

The role of glutamate in the regulation of bone mass and architecture

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Abstract

Communication between the cells in bone underlies the way that the tissue functions physiologically, and in nearly all pathologies, the pathogenesis of skeletal diseases. The number of molecules involved in intercellular signalling in bone grows constantly and it is perhaps unsurprising that the list includes many with functions in other tissues. In recent years, evidence has accumulated to show that molecules involved in neurotransmission have paracrine roles in the skeleton. The focus of this review is the excitatory amino acid glutamate and its role in regulating bone formation and resorption. Specifically, this article will concentrate on the functional role of the system, and the reasons why mechanisms like synaptic transmission are relevant to what might appear to be a slow responding tissue, as the sites of expression of glutamate signalling components in bone have been reviewed already. While there is strong evidence for a regulatory role for glutamate in osteoblast and osteoclast differentiation and function *in vitro*, *in vivo* data is less advanced. Preliminary data from *in vivo* systems does however suggest that glutamate has a physiological function in the skeleton.

Keywords: Glutamate, Bone, Memory, Exercise

Introduction

The extracellular matrix of bone is the component that performs the function of the skeleton to provide a rigid and relatively incompressible material for structural purposes. Those purposes include protection of vital soft tissues and provision of rigid levers to allow muscle contraction the ability to move the body. Even the non-structural roles of bone in mineral homeostasis and support of the bone marrow environment are performed by the extracellular matrix. However, that is not to imply that cells are unimportant once bone has formed. Modelling, remodelling and repair all occur during life, as a result of the orchestrated activity of populations of cells. The major players in those processes are the osteoblasts and osteoclasts, but their precursors

clearly affect the presence of mature functional bone forming and bone resorbing cells¹. In addition, other cells in the marrow and bone environment such as megakaryocytes² and osteocytes³ have the ability to influence osteoblasts and osteoclasts, as do numerous more remote cells responsible for endocrine and neuronal control of the skeleton endocrine through growth and during ageing.

Within the bone microenvironment though, a more restricted range of paracrine signalling molecules regulate bone formation and resorption, and the number of these increases regularly as a result of research. Relatively recent key players in bone formation and resorption include the Wnt pathway members⁴ and the RANK ligand and receptor family⁵. In both those cases, local levels of ligands provide a microenvironmental milieu that influences small populations of osteoblasts or the recruitment of osteoclast precursors to hierarchically distinct sites. However, cell communication in the skeleton functions at a more discrete level again. Interactions between small numbers of cells or even pairs of cells are able to influence the skeleton^{6,7}. It has been known for decades that gap junctions exist between osteocytes, and between osteocytes and osteoblasts⁸. Blockade of this signalling affects responses of bone to mechanical loading, showing that it is physiologically relevant⁹.

The purpose of this review is to consider the role of the

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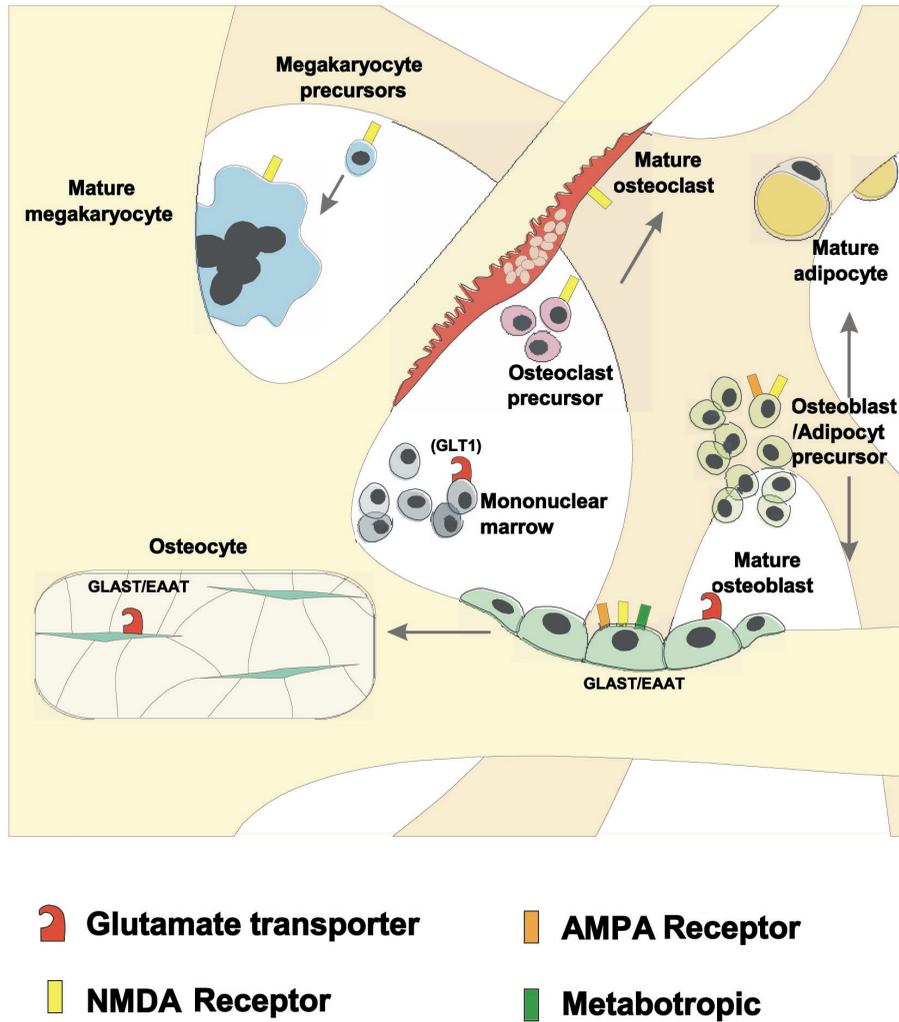


Figure 1. Sites of expression of components of the glutamatergic signalling mechanism within bone. Osteoblasts express pre- and post-synaptic components and glutamate, while osteoclasts, osteocytes and megakaryocytes express a more restricted subset of post-synaptic receptor components. (Reprinted from Trends Pharmacol Sci "Glutamate signalling in non-neuronal tissues" Vol 22: Skerry TM and Genever P, pages 174-181, Copyright (2001), with permission from Elsevier).

excitatory amino acid glutamate as a local paracrine signalling molecule. There are already several reviews of the literature on this subject, so it is not my intention to reiterate those¹⁰⁻¹³. Many of the published data in the area are connected with the expression of different components of the glutamate signalling mechanisms and those can be summarised in Figure 1.

Regulation of glutamate signalling components in bone

While the literature on sites of expression of different components of the glutamate signalling system in bone is relatively comprehensive¹⁴⁻¹⁸, the mechanism by which they may function to regulate bone mass remains controversial. In one

paper, it is concluded that there is no physiological role for glutamate signalling in bone because mice lacking the glutamate transporter GLAST had no detectable skeletal phenotype¹⁹. Responses to this paper pointed out the difficulty of proving such an argument on the absence of an effect conclusively, as well as technical flaws which made the original paper open to different interpretation^{20,21}. However, there have been several studies linking changes in bone mass in response to mechanical loading and other osteotropic stimuli with changes in expression of glutamate signalling components in bone. In our first report suggesting a signalling role for glutamate in bone, we detected the mechanically regulated expression of the GLAST transporter in osteocytes by mRNA analysis and in osteoblasts and osteocytes by immunocytochemistry¹⁴. More recently, Szczesniak et al. have showed that similar mechanical loading downregulated

expression (as detected by immunocytochemistry) of ionotropic glutamate receptors in both osteoblasts and osteoclasts¹⁸. However, Ho et al. have also reported down-regulation of glutamate receptors in osteoblasts but not osteoclasts (by Western blotting and immunocytochemistry) in response to disuse that induced bone loss and changes in expression of markers of osteoblast function²². It is possible that the respective 4-day and 3-week experimental periods of the two animal studies respectively led to alterations in the cell populations that was then associated with a change in glutamate receptor expression. More recently it has been shown that glucocorticoids regulate glutamate receptors and transporters in osteoblastic cells¹², while papers from Stone's lab have shown the possible involvement of glutamate receptors in bone disease in association with metabolites of the kynurenine pathway²³. Interestingly, there were significant reductions in kynurenine pathway metabolites with the ability to activate glutamate receptors in osteoporotic patients compared with healthy controls and treated patients, suggesting links in humans between glutamate signalling and regulation of bone²⁴.

The difficulty in interpreting these studies arises from two issues. First, the different stimuli, loading, disuse and glucocorticoids appear to have some similar and some different effects on receptor expression in some but not all cells. Second and more important, changes in expression of a receptor or other signalling component do not in themselves provide a causal link between the stimulus and the observed changes in expression.

Functional studies – general principles

To show functional evidence for a role for glutamate in the skeleton, interventional studies are required in which regulation of the system is associated with subsequent changes in cell, tissue or whole body skeletal properties. Such studies have been performed *in vitro* and will be described in later sections of this review. Studies *in vivo* may seem simple to perform but are made difficult by the fact that many available antagonists for glutamate receptors can have effects in numerous sites in the body. In addition to their crucial role in the CNS, it is well catalogued that glutamate receptors have been detected in tissues as diverse as the gut, pancreas, spleen, skin, adrenal, and pituitary^{10,25}. The importance of this is that modulators of glutamate receptor function given systemically may induce indirect effects on the bone via its innervation or influences on other systems that alter systemic osteotropic homeostasis. Specifically, if drugs cross the blood-brain barrier, they have profound effects on animal and human behaviour. NMDA receptor antagonists such as MK801 and phencyclidine (which is taken recreationally as angel dust) do exactly that and reduce activity, feeding and water intake in rodents to a serious extent (Skerry, unpublished). The potential effects of such agents on efferent nerve signalling to bone is not clear but is a further potential complication. This causes serious

concerns to us over the specificity of data acquired on bone changes in response to such agents, and we have not published such findings for that reason. (In our hands, agents that depress body functions generally lead to reductions in bone formation and increased bone resorption). A better alternative is to use agents that do not cross the blood-brain barrier, and some of those will be reported later, but it is important to realise that those agents have the ability to regulate physiology in the other tissues. Modulation of glutamate receptor signalling in the gut, testis and pancreas (for example) all have clear potential to alter bone metabolism by regulation of nutrient uptake, sex hormone status and insulin secretion, respectively, and studies should be well controlled and interpreted with caution. The widespread availability of knockout mice offers the cleanest system to evaluate the role of glutamate in bone *in vivo*, using systems such as Cre-lox to provide tissue specific deletions of genes. Evidence from such studies is now beginning to accumulate and the remainder of this review will focus on functional analysis from cell to whole animal.

Functional analysis of glutamate signalling in osteoclasts

Very soon after our discovery of regulated expression of GLAST in bone and the suggestion that glutamate receptors were expressed in bone¹⁴, Chenu's group provided evidence for regulation of glutamate signalling to influence osteoclast function *in vitro*¹⁵. These studies used chemical antagonists and antibodies to the NMDA receptor in studies using isolated osteoclasts, and showed reductions in bone resorption. Subsequent studies from the same group²⁶ used electrophysiological techniques to show that both glutamate and NMDA activated inward currents in mature rabbit osteoclasts, with features that were comparable to the current voltage relationships obtained in similar studies in neurones. We performed studies on a co-culture model of mouse osteoclastogenesis and showed that in that system osteoclast differentiation, but not mature cell function, was regulated by two different NMDA receptor antagonists²⁷, MK801 and PCP. More recently, Szczesniak et al. have confirmed the results of Chenu's group, and showed that inhibitors of both NMDA and Kainate receptors reduced osteoclastic resorption (by isolated primary rabbit osteoclasts)¹⁸. However, there is still no clear resolution to the question regarding the expression and function of glutamate receptors on mature osteoclasts or precursors as the data in the studies to date all appear robust. A more recent report raised further questions as Hinoi et al. detected expression of metabotropic but not the ionotropic glutamate receptors shown previously in mature osteoclasts and RAW 264.7 cells^{16,27,28} although they did show expression of a glutamate transporter, VGLUT-1 (known formerly as BNPI) and regulated glutamate release from those cells²⁹. Several chemical antagonists of glutamate transport had clear effects in inhibiting bone resorption. While analysis of osteoclasts from a small number of mice

lacking the VGLUT-1 transporter revealed reductions in KCl induced- glutamate release, there were no changes in bone resorption *in vitro*. Interestingly though, the VGLUT-1^{-/-} mice had lower bone mass, with large reductions in trabecular bone volume. The authors suggest that the action of glutamate is to provide an autocrine suppressive feedback on osteoclast function acting via the metabotropic glutamate receptor MGlur8. The VGlut-1^{-/-} mice are suggested to have low bone mass as a result of a consequent increase in osteoclastic resorption. Without dynamic histomorphometry, that suggestion remains to be confirmed. As we have shown that VGLUT-1 (aka BNPI) is expressed in osteoblasts³⁰, it is not possible to rule out more complex explanations for the skeletal phenotype of the VGLUT-1^{-/-} mouse involving more than effects on osteoclasts.

Functional analysis of glutamate signalling in osteoblasts

Evidence for a functional role for glutamate signalling in osteoblasts has followed a similar pattern as in osteoclasts. Studies showing the expression of different glutamate signalling components^{12,16,31-33} has been succeeded by *in vitro* experiments. Several patch clamp electrophysiological studies have been performed and show that in primary and osteoblast cell lines, glutamate and the specific pharmacological receptor ligands induce rapid inward currents which mirror those seen in neuronal cells under similar conditions^{31,34,35}. Receptor antagonists are able to induce dose-dependent suppression of those changes. In a longer timescale (1-3 minutes), we have shown that treatment of osteoblastic SaOS cells with glutamate induces an increase in intracellular calcium, which is blocked by the NMDA receptor antagonist AP5. In addition, it has been shown that treatment of rat osteoblast-like cells with glutamate reduced their electrical coupling of approximately one-third of cells within a few minutes of treatment³⁶. Since it is known that functional coupling of bone cells affects skeletal responsiveness, there are mechanisms by which such an effect could have physiological implications⁹.

In the longer timescale, *in vitro* studies become harder to interpret because most cell culture media contain high levels of glutamate. For short duration studies, it is possible to maintain cells in glutamate free media, but their ability to survive for long periods and respond to regulatory influences is not clear. For this reason, studies with durations that are over 1 day have tended to use antagonists at selective doses rather than agonists. Long-term agonist studies are also complicated by the release of glutamate from osteoblasts³⁷ and osteoclasts³⁸, which may compete with any added receptor ligands even if the media are glutamate-free.

Studies *in vitro* of the role of glutamate on osteoblast function have generally focused on the use of NMDA receptor antagonists and measurement of markers of osteoblastic phenotype such as alkaline phosphatase. Hinoi et al. showed that both MK801 and a receptor glycine domain antagonist

(glycine is a co-agonist for NMDA receptors) had the effect on osteoblasts over long periods (7-28 days) of depressing alkaline phosphatase and elevations of intracellular free calcium without affecting cell survivability³⁹. Sustained treatment downregulated CBFA-1 expression suggesting a role for glutamate in regulation of differentiation state. In current studies we have shown that NMDA and AMPA receptor antagonists inhibited osteoblastic differentiation processes in rat bone marrow cultures, suggesting the same conclusion, with the specific finding that the cultures became less osteogenic and contained more adipocytes (Figure 2) (Skerry, Taylor and Dobson, unpublished).

We have also investigated the longer-term consequences of interference with glutamate signalling in cells from differentiating skeletal elements using limb bud micromass culture systems. Cultures prepared from the limb buds of day 10.5 dpc mouse embryos, were allowed to develop and chondrogenic differentiation was assessed by alcian blue staining (Figure 3) and mRNA analysis.

In vivo studies

As stated previously, there are several difficulties in using chemical antagonists *in vivo* to modulate bone's glutamate signalling mechanisms without profound side effects. In a similar way, the bones of mice lacking the gene for a crucial subunit of the NMDA receptor are a flawed model for bone studies because it is known that there are profound influences of movement on bone development. It is not clear whether the NMDAR1^{-/-} mice move normally *in utero*, but they are weak and move little as neonates⁴⁰, so it is likely that any skeletal phenotype exhibited at birth is not wholly a direct consequence of changes in bone cell signalling. As the mice survive for less than 24 hours after birth, their skeletal responses to challenge cannot be studied and they are not of great value in bone biology studies. We have adopted two approaches to *in vivo* studies, the use of careful drug studies, and tissue specific gene knockouts. We have used drugs that do not cross the blood-brain barrier, as that effectively removes CNS-mediated behavioural and innervation-related effects.

We administered the AMPA antagonist NBQX and the NMDA receptor antagonist AP5 to groups of 6-week-old CD-1 mice by osmotic minipumps⁴¹. After 8 days of infusion (at 5 mg hr⁻¹) the mice were killed and the bones analysed by microCT. There were interesting differences in the response of the bones to the different drugs. Trabecular thickness was reduced by 50% in the NBQX-treated animals, but not those receiving AP5, while at midshaft sites, AP5 reduced cortical thickness by 40% and NBQX increased thickness by 30%. These differences were significant and though full analysis is still in progress, there were concomitant changes in other parameters such as bone volume, trabecular separation, structure model index (SMI) and degree of isotropy, all of which are important determinants of bone strength, which point to different roles for NMDA and AMPA receptors in regulation

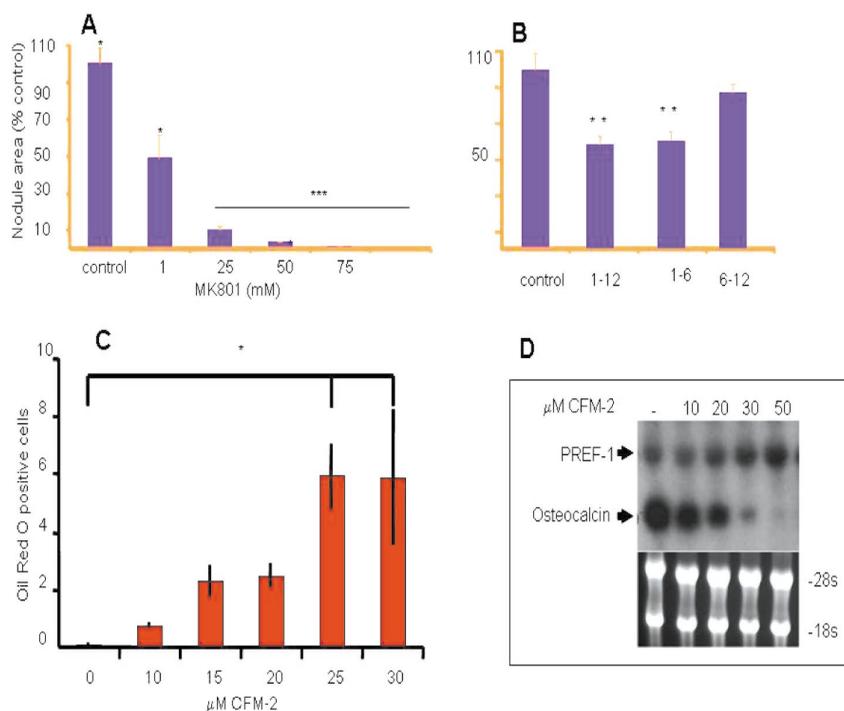


Figure 2. Effects of glutamate receptor antagonists on rat osteoblast differentiation. **A.** Dose-dependent inhibition by MK801 of mineralised nodule formation from marrow cultures under osteogenic conditions after 12 days in culture. **B.** Effect of MK801 for days 1-12, 1-6 and 6-12 compared with untreated controls. **C.** Numbers of oil red O stained cells in cultures in response to treatment with the AMPA receptor antagonist CFM-2. **D.** Reciprocal upregulation of the adipocyte marker PREF-1 and downregulation of osteocalcin expression in response to CFM2.

of cortical and trabecular bone mass and architecture.

In groups of female mice, ovariectomised at age 30 days, and treated with the same agents by minipump for 43 days, there were profound effects of the antagonists both of which were associated with highly significant increases in tibial trabecular bone loss of around 60% compared with ovariectomised vehicle-treated controls (Burford and Skerry, unpublished).

Lastly, we have recently begun a programme to use tissue specific knockout mice to abrogate glutamate signalling in specific cell populations. These studies are limited by the availability of floxed mouse strains, but early results show that when mice expressing the Cre recombinase under the control of the osteocalcin promoter are crossed with mice where a key region of the NMDA1 subunit is flanked by lox-p sites (officially designated *Grin1*), then F2 offspring with the genotype *OCCre⁺Grin1^{flox/flox}* fail to express functional NMDA receptors in their osteoblasts and are born with grossly stunted skeletons (Figure 4).

Why does bone need a rapid sensing/signalling system?

When the complexity of the glutamate signalling system (and other neurotransmitter signalling systems) is considered, it is not clear immediately why the skeleton should

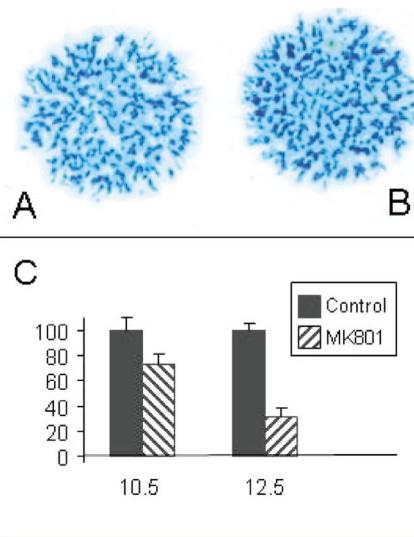


Figure 3. Effect of MK801 treatment on chondrogenic differentiation in micro-mass cultures from 10.5 dpc embryos: **A.** MK801 100 mM; **B.** Control. **C.** Densitometric analysis of Alcian blue stained area (% of control) in micro-mass cultures from 10.5 and 12.5 d.p.c. embryos after treatment with MK801. (Horner and Skerry, unpublished). There was a significant inhibitory effect of MK801 on chondroid matrix production, and a reduction in expression of chondrogenic markers such as Sox-9.

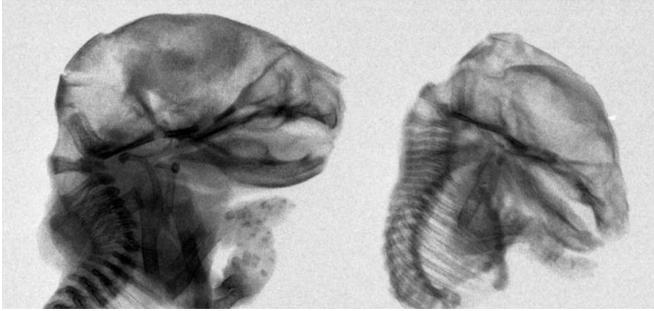


Figure 4. Radiographic images of wild type (left) and OC⁺Cre Grin1^{flox/flox} (right) mice showing delayed development, thin bone structure and poor mineralisation of the axial and appendicular bones.

need those properties. It is generally considered that the skeleton responds slowly to changes and modulates the cell populations responsible for formation and resorption so that at each of the many remodelling sites in the skeleton, long-term small changes accrue into significant effects on bone architecture. That view is not accurate when the response of bone to mechanical loading is considered. Four specific observations suggest that the skeleton has a requirement for a rapid sensing system that can retain information on the effects of previous loading.

The skeleton responds differently to different types of loading. While strain magnitude is a powerful determinant of bone's response to load⁴², the rate of application of strain is a similarly powerful stimulus^{43,44}. Slow rates of strain are either of low potency, or are not perceived by bone, while high rates of strain have profound physiological effect. Strain rates *in vivo* may easily exceed 200,000 microstrain per second in the event of unforeseen movements such as trips or landing from jumps, and those events persist for only millisecond timescales (Hillam and Skerry, unpublished). As the skeleton is able to differentiate between slow and fast rate events, its perception systems must be able to detect such brief events.

Only brief periods of exercise are necessary to saturate the osteogenic response to a single bout of daily loading⁴⁵. In the first report suggesting this, using a model of disuse in bird wings, Rubin and Lanyon showed that 72 seconds of osteogenic loading each day, when for the rest of each 24-hour period, the bone was protected from functional loading, was sufficient to produce a maximal bone forming response that was not increased by further loading. In that paper, an additional finding which in retrospect seems still more remarkable was that only 8 seconds (4 cycles of loading) in each 24 hours was sufficient to prevent the bone loss due to disuse seen in non-loaded animals. These findings show that the skeleton is constantly sensitive to very brief periods of mechanical load and able to sense and act upon or record very transient events.

A single loading event will produce a saturated response

after a relatively short period of loading, but if that series of load cycles is divided into 2, 3 or 4 separate bouts of loading within a single 24-hour period, then its effect is not only sustained, but it is potentiated⁴⁶. This suggests again that the fact of a previous loading event is recorded by bone cells and modulates their effect to subsequent events.

Insertion of rest periods between individual load cycles potentiates the effect of a fixed number of load cycles, or maintains the effect of a fixed time of loading despite large differences in numbers of cycles^{47,48}. Specifically, the effect of 400 load cycles applied to bone at 1Hz can be maintained by applying 40 cycles with rest periods of 10 seconds between each. Alternatively, if 400 cycles are applied with rest periods between them, then their effect is significantly increased.

These observations all point to the fact that the skeleton is rapidly and constantly sensitive to very brief loading events and retains information on their occurrence in order to influence the response to subsequent events. It is therefore necessary for there to be a biological system that can perform such perception/memory events. It is well known that the molecular basis of memory and learning involves so-called synaptic plasticity, a process comprising long-term potentiation and depression, in which repeated glutamate signalling events modulate the pre- and post-synaptic cell processes by altering exocytotic events and receptor activation processes. Preliminary data suggests that the bone cells express the necessary components for LTP and that the response of bone cells to repeated bouts of loading *in vitro* are dependent upon LTP-like processes⁴⁹⁻⁵¹. This argument does not strengthen the case for a functional role for glutamate signalling in bone, but it does answer the question as to why the skeleton should need such a system.

Summary and conclusions

While the data on the expression of glutamate signalling components in bone cells *in vitro* is very strong despite some unresolved difference in detail, the functional data is not complete. Studies *in vitro* provide clear evidence that glutamate receptors function in bone cells in a very similar manner to their role in the CNS. Manipulation of pre- and post-synaptic components in culture systems has clear effects on bone formation and resorption *in vitro*. Data *in vivo* is emerging slowly, but it is important to interpret antagonist studies carefully. The recent availability of mice lacking relevant genes is advancing progress rapidly, and it seems likely that there will soon be clear evidence regarding a physiological role for glutamate signalling in bone. Whether the subject develops into a target process for prevention or treatment of bone diseases depends on the parallels between experimental species and man (though several papers report expression of glutamate receptors in human bone cells) and other considerations beyond biology.

The issue of side effects of drugs even if they do not cross the blood-brain barrier remains to be clarified, as potential actions in other tissues may be unacceptable clinically.

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