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Review Article

Hylonome

# The role of dopamine and serotonin in regulating bone mass and strength: Studies on dopamine and serotonin transporter null mice

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**Abstract**

Neurotransmitter regulation of bone metabolism has been a subject of increasing interest and investigation. Dopamine (DA) has been reported to have effects on calcium and phosphorus metabolism. The dopamine transporter (DAT) is believed to control the temporal and spatial activity of released DA by rapid uptake of the neurotransmitter into presynaptic terminals. We have evaluated the histologic and biomechanical properties of the skeleton in mice homozygous for deletion of the DA transporter gene (DAT $^{-/-}$) to help delineate the role of DA in bone biology. We have demonstrated that DAT $^{-/-}$ mice have reduced bone mass and strength. DAT $^{-/-}$ animals have shorter femur length and dry weight, and lower ash calcium content. Cancellous bone volume in the DAT $^{-/-}$ proximal tibial metaphysis is significantly decreased with reduced trabecular thickness. DAT $^{-/-}$ vertebrae have lower cancellous bone volume as a consequence of increased trabecular spacing and reduced trabecular number, and cortical thickness and bone area in the femoral diaphysis are reduced. The ultimate bending load (femoral strength) for the DAT $^{-/-}$ mice is 30% lower than the wild-type mice. Thus, deletion of the DAT gene results in deficiencies in skeletal structure and integrity. Since serotonin (5-HT) plays a role as a regulator of craniofacial morphogenesis, we explored the expression and function of 5-HT receptors and the 5-HT transporter (5-HTT) in bone. Primary cultures of rat osteoblasts (rOB) and a variety of clonal osteoblastic cell lines including ROS 17/2.8, UMR 106-H5 and Py1a show mRNA expression for the 5-HTT, and the 5-$HT_{1A}$, 5-$HT_{1D}$, 5-$HT_{2A}$ and 5-$HT_{2B}$ receptors by RT-PCR analysis and immunoblot. A relatively high density of nanomolar affinity 5-HTT binding sites is present in ROS 17/2.8 and UMR 106-H5 cells. The maximal [$^3$H]5-HT uptake rate in ROS cells was 110 pmol/10 min/well, with a $K_m$ value of 1.13 μM. In normal differentiating rOB cultures, 5-HTT functional activity was observed initially at day 25, and activity increased by almost eight-fold at day 31. In mature rOB cultures, the estimated density of [$^{125}$I]RTI-55 binding sites was 600 fmol/mg protein. PMA treatment caused a significant 40% reduction in the maximal uptake rate of [$^3$H]5-HT, an effect prevented by pretreatment with staurosporine. 5-HT potentiates the PTH-induced increase in AP-1 activity in UMR 106-H5 cells. In 5-HTT $^{-/-}$ animals, cancellous bone volume (BV/TV) in the lumbar vertebrae is reduced, with a trend toward decreased trabecular thickness and trabecular number. These results demonstrate that osteoblastic cells express a functional serotonin system, with mechanisms for responding to and regulating uptake of 5-HT, and disruption of the 5-HTT gene may cause osteopenia.

**Keywords:** Dopamine, Serotonin, Neurotransmitter, Bone, Osteoblast

Neurotransmitter regulation of bone metabolism has been a subject of increasing interest and investigation. Collectively, anatomical and *in vitro* studies suggest that bone metabolism may be influenced by the nervous system. For example, bone and periosteum are innervated by both sympathetic and sensory nerves[1-10]. Anatomic studies of nerve terminals innervating bone have revealed the presence of several neuropeptides including calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide, substance P and neuropeptide Y[11]; glutamate-containing terminals have also been described in a dense and intimate network in bone tissue[12]. Fann et al. demonstrated that bone morphogenetic proteins (BMP-2, BMP-6) induce mRNAs for some neuropeptide and neurotransmitter synthetic enzymes *in vitro*[13]. VIP stimulates $PGE_2$ and cAMP production in

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human osteoblast-like cells[14]. These immunohistochemical and biochemical studies of nervous system components in bone may not only reflect sensory and vascular regulatory functions for neurotransmitters, but potentially neurohormonal control of bone cell activities.

Complementary to these findings are reports of the effects of neurotransmitter transporter expression/deletion on bone function. We have recently explored the effects of deletion of the dopamine transporter (DAT) in mice[15]. Dopamine (DA) has been reported to have effects on calcium and phosphorus metabolism. The dopamine transporter (DAT) is believed to control the temporal and spatial activity of released DA by rapid uptake of the neurotransmitter into presynaptic terminals. We have evaluated the histologic and biomechanical properties of the skeleton in mice homozygous for deletion of the DA transporter gene ($DAT^{-/-}$) to help delineate the role of DA in bone biology. We have demonstrated that $DAT^{-/-}$ mice have reduced bone mass and strength. $DAT^{-/-}$ animals have shorter femur length and dry weight. Ash calcium content of the femur is 32% lower in the $DAT^{-/-}$ mice than in the wild-type animals. Cancellous bone volume in the proximal tibial metaphysis is significantly lower in the $DAT^{-/-}$ animals ($p<0.04$). There is a 32% reduction in trabecular thickness ($p=NS$). For the vertebrae, cancellous bone volume was again lower in the $DAT^{-/-}$ animals compared to wild type as a consequence of increased trabecular spacing ($p<0.05$) and reduced trabecular number ($p<0.05$). Cortical thickness and bone area in the femoral diaphysis were reduced in the $DAT^{-/-}$ animals. The ultimate bending load (femoral strength) for the $DAT^{-/-}$ mice is 30% lower than the wild-type mice ($p=0.004$). Thus, deletion of the DAT gene results in deficiencies in skeletal structure and integrity.

DAT is a member of a highly homologous family of neurotransmitter transporters for bioactive amines, which includes the serotonin transporter. These transporters allow intracellular accumulation of neurotransmitters by reuptake from the extracellular fluid through a sodium/chloride dependent cotransport process (for review[16]). Presynaptic transporters that reduce neurotransmitter concentrations in the synapse are a major mechanism for terminating synaptic transmission[17]. It is the augmentation of synaptic activity, by inhibition of sodium-dependent monoamine transport, which forms the basis for the mechanism of action of important antidepressant drugs. Neurotransmitter transporters are also expressed in non-neural tissue, including kidney, liver, muscle and intestine, where they are thought to play a role in cellular signaling, metabolism and organ function (for example[18]). In osteoblast and osteocyte cells, expression and regulation of the excitatory amino acid glutamate/aspartate transporter by mechanical loading has been described[4].

Serotonin (5-HT) has been demonstrated to play a role as a regulator of craniofacial morphogenesis, which may in part be mediated by the 5-HT transporter (5-HTT). In particular, 5-HT has been shown to influence development of craniofacial mesenchyme[19]. The 5-HTT has been localized in developing craniofacial mesenchyme of the mouse[20] where it may influence the morphogenic effects of 5-HT by transporting the neurotransmitter toward epithelial uptake sites. Whole-embryo culture studies have demonstrated that craniofacial malformations may result from 5-HT uptake inhibitors[21], as well as 5-HT agonists and antagonists[22].

Since serotonin (5-HT) plays a role as a regulator of craniofacial morphogenesis, we explored the expression and function of 5-HT receptors and the 5-HT transporter (5-HTT) in bones[23]. Primary cultures of rat osteoblasts (rOB) and a variety of clonal osteoblastic cell lines including ROS 17/2.8, UMR 106-H5 and Py1a show mRNA expression for the 5-HTT, and the $5\text{-}HT_{1A}$, $5\text{-}HT_{1D}$, $5\text{-}HT_{2A}$ and $5\text{-}HT_{2B}$ receptors by RT-PCR analysis. Protein expression of the $5\text{-}HT_{1A}$, $5\text{-}HT_{2A}$ and $5\text{-}HT_{2B}$ receptors was confirmed by immunoblot. 5-HTT binding sites were assessed in ROS 17/2.8 and UMR 106-H5 cells by binding of the stable cocaine analog [$^{125}$I]RTI-55, which showed a relatively high density of nanomolar affinity binding sites. Imipramine and fluoxetine, antagonists with specificity for 5-HTT, show the highest potency to antagonize [$^{125}$I]RTI-55 binding in ROS and UMR cells. GBR-12935, a relatively selective dopamine transporter antagonist, had a much lower potency, as did desipramine, a selective norepinephrine transporter antagonist. The maximal [$^{3}$H]5-HT uptake rate in ROS cells was 110 pmol/10 min/well, with a $K_m$ value of 1.13 nM. Imipramine and fluoxetine inhibited specific [$^{3}$H]5-HT uptake with $IC_{50}$ values in the nanomolar range. In normal differentiating rOB cultures, 5-HTT functional activity was observed initially at day 25, and activity increased by almost eight-fold at day 31. In mature rOB cultures, the estimated density of [$^{125}$I]RTI-55 binding sites was 600 fmol/mg protein. Functional down regulation of transporter activity was assessed after PMA treatment, which caused a significant 40% reduction in the maximal uptake rate of [$^{3}$H]5-HT, an effect which was prevented by pretreatment with staurosporine. The affinity of 5-HT for the transporter was significantly increased following PMA treatment. We assessed the functional significance of expression of the 5-HT receptors by investigating the interaction between 5-HT and parathyroid hormone signaling. 5-HT potentiates the PTH-induced increase in AP-1 activity in UMR cells. Finally, in $5HTT^{-/-}$ animals, cancellous bone volume (BV/TV) in the lumbar vertebrae is reduced, with a trend toward decreased trabecular thickness and trabecular number. These results demonstrate that osteoblastic cells express a functional serotonin system, with mechanisms for responding to and regulating uptake of 5-HT.

We have demonstrated that the 5-HTT and multiple 5-HT receptors are expressed in a variety of osteoblastic cells. This is the first report of expression of 5-HT receptors and the 5-HTT in bone. Serotonin thus joins other neurotransmitters which have been described as having receptors and/or transporters expressed in osseous tissue[4,24]. Neurotransmitter expression in nerve terminals penetrating densely into bone

has been described[11,12]. Therefore, locally produced neurotransmitters may act as signaling molecules and have direct effects on bone cells. Combined, these results demonstrate that osteoblastic cells express both a mechanism for responding to and regulating uptake of 5-HT, and thus represents a functional serotonin system in bone.

Previously, 5-HT has been demonstrated to play a role as a regulator of craniofacial morphogenesis. Early craniofacial morphogenesis involves intercellular interactions which regulate neural crest cell migration[25], mesenchymal growth[26], and differentiation[27]. It has been suggested that 5-HT may regulate migration of cranial neural crest cells and their mesenchymal derivatives in the mouse embryo[28]. Furthermore, the 5-HTT has been localized in developing craniofacial mesenchyme of the mouse[20] where it may mediate the morphogenic effects of 5-HT by transporting the neurotransmitter toward epithelial uptake sites. Shuey et al. have found that inhibition of 5-HT uptake into craniofacial epithelia interferes with serotonergic regulation of epithelial-mesenchymal interactions important for normal craniofacial morphogenesis[21]. 5-HT has also been shown to directly influence craniofacial mesenchyme[19]. In mandibular mesenchyme cells, 5-HT has been found to promote expression of cartilage core protein by activation of $5\text{-HT}_3$ or $5\text{-HT}_{1A}$ receptors, to inhibit production of tenascin (an extracellular matrix molecule produced by perichondrial and periosteal cells[29], and to either promote or inhibit synthesis of the calcium binding protein S-100[30]. These studies suggest that 5-HT may have profound effects on craniofacial development at a variety of levels.

5-HT receptors are linked to a variety of signal transduction pathways including multiple G-protein associated effector systems, phospholipase C activation and $Ca^{2+}$ release (for review[31]). We do not yet know which effector system(s) are important for mediating serotonin effects in osteoblasts. Also, we have not characterized potential expression in osteoclastic cells.

We noted some heterogeneity in the size of proteins recognized by our antibodies raised against the $5\text{-HT}_{2B}$ receptor. Specifically, the osteoblastic osteosarcoma cell lines ROS 17/2.8 and UMR 106-H5 express an immunoreactive protein of ~80 kDa which is not seen in rat hippocampus or primary rat calvarial osteoblasts. Isoforms of other 5-HT receptors have been described which arise due to either alternative splicing or differences in glycosylation. For example, $5\text{-HT}_3$ receptor splice variants have been reported in the rat, and the expression of these isoforms is regulated in the central nervous system during development[32]. A splice variant of the $5\text{-HT}_4$ receptor has been described which displays some differential responsivity to antagonists in comparison to other $5\text{-HT}_4$ isoforms[33]. Both the $5\text{-HT}_{1A}$[34] and the $5\text{-HT}_{2C}$ receptors[35] have isoforms which arise from different glycosylation patterns. We are not aware of any evidence for alternative splicing or differential glycosylation of $5\text{-HT}_{2B}$ receptors, but it is an intriguing possibility to explain our findings. Future work in our lab will explore this issue.

We also describe expression of 5-HTT in all osteoblastic cells examined. In the differentiating primary cultures of fetal rat calvarial osteoblasts (rOB), the expression of 5-HTT mRNA was observed in confluent cultures. However, functional 5-HTT activity was not detected until relatively late in culture (day 25), a time at which the cells are secreting osteocalcin and undergoing mineralization[36]. This result suggests that transporter uptake of 5-HT may influence events late in the differentiation paradigm of the osteoblast. We also show that 5-HTT activity is down regulated by treatment with PMA, an activator of the protein kinase C system. This post-translational modulation of 5-HTT function via activation of second messenger pathways may be a mechanism for short-term functional regulation or plasticity of the serotonin uptake system in osteoblasts.

We have also begun to explore the functional correlates of 5-HT receptor and 5-HTT expression in osteoblastic cells. 5-HT regulates immediate early gene responses in a number of cell types and tissues. We have demonstrated that a six hour incubation with 5-HT potentiates the PTH-induced increase in AP-1 activity in osteoblastic cells. This has physiologic relevance in that PTH induces collagenase production in osteoblastic cells through a mechanism which involves an AP-1 consensus-binding sequence[37]. Interestingly, the stimulation correlates with CREB binding activity and implicates phosphorylation of CREB as a possible mechanism for transcriptional activation of interstitial collagenase. This suggests a testable hypothesis for the mechanism whereby serotonin potentiates the PTH effect on AP-1 activity, and potentially on regulation of collagenase expression.

Expression in bone cells of another neurotransmitter transporter was recently described[4]. Messenger RNA and protein for the excitatory amino acid glutamate/aspartate transporter was found in osteoblasts and osteocytes. Regulation of the glutamate transporter protein was demonstrated by mechanical loading: mechanical loading of rat ulna produced a down regulation of glutamate transporter in cortical bone, while up regulation of the protein occurred on the periosteal surface. This data suggests that the glutamate transporter may be involved in coupling mechanical loading to skeletal modeling; thus the authors proposed that regulation of glutamate transport may be an early response of osteocytes to mechanical loading of bone[4]. Furthermore, mRNA expression for a range of glutamate receptor subtypes in various osteoblast cell lines and primary cultures and in osteoclasts[1,38] has been described. In addition, functional glutamate receptors have been demonstrated in mammalian osteoclasts[24], and specific N-methyl-d-aspartate (NMDA) antagonists have been shown to prevent formation of the osteoclast sealing zone required for bone resorption[39]. It has also been shown that glutamate-containing fibers, among others, are present as a dense and intimate network in bone tissue[12]. Together, these

studies suggest a functional system for glutamate in bone.

We have demonstrated that disruption of the dopamine transporter (DAT) gene in mice results in deficiencies in skeletal structure and integrity by mechanisms yet to be defined (see above)[15]. These studies, combined with the results presented here, suggest that neurotransmitters, through their respective transporters and receptors, may play a significant but underappreciated role as signaling molecules that modulate skeletal health. Further, they suggest that one level of neurotransmitter action may be through direct or indirect effects on differentiating osteoblasts/osteocytes. Our findings regarding expression of 5-HT receptors and the 5-HTT in immortalized and transformed osteoblastic cells have been confirmed in normal differentiating rat osteoblasts. We do not know the functional correlates of our findings *in vivo*, however. Future work in our laboratory will be directed to investigate the biological role of serotonin in the regulation of osteoblast differentiation.

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