data suggest a beneficial effect of 5-HT_{2B} receptor activation on bone formation. This was subsequently confirmed in primary osteoblasts harvested from mice with a null mutation in the gene encoding the 5-HT_{2B} receptor, with osteoblasts from these animals demonstrating a marked reduction in *in vitro* proliferation⁶¹.

The combined observations in osteoblastic and osteocytic cells indicate that these cell types possess functional pathways for responding to 5-HT, and that 5-HT may potentially benefit bone formation. However, addition of a 5-HT analogue to mouse-derived osteoblasts inhibited their release of NO when exposed to mechanical stimulation⁶⁰. NO appears to be an important signaling molecule in the osteogenic response to mechanical stimuli⁶², suggesting that 5-HT may actually have detrimental effects on bone formation. Whichever is the case, one thing lacking from the experiments utilizing osteoblasts and osteocytes is investigation of the *in vitro* effects of 5-HTT inhibition. Simple addition of 5-

HT to culture medium is mostly likely not reflective of the role of the 5-HTT, which will modulate 5-HT receptor activation by regulating extracellular 5-HT levels and contribute to the cellular internalization of 5-HT. One study did investigate the effect of 5-HTT inhibition using an SSRI (fluoxetine; 0.001-10 μ M)⁴⁶. The SSRI had a small stimulatory effect on MC3T3-E1 cell proliferation when used at a low concentration (0.01 μ M), but had a large inhibitory effect when introduced at high concentrations (1-10 μ M). This bimodal effect may be explained by the investigation of both clinically therapeutic (0.01 μ M) and supra-therapeutic (1-10 μ M) doses.

In terms of the potential effects of 5-HT and 5-HTT inhibition on bone resorption pathways, cells (RAW264.7 and hPBMC stimulated with RANKL) treated with 5-HT (0.01-50 μ M) or an SSRI (fluoxetine; 0.001-10 μ M) increased their differentiation into osteoclast-like cells and increased their bone-resorption activity⁴⁶. This suggests a stimulatory effect of both 5-HT and 5-HTT inhibition on bone resorption. However, osteoclast differentiation and activity were decreased at the highest concentration of the SSRI (fluoxetine; 10 μ M)⁴⁶. Similarly, an alternative study found high doses of an SSRI (fluoxetine; 1 or 3 μ M) to inhibit osteoclast formation from RAW264.7 and bone marrow derived cells⁴³. This did not result from either the inhibition of proliferation or stimulation of apoptosis, leading the investigators to conclude that 5-HTT inhibition reduced osteoclast differentiation.

***In vivo* effects of 5-HT and 5-HTT inhibition**

Overall, the *in vitro* investigations into 5-HT and 5-HTT effects confirm the presence of functional 5-HT pathways; however, they do not strongly indicate whether these pathways are beneficial or detrimental to bone as the studies provide contrasting findings. Nevertheless, the identification of functional 5-HT pathways in bone cells has stimulated interest into the *in vivo* skeletal effects of 5-HT and 5-HTT inhibition. This interest is considerable given the widespread use of agents that specifically target the 5-HTT for the treatment of affective disorders. Reflecting the heightened interest into

this line of inquiry, the NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis and Therapy⁶³ recommended that there be further research efforts into the relationship between neuropsychiatric disorders, medications for these disorders and bone health.

Current understanding of the *in vivo* effects of 5-HT and inhibition of the 5-HTT is in its infancy. This is evident by the fact that preclinical studies investigating the roles of 5-HT and 5-HTT inhibition in animal models have provided contrasting evidence. Some studies have suggested that 5-HT and 5-HTT inhibition are beneficial to bone^{61,64-66}, whereas others suggest the complete opposite⁶⁷⁻⁷⁰. There are numerous possible explanations for these contrasting findings, as detailed below.

Evidence for *in vivo* beneficial effects of 5-HT and 5-HTT inhibition

Some recent preclinical studies suggest that heightened extracellular 5-HT may be beneficial to bone^{61,64-66}. The most direct evidence for this was provided by Gustafsson et al.⁶⁵ who injected growing (2-month-old) rats for three months with high daily subcutaneous doses of 5-HT (5 mg/kg). 5-HT treatment was found to bring about small skeletal benefits, with treated animals having elevated whole-body areal bone mineral density (aBMD) which purportedly resulted from a reduction in bone area (B.Ar) rather than a change in bone mineral content (BMC). This suggests that exogenous 5-HT reduced growth – a hypothesis partially supported by the observations that 5-HT administration substantially reduced body mass and reduced trabecular bone volume (BV) within the distal femur. However, no skeletal growth measures were provided (such as bone lengths or formation rates), localized (femoral) aBMD did not differ between groups, and the reduced body mass with exogenous 5-HT was likely due to adverse effects within the GI tract and CV system^{65,71}. Similarly, the reduction in trabecular BV with 5-HT was no longer present when values were appropriately normalized to tissue volume (TV). Thus, it is unclear whether prolonged 5-HT administration actually resulted in much of a skeletal effect.

Other direct evidence for a potential beneficial effect of 5-HT on the skeleton was provided by Collet et al.⁶¹. They found mice with a null mutation in the gene encoding for the 5-HT_{2B} receptor to have significantly reduced whole-body and femoral aBMD, and altered trabecular architecture within the proximal tibia. These observations resulted from a reduction in bone formation as opposed to an increase in bone resorption, and suggest that the 5-HT_{2B} receptor and intact 5-HT signaling is important in bone biology. However, these data possess a number of caveats. The skeletal phenotype was present only in female mice and was absent in males. Also, the model involved lifelong global removal of the 5-HT_{2B} receptor in every tissue it was expressed, which included the brain, stomach, intestine, myocardium and pulmonary smooth muscle⁷². Removal of the 5-HT_{2B} receptor in

these tissues may have resulted in indirect negative effects on the skeleton. Suggesting a more global effect of null mutation of the 5-HT_{2B} receptor gene is the fact that one-third of embryonic mutant mice died during mid-gestation and one-third died at birth from cardiac defects. Those mice that did survive beyond birth apparently had a normal lifespan, albeit with a persistent cardiac phenotype⁷³.

Indirect evidence for a potential beneficial effect of 5-HT on bone has been provided by studies investigating the skeletal effects of 5-HTT inhibition. As 5-HTT inhibition causes an increase in extracellular 5-HT, it would be logical to predict that if 5-HT has a beneficial effect then 5-HTT inhibition may replicate this effect. Two studies provide preliminary, yet disputable, evidence for this theory^{64,66}. Battaglini et al.⁶⁴ treated Swiss-Webster mice for six weeks with daily intraperitoneal injections of a SSRI (fluoxetine; 10 mg/kg). They reported no effect of the SSRI on cortical bone parameters, but increased trabecular BV/TV, and concluded that 5-HTT inhibition had an anabolic effect on trabecular bone. However, the data were limited to a single measure (BV/TV assessed using micro-computed tomography [μ CT]) at a single skeletal site (femur) in a single cohort of animals (sham-ovariectomized [OVX]). μ CT is a sensitive skeletal measure; however, it can be influenced by sampling issues, particularly in bones such as the femur where BV changes dramatically at different sites within the bone. To confirm a true anabolic effect of 5-HTT inhibition, μ CT measures should have been coupled with complementary measures, such as histomorphometric measures of bone formation. Additional concerns about this particular study include the facts that a corresponding anabolic effect of 5-HTT inhibition on vertebral BV/TV was not found, 5-HTT benefits were not observed in OVX animals, the published manuscript did not report the number of animals investigated or provide an indication of the variability (standard deviations or equivalent) within the obtained results, and the study used animals within a wide, nondescript age range (8-14 weeks). These limitations suggest that the study's results need to be interpreted cautiously until they are independently confirmed.

The second study providing indirect evidence for a potential beneficial effect of 5-HT on bone was performed by Yirmiya et al.⁶⁶. These investigators used chronic mild stress to create a depressive state in mice. Depression resulted in negative skeletal changes, including decreases in trabecular BV/TV and number (Tb.N) in the lumbar vertebrae and distal femur which resulted from a decrease in bone formation. Supporting a potential beneficial effect of 5-HTT inhibition on bone status, administration of a tricyclic antidepressant (imipramine; 10 mg/kg) ameliorated the negative skeletal effects of the chronic mild stress. This is an interesting finding; however, it provides inconclusive evidence for a beneficial skeletal effect of 5-HTT inhibition. While imipramine is a relatively specific inhibitor of the skeletal 5-HTT^{44,45}, its administration in the study performed by Yirmiya et al.⁶⁶ was associated with attenuation of stress-induced depressive behaviors (including decreased locomotor activity [social

exploration]). This change in the routine loading environment of the skeleton may explain the observed benefit of 5-HTT inhibition in this model.

Evidence for *in vivo* detrimental effects of 5-HT and 5-HTT inhibition

In contrast to the disputable preclinical evidence suggesting that 5-HTT inhibition may be beneficial to bone, there is a growing body of evidence from independent research groups demonstrating that 5-HTT inhibition may actually be detrimental to skeletal health. This work was stimulated by the initial observation that mice with a null mutation in the gene encoding for the 5-HTT possess a consistent skeletal phenotype of reduced mass, altered architecture and inferior mechanical properties⁶⁹. This phenotype apparently resulted from a reduction in bone accrual, as evident by a decrease in bone formation rates without a concomitant change in bone resorption.

There are a number of putative explanations for the skeletal phenotype observed in mice genetically lacking the 5-HTT. Previous reports suggested that 5-HT may influence mechanotransduction^{60,74}, so mice lacking the 5-HTT could have reduced skeletal responsiveness to mechanical loading and, thus, reduced bone mineral accrual. However, no influence of 5-HTT null mutation was found on skeletal mechanosensitivity using an established loading model⁶⁹. An established effect of gene-mediated inhibition of the 5-HTT in mice is heightened anxiety-like behavior, which manifests in a hypoactive locomotor behavioral phenotype⁷⁵⁻⁷⁷. As bone tissue is mechanosensitive, a reduction in cage activity could have contributed to the reduced bone mass observed in weight-bearing bones in the 5-HTT null mutant mice. However, it is unlikely that reduced activity would have any effect on bone in the cranium, yet bone formation and BMC were reduced in the cranial bones of mice lacking the 5-HTT⁶⁹. This suggests that the reduced bone formation in 5-HTT null mice was not the result of reduced physical activity. The function of the 5-HTT in regulating peristalsis within the GI tract may have contributed to the skeletal phenotype. 5-HTT null mice have altered colorectal motility⁷⁸; however, this does appear sufficient to significantly alter their nutritional status. The bowel continues to function in 5-HTT null mice⁷⁸, and these mice gain equivalent weight and have comparable survival to wild-type littermates⁷⁹. Another consideration for the observed phenotypes is the effect of 5-HTT gene null mutation on circulating levels of skeletally relevant hormones and biochemical mediators. 5-HT pathways directly modulate numerous hormonal and biochemical pathways, including those in the hypothalamic-pituitary-adrenal axis^{80,81}. However, 5-HTT null mutant mice did not display significant differences in any of the skeletally-relevant hormones or biochemical markers assessed⁶⁹, which is consistent with previous reports that found no genotype effects on plasma corticosterone or adrenocorticotrophic hormone levels unless the animals were stressed^{82,83}.

A final putative explanation for the observed skeletal phenotype in mice with a null mutation in the 5-HTT gene is that absence of the transporter had negative effects during embryonic skeletogenesis. The 5-HTT has widespread distribution during ontogeny⁴⁷ and it affects the embryonic development of numerous tissues⁸⁴. As eluded to earlier this may include the skeleton, with 5-HTT inhibition during morphogenesis potentially contributing to craniofacial deformations. However, 5-HTT null mice do not show any major post-natal anatomical anomalies⁷⁹ and do not have any differences in long bone length or growth plate height⁶⁹. Similarly, clinical studies into drug-mediated antagonism of the 5-HTT have shown *in utero* exposure does not increase the risk of birth defects or result in poor perinatal condition⁸⁵⁻⁸⁷. These findings suggest that the observed skeletal phenotype in mice with a null mutation in the 5-HTT gene did not result from 5-HTT effects on prenatal bone development or postnatal longitudinal bone growth.

Further evidence for a potential detrimental effect of 5-HTT inhibition has been provided by studies into the skeletal effects of pharmaceutical antagonists of the 5-HTT⁶⁷⁻⁷⁰. These studies found that rodents treated with daily doses of a SSRI (fluoxetine; 5-20 mg/kg) exhibited reduced bone accrual and mechanical properties. This was initially shown in rapidly growing (4-week-old) C57BL/6J mice⁶⁹, leading Battaglini et al.⁶⁴ to suggest that their observed anabolic skeletal effect of 5-HTT inhibition resulted from the use of adult (8-14-week-old) mice with an alternative genetic background (Swiss-Webster). However, 8-week-old mice are not considered skeletally mature⁸⁸, and adult (15-week-old) Swiss-Webster mice treated for four weeks with intraperitoneal injections of a SSRI (fluoxetine; 5 or 20 mg/kg) have recently been shown to have reduced gains in areal and volumetric BMD, and negatively altered trabecular architecture at multiple skeletal sites⁶⁸. These effects were dose-related such that there were increasing negative skeletal effects with increasing SSRI dose. Additional support for a detrimental effect of 5-HTT inhibition on the adult skeleton was provided by Bonnet et al.⁶⁷ who treated 12-week-old C57BL/6J mice for four weeks with subcutaneous injections of a SSRI (fluoxetine; 10 mg/kg). Using the same SSRI and dose as Battaglini et al.⁶⁴, Bonnet et al.⁶⁷ found 5-HTT inhibition to cause structural and mechanical deterioration within the femur.

The observations with drug-mediated inhibition of the 5-HTT extend to those found in mice with lifelong null mutation of the 5-HTT gene by demonstrating that the negative skeletal effects of 5-HTT inhibition are not solely due to influences on *in utero* and early postnatal skeletal development. However, the hypoactive phenotype caveat associated with null mutation of the 5-HTT gene persists with pharmaceutical inhibition of the 5-HTT. Mice treated with SSRIs display reduced physical activity levels^{67,89}. This may explain why the negative skeletal effects of SSRIs have been restricted to weight-bearing bones (femur and lumbar vertebrae) and have not been found at non-weight-bearing sites (cranium)⁶⁹;

Bone cell type	Functional 5-HTT	Functional 5-HT receptors	Potential synthesis of 5-HT*
Osteoblasts	Yes	Yes	Yes
Osteocytes	Yes	Yes	Yes
Osteoclasts	Yes	Yes	Yes
*As suggested by presence of tryptophan hydroxylase.			

Table 1. 5-HT machinery identified the major bone cell types.

however, preliminary evidence suggests that physical inactivity does not completely account for the negative skeletal effects of SSRIs. Bonnet et al.⁶⁷ found physical activity in mice to be significantly reduced with both SSRI (fluoxetine; 10 mg/kg) and tricyclic antidepressant (desipramine; 20 mg/kg) treatment. Although the SSRI-treated animals had significant structural and mechanical deterioration within the femur, animals treated with the tricyclic antidepressant did not. As desipramine is a selective antagonist of the norepinephrine transporter, having 400-fold higher affinity for the norepinephrine transporter than the 5-HTT⁹⁰, the findings of Bonnet et al.⁶⁷ can be interpreted as showing that either 5-HTT inhibition is detrimental to bone or that inhibition of the norepinephrine transporter is beneficial. Suggesting the former, we recently completed a study wherein an SSRI (fluoxetine; 20 mg/kg) caused reduced bone gain and structural deterioration in growing mice, independent of drug effects on animal activity levels. Control of drug effects on activity levels was achieved by introducing the SSRI to both tail-suspended and cage control animals. As there were no significant interactions between the two loading environments, altered loading was not responsible for the skeletal phenotype observed with 5-HTT inhibition (*unpublished data*).

The above preclinical animal studies combined with evidence provided by initial clinical studies (see accompanying paper discussing clinical study findings¹⁹) suggest that 5-HTT inhibition has a negative skeletal effect. However, it remains unclear whether this effect is due to changes in bone formation or resorption, or a combination of both. The presence and functionality of the 5-HTT in both osteoblasts and osteoclasts suggests that 5-HTT inhibition has the potential to influence both bone formation and resorption. This has indeed been the case as bone formation is negatively impaired in growing animals treated with an SSRI (fluoxetine; 5 or 20 mg/kg) or possessing a null mutation in the 5-HTT gene⁶⁹, and both bone formation and resorption are influenced in adult animals treated with an SSRI (fluoxetine; 5 or 20 mg/kg)⁶⁸.

Conclusions and future directions

There is increasing evidence from preclinical *in vitro* and *in vivo* studies that 5-HT and the 5-HTT may have a role within the skeleton. Functional pathways to respond to and

uptake 5-HT have been identified in the three major bone cell types – osteoblasts, osteocytes and osteoclasts (Table 1). Activation or inhibition of these pathways has been found to result in a number of bone cell responses. The majority of these responses *in vitro* have been consistent with an osteogenic or anti-resorptive effect of 5-HT and 5-HTT inhibition, suggesting that drugs that antagonize the 5-HTT may have beneficial effects on the skeleton. However, the *in vitro* effects of 5-HT and 5-HTT inhibition remain disputable as they have been under-explored and are inconsistent with the majority of the *in vivo* evidence.

Some *in vivo* evidence suggests that 5-HT and the inhibition of the 5-HTT is beneficial to the skeleton; however, the majority of the *in vivo* evidence indicates the opposite. The latter evidence includes studies that performed in-depth analyses at multiple skeletal sites in various strains of animals that were treated with more than one drug dose. These studies have found 5-HTT inhibition to produce a consistent skeletal phenotype of reduced bone mass and density, and altered architecture. This phenotype was found to result from changes in both bone formation and resorption.

The *in vivo* studies demonstrating a negative effect of 5-HTT inhibition on bone are consistent with evidence provided by initial clinical studies; however, they possess a number of limitations. The primary limitation is that in each *in vivo* study 5-HTT inhibition was global rather than localized within the skeleton. As the 5-HTT has significant roles within the CNS, GI tract and CV system, it possible that 5-HTT inhibition in these systems resulted in or contributed to the observed skeletal phenotype. To address this and conclusively establish that 5-HTT inhibition has a direct skeletal effect, there is a need to develop techniques that selectively inhibit the 5-HTT in the skeleton without influencing its function in alternative systems. This may be achieved by creating a bone-specific null mutant of the 5-HTT.

In addition to investigating the isolated skeletal effects of the 5-HTT, it is also important to determine how 5-HTT inhibition actually influences bone cells. The 5-HTT is a transporter and as such does not independently influence cellular activity. 5-HT needs to be present locally within the skeleton for the 5-HTT to influence bone cell activity. Thus, the source of 5-HT for bone cells needs to be conclusively elucidated. Demonstration of the presence of 5-HT near bone cells would allow 5-HTT inhibition to potentially

increase extra-cellular and decrease intra-cellular 5-HT levels by limiting bone cell uptake of 5-HT. This may be sufficient to influence the activity of 5-HT receptors on bone cell membranes, thereby producing a cellular response.

Overall, there is mounting evidence for a skeletal effect of 5-HTT inhibition, and several aspects of this effect remain to be investigated and described in detail. Areas of future research include signaling pathways for 5-HTT and 5-HT receptors, sources of 5-HT in bone, and the development of a method for isolating the 5-HTT inhibitory effect to the skeleton.

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