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*Review*

# Glutamatergic System in Bone Physiology

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**Abstract.** *Bone is a highly innervated tissue consisting of nerve fibers, which contain many neurotransmitters including glutamate. Recently, a number of studies have identified functional glutamate receptors in osteoblasts and osteoclasts, implying that the glutamatergic system has an apparent regulatory role in bone physiology. This review outlines the evidence which suggest that the glutamatergic system regulates bone physiology.*

Glutamate is the major excitatory neurotransmitter found in abundance in nature. However, since the blood-brain barrier has a very low permeability to glutamate, the brain must synthesize it *de novo*. In glial cells, glutamate taken up from the extracellular fluid may be converted to glutamine, by glutamine synthetase, and then it is released into the extracellular space. Glutamine, in its turn, can be taken up by neurons, converted to glutamate, packaged into synaptic vesicles and used as a neurotransmitter (Figure 1). Notably, glutamine of the extracellular space can function as a carrier of excess ammonium across the blood-brain barrier (1-5).

In the CNS, glutamate is important for synaptic plasticity and long-term potentiation (LTP). These are mechanisms by which transient or brief signalling events can potentiate subsequent trains of depolarizations. At a molecular level, the initiation of LTP in neurons is dependent on an increase in intracellular calcium ($Ca^{++}$), which is caused by the activation of a postsynaptic type of glutamate receptors (Glu.Rs) on depolarized cells (6, 7). A high concentration of glutamate, acting on a specific category of Glu.Rs, can induce an influx of cations and a collapse of mitochondrial function that leads to necrosis, so called excitotoxicity. This neurotoxic action has recently been linked with the pathophysiology of hypoxic injury, stroke and epilepsy.

Glutamate, in response to a presynaptic depolarization event, is packaged into vesicles and is released into the synaptic cleft. This extracellular glutamate remains contained within the synaptic cleft and acts on a variety of glutamate receptors presented on the postsynaptic cell. Simultaneously presynaptic transporters, located on the plasma membrane of both glial cells and neurons, are responsible for the reuptake of the glutamate from the cleft, enabling its recycling, thus provoking the cessation of the signalling episode (8, 9). Glutamate transporters acting into the synaptic cleft have been also implicated in a wide range of diseases, such as Alzheimer, Parkinsons, glaucoma and inflammation, where the glutamate concentration is apparently disrupted (10, 11).

The Glu.Rs can be divided into 2 categories: the ionotropic (iGlu.Rs) and the metabotropic (mGlu.Rs) receptors (12, 13) (Table I). The iGlu.Rs are directly gated ion channels having only excitatory action (12), whereas the mGlu.Rs are transmembrane receptors that are coupled with G-protein, activating intracellular signal transduction pathways (14, 15).

The iGlu.Rs are subdivided into 3 groups, NMDA, AMPA and Kainate receptors, based on their pharmacology and structural similarities. They are named according to the types of synthetic agonist that activates them (N-methyl-D-aspartate, a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid and kainate). These receptors are multimeric assemblies of 4 or 5 subunits (Figure 2). Each subunit possesses 3 transmembrane domains (TMI, TMIII and TMIV) and one region (TMII) that forms a re-entrant loop, giving this receptor subunit an extracellular N-terminus and intracellular C-terminus domain. The long loop between TMIII and TMIV is exposed to the cell surface and forms part of the binding domain (12, 16-19).

NMDA receptor (NMDA.R) is a hetero-oligomeric protein. It is composed of 2 classes of subunits, 2 NR1

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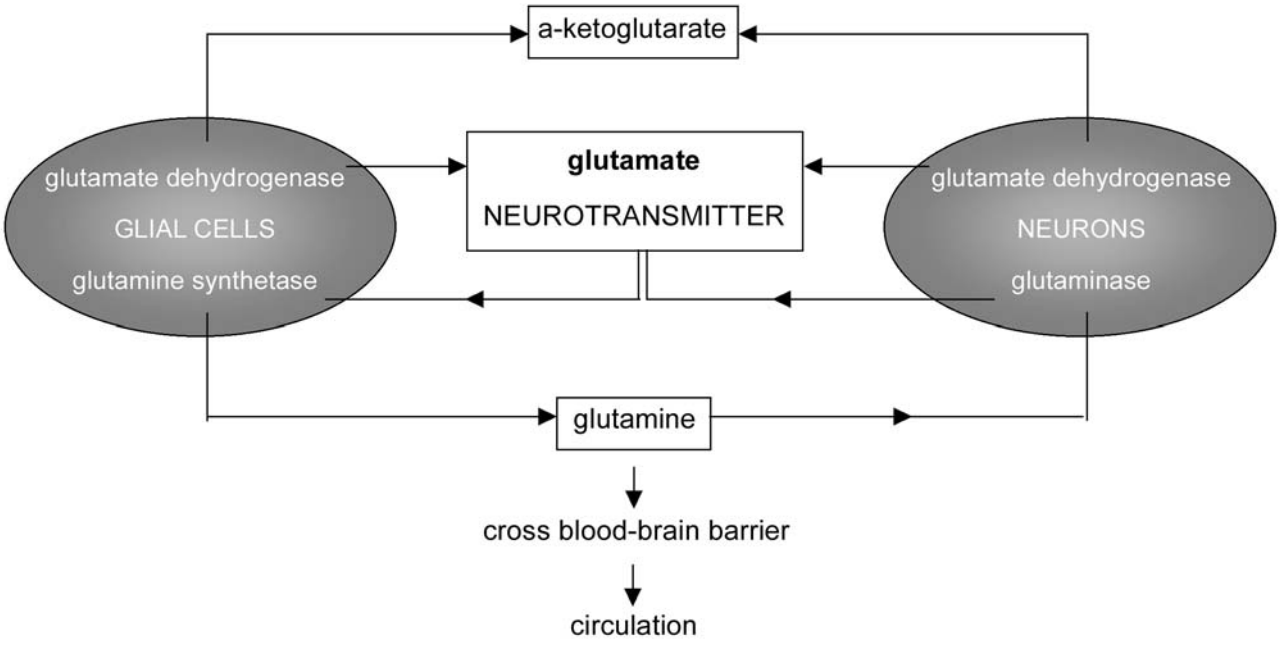


Figure 1. *In neurons and glial cells, α-ketoglutarate is converted to glutamate by glutamate dehydrogenase (reverse reaction). Glutamate released from a neuron is taken up by glial cells. There glutamate may be converted to α-ketoglutarate by glutamate dehydrogenase, or it may be amidated to glutamine by glutamine synthetase. Glutamine, in its turn, is taken up by neurons. Neurons convert glutamine back to glutamate by glutaminase.*

subunits and 2-3 NR2 subunits, that coassemble to form a tetramer (20) or a pentamer (21). The expression of both subunits is required to form functional channels. There are 4 separate genes producing 4 NR2 subunits (NR2A-D) (17, 18, 22-24). The NR1 subunit exists as multiple splice variants, produced by differential splicing of the mRNA derived from a single gene (17). There is a third subunit, NR3, which is a regulatory subunit that decreases the NMDA channel activity. NR3 exists as NR3A and NR3B, products from different genes (25). NMDA.R requires a co-agonist, glycine. The glycine binding site is found on the NR1 subunit. The NR2B subunit also possesses a binding site for polyamines. The glutamate binding domain is formed at the junction of the NR1 and NR2 subunits. At resting membrane potentials, NMDA.Rs are inactive. This is due to a voltage-dependent block of the channel pore by magnesium ions, preventing ion flows through. When the membrane is depolarized, $Mg^{2+}$ is expelled from the channel, allowing $Na^{+}$ and $Ca^{2+}$ to enter. Thus both glutamate and depolarization are needed to open the channel (7, 17). The NMDA.Rs are effectively blocked by APV (2-amino-5-phosphonovaleric acid), PCP (phencyclidine) and MK801 (dizocilpine) (12).

The AMPA and Kainate receptors are often referred to together as the non-NMDA receptors. The AMPA receptors are composed of subunits Glu.R1-4, which are products of separate genes. All AMPA subunits exist as 2 splice variants (flip and flop). The alternative splice cassette is found at the C-terminal end of the loop between TMIII and TMIV. Native AMPA.R channels are impermeable to $Ca^{2+}$, a function controlled by the Glu.R2 subunit. The calcium permeability is determined by the post-transcriptional editing of the Glu.R2 mRNA, which changes a single amino acid in the TMII region from glutamine (Q) to arginine (R). Glu.R2(Q) is calcium permeable while Glu.R2(R) is not. The Kainate receptors are composed from multimeric assemblies of Glu.R5-7 and KA-1/2 subunits. They also undergo both splice variant and RNA editing. They are impermeable to $Ca^{2+}$. The drug CNQX (6-cyano-7-nitroquinoxalone-2,3-dione) blocks both the AMPA.R and Kainate receptor (26, 27).

The mGlu.Rs are G-protein coupled receptors that have been subdivided into 3 groups, group I, II and III, based on sequence similarity, pharmacology and intracellular signalling mechanisms. mGlu.Rs possess a 7-transmembrane domain with an extracellular N-terminus and intracellular C-terminus domain. Group I mGlu.Rs (mGlu.R1, mGlu.R5) are coupled to PLC, while group II (mGlu.R2, mGlu.R3) and III (mGlu.R4, mGlu.R6, mGlu.R7, mGlu.R8) are negatively coupled to adenyl cyclase. These 8 mGlu.Rs are products from different genes. The mGlu.Rs of Group III have 67-70% protein sequence similarity and 42-45% similarity with the mGlu.Rs of Group I and II. Alternative splice variants are also found for each mGlu.R. The mGlu.Rs can be

Table I. *Glutamate Receptors (Glu.Rs).*

| ionotropic Glutamate Receptors (iGlu.Rs) | | metabotropic Glutamate Receptors (mGlu.Rs) | | |
|---|---|---|---|---|
| directly gated ion channels<br>excitatory action<br>multimeric assemblies of 4-5 subunits<br>each subunit possesses 4 TMs domains<br>3 groups: NMDA, AMPA, Kainate receptors | | G-protein coupled receptors<br>excitatory or inhibitory action<br>transmembrane receptors<br>possess a 7 TM domain<br>3 groups: group I, II, III | | |
| NMDA Receptor | non-NMDA Receptors (AMPA, Kainate) | group I | group II | group III |
| flow of ions: $Ca^{2+}$, $Na^{+}$, $K^{+}$ | impermeable to $Ca^{2+}$ | mGlu.R1 | mGlu.R2 | mGlu.R4 |
| tetramer or pentamer | specific antagonists: CNQX, NBQX, DNQX | mGlu.R5 | mGlu.R3 | mGlu.R6 |
| (2x) NR1, (2-3x) NR2, (1x) NR3 subunits | | | | mGlu.R7 |
| 1 gene – 1 NR1 subunit: splice variants | | | | mGlu.R8 |
| 4 genes – 4 NR2 subunits (NR2A, B, C, D) | AMPA Receptors: | coupled to PLC | negatively coupled to cAMP | |
| 2 genes – 2 NR3 (NR3A, B): regulatory subunit | 4 genes: Glu.R1-4 subunits | | | |
| co-agonist: glycine (NR1 subunit) | 2 splice variants: flip/flop | | | |
| : polyamines (NR2B subunit) | permeable/impermeable to $Ca^{2+}$ | | | |
| voltage sensitive: $Mg^{2+}$ | Kainate Receptors: | 8 genes – 8 mGlu.Rs | | |
| Glu binding domain: at the junction of NR1-NR2 | Glu.R5-7, KA-1/2 subunits | splice variants | | |
| specific antagonists: MK801, APV, PCP | splice variants | | | |

selectively activated by ACPD (trans-(1S,3R)-1-amino-1, 3-cyclopentanedicarboxylic acid) (14, 15, 28) (Figure 3).

A number of proteins have been found to associate with iGlu.Rs (calmodulin, CaMKII, PKA, PKC and Yotio) and some cluster to specific iGlu.R family members. This clustering acts to co-localize iGlu.R with specific intracellular signalling molecules, thus increasing the efficiency of transducing the signal and also explaining how a single episode of glutamate signalling triggers numerous different intracellular signalling cascades (8, 29-31).

One postsynaptic protein important for the clustering of Glu.Rs is PSD-95 (postsynaptic density protein of 95 kDa). PSD-95 is a cytoplasmic protein that contains 3 repeated domains, important for protein-protein interaction. These so-called PDZ domains bind a number of cellular proteins. In PSD-95, the PDZ domains interact with the C-terminal of the NR2 subunit of NMDA.R and the shaker type of voltage-gated $K^{+}$ channel, thereby localizing and concentrating these proteins together at postsynaptic sites (32). Also PSD-95 localized intracellular effector proteins that act downstream of the NMDA.R. One such protein is nNOs, which catalyzes the production of NO downstream of $Ca^{2+}$ influx through NMDA.R (33). The C-terminus region of the NR1 is anchored to the actin cytoskeleton by its direct interaction with a-actin 2 (34). AMPA.Rs interact with a distinct PDZ domain protein called GRIP (31) and mGlu.Rs interact with another PDZ domain protein called HOMER (35). Activation of Group I mGlu.R causes activation of the protein tyrosine kinase Src, by the PKC pathway. Src is responsible for the tyrosine phosphorylation of NMDA.R (2A and 2B), thus Src up-regulates the activity of NMDA.R (36).

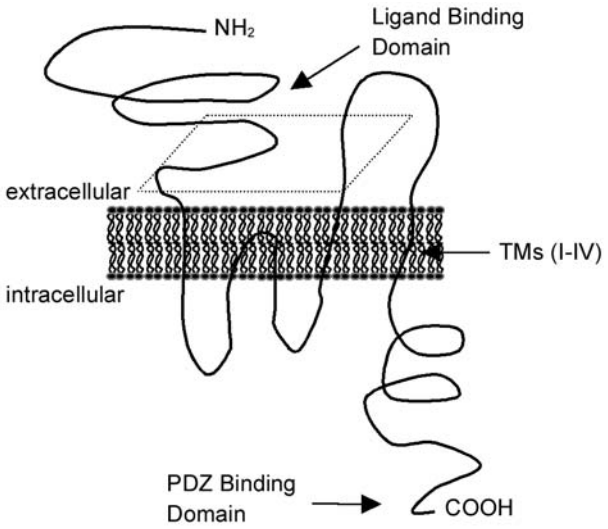


Figure 2. *Ionotropic Glutamate Receptor (iGlu.R). The subunit of iGlu.R possesses four transmembrane domains (TMI-IV), with an extracellular N-terminus and intracellular C-terminus domain. The TMII forms a re-entrant loop. The loop between TMIII and TMIV is exposed to the cell surface and forms part of the binding domain. The PDZ domain interacts with the C-terminal of the NR2 subunit of NMDA receptor.*

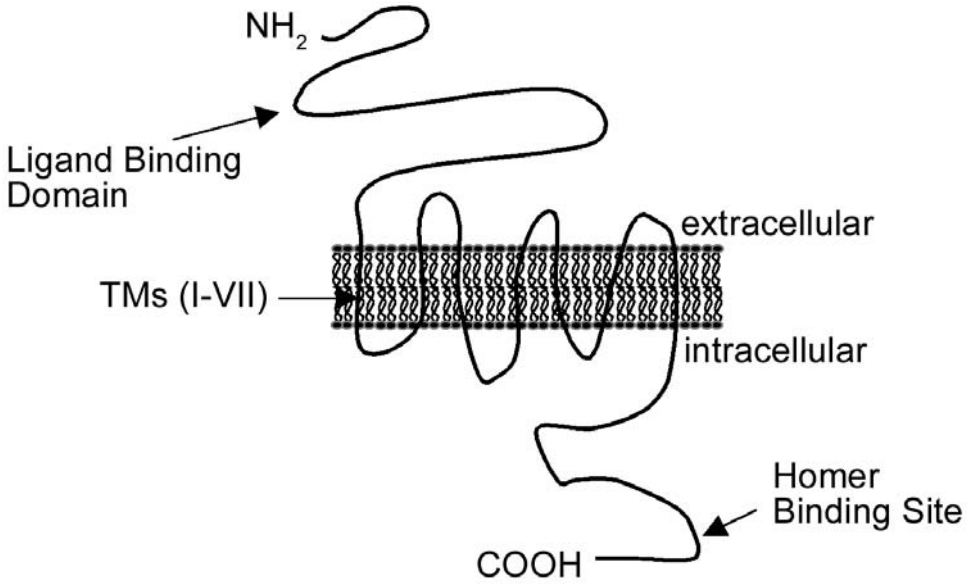


Figure 3. *Metabotropic Glutamate Receptor (mGlu.R). The mGlu.Rs possess seven transmembrane domains (TMI-VII) with an extracellular N-terminus and intracellular C-terminus. The ligand binding domain is localized near the N-terminus. The PDZ domain protein (HOMER) interacts with the C-terminal of mGluRs.*

In addition to the Glu.Rs there is the glutamate transporter family, which has been functionally characterized and classified into 3 groups based on their substrate specificity (37). The first ($C_4$-dicarboxylate transporter) is found in bacteria, while the glutamate/aspartate transporters and the neutral amino acid transporters are found in bacteria and eukaryocytes. Sequence analysis of the members of this family suggests a topology common to all the members of this family of polytopic membrane proteins, although the similarity of their amino acid sequences ranges only between 25 and 50% (38).

The bacterial $C_4$-dicarboxylate carriers transport the tricarboxylic acid cycle intermediates succinate, fumarate and malate (39, 40). The high affinity substrates for the mammalian neutral amino acid transporters are alanine, serine, cysteine and threonine. However, some members (ACST2 from mice, humans and rabbits) show a broader substrate specificity and accept glutamine and asparagine with high affinity and several other amino acids, including glutamate with lower affinity (41-44). All glutamate transporters use L-glutamate and L-aspartate as high-affinity substrates (45-47).

The concentration of the neurotransmitter L-glutamate in the excitatory synapses of the human CNS is regulated by $Na^+$-coupled L-glutamate/L-aspartate transporters, which form the EAAT (excitatory amino acid transporters) family. Five different transporters of this family have been identified and cloned to date: EAAT1 (human) /GluT-1/SLC1A3 (human) /GLAST-1 (rat) (45, 48), EAAT2 (human) /SLC1A2 (human) /GLT-1 (rat) (46), EAAT3 (human) /SLC1A1 (human) /EAAC-1 (rabbit) (47), EAAT4 (human) (49), EAAT5 (human) (50). These 5 transporters, products from different genes, share 50-60% amino acid sequence identity with each other and show some homology with the neutral amino acid transporters ASCT1 and ASCT2 and bacterial glutamate transporters (37). The 5 EAATs are expressed by different cell types and are susceptible to different glutamate uptake inhibitors. They are located on the plasma membrane of glial cells and neurons (51). Studies confirmed the presence of six membrane-spanning segments in the N-terminal half of these transporters (45-47). It was suggested that segment 7 may form a re-entrant loop with both ends positioned at the extracellular side of the membrane (52).

## The role of the glutamatergic system in bone cells

Apart from the CNS, functional glutamate receptors have been identified in the adrenal medulla, lung, pancreatic islet β cells, megakaryocytes, guinea-pig ileum, keratinocytes, heart and bone (53-58). However, since the majority of the commercially available glutamate receptor antagonists are specifically designed to readily cross the blood-brain barrier and modulate neurological function, the role of glutamatergic signalling in non-neuronal tissues can be investigated only *in vitro*. Moreover, because glutamate is a

prerequisite for normal cell growth (thus it is impossible to culture cells in the absence of glutamate), the *in vitro* studies can use only non-competitive antagonists (8, 59).

Nevertheless, the first description of glutamatergic signalling in bone was reported by Mason *et al.* (1997), who demonstrated the expression of GLAST mRNA in active cuboidal osteoblasts and osteocytes, that was regulated by mechanical loading of rat bone, *in vivo* (57). Bone cells are regulated by circulating hormones and by a number of local humoral factors, including prostaglandins, growth factors, cytokines and nitric oxide (60-66). Therefore, it was suggested that glutamate may be either a paracrine and/or autocrine regulator in bone physiology.

The origin of glutamate in bone is still unknown. Recently, it has been demonstrated that bone cells have the machinery required for glutamate signalling and numerous pre- and post-synaptic components of neuronal glutamate signalling have been identified in bone cells (67-71). NMDA.Rs were the most studied in mature osteoblasts and osteoclasts. A number of electrophysiological studies showed that osteoblastic NMDA.Rs display very similar functional characteristics to those seen in neuronal systems (72, 73). Mammalian (rabbit) osteoclasts express NR1, NR2B and NR2D subunits (9, 74). Rat calvaria and MG-63 osteoblast-like cells also expressed several NR2 subunits, namely NR2A/B/D (74). Primary and clonal osteoblasts contain glutamate-filled vesicles similar to those seen in glutamatergic neurons (71). The NMDA.R clustering protein PSD-95 mRNA was demonstrated in bone marrow cells, osteoblastic cell lines and mammalian osteoclasts (74, 75). The presence of AMPA and Kainate receptors have been less studied, although they were also present in bone cells. In addition, mGlu.R1b (group I) was identified in rat femoral osteoblasts. Hinoi *et al.* demonstrated the expression of group III mGlu.R (mGlu.R4 and mGlu.R8) in primary osteoblasts isolated from rat calvaria (72, 76). In addition, 2 glutamate transporters have been identified in bones. GLAST-1 mRNA was localized in active cuboidal osteoblasts as well as in osteocytes, while GLT-1 was expressed in bone marrow cells. Recently, a splice variant of GLAST-1 (GLAST-1a), lacking exon 3, was identified in bone (57, 77).

The role of the glutamatergic system in osteoblasts has been studied *in vitro*. An NMDA antagonist inhibits cell differentiation of osteoblasts. In addition, the MK801 antagonist of NMDA.R causes a dose-dependent inhibition of bone formation, *in vitro* (78, 79). Apparently, there is a cross-talk between the different Glu.Rs types, because mGlu.Rs act to regulate the activity of iGlu.Rs (28). This was confirmed in studies where mGlu.R1b receptor negatively regulated the activity of the rat femoral osteoblastic NMDA.R (72). Recent findings also suggest that the NMDA.R2C subunit may be required for the inhibitory modulation of NMDA.R (80). This inhibitory pathway acts *via* DAG and PKC activation. PTH also uses this pathway (81, 82). Notably, it was found that, after application of NMDA, the $[Ca^{2+}]$ i response was allowed to develop. Therein 100 nM PTH produced an effect similar to that of ACPD (mGlu.R agonist), causing $[Ca^{2+}]$ i to fall rapidly to a point approximately midway between the basal and NMDA-enhanced levels (72). This was suggestive of potential interactions among Glu.Rs and PTH regulation in bone cells.

Furthermore, sustained exposure to MK801 inhibited the expression of (at both the mRNA and protein level) bone differentiation CBFA1 transcription factor. CBFA1 is a transcription factor essential for the growth of osteoblasts. CBFA1 is shown to regulate the expression of several genes such as collagenase-3, type (1) collagen, osteoprotegerin, bone sialoprotein, osteopontin, alkaline phosphatase and osteocalcin. Therefore, the findings that MK801 inhibited alkaline phosphatase and osteocalcin expression argue in favor of the idea that MK801 can suppress the expression of particular genes, which are essential for differentiation and maturation of osteoblasts, *via* modulation of CBFA1 DNA binding (83).

In addition, NMDA.Rs are expressed throughout the differentiation of osteoclasts and antagonists of NMDA.Rs acted to modulate both osteoclastogenesis and osteoclast activity (84). MK801 reduced the number of resorption pits on cortical bone slices without affecting the osteoclast attachment onto bone and osteoclast survival. In addition, it rapidly decreased the percentage of osteoclasts with actin ring structures, a characteristic associated with actively resorbing osteoclasts (84, 85). Moreover, studies using the co-culture system of maturing osteoclastic precursors by simultaneously culturing them with pre-osteoblasts have shown that non-competitive NMDA.R antagonists inhibited osteoclastogenesis, *in vitro*. The effects of NMDA.R antagonists may be due to modulating the osteoblastic activity/phenotype of pre-osteoblast present within these cultures, which would have the secondary effect of inhibiting osteoclastogenesis (8, 59). Furthermore, the protein tyrosine kinase Src was shown to increase the tyrosine phosphorylation of NMDA.R (2A and 2B), thus Src probably up-regulates the activity of NMDA.R. Intrestingly, because Src is highly expressed in osteoclasts, it has been postulated that the control of the Src protein in osteoclasts may provide a new therapeutic approach to prevent osteoporosis (86, 87).

## Conclusion

A number of studies have identified a possible role for the glutamatergic system in bone physiology, mainly because glutamate receptors have been identified in bone cells (osteoblasts and osteoclasts). However, the origin of glutamate in bone is still ambiguous. The fact that bone is

highly innervated and that skeletal nerve fibers contain many neuromediators implies that there is a neuronal control of bone formation and bone remodeling. Further work is required to characterize cell lines of osteoblast-like and osteoclast phenotype, to determine the molecular mechanisms of glutamatergic action on bone cells, *via* iGlu.Rs and/or mGlu.Rs activated intracellular signal transduction pathways. The latter could possibly provide the basis of novel therapeutic approaches to treat bone diseases.

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