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Osteoblastic glutamate receptor function regulates bone formation and resorption

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Abstract

Previous studies showed that a variety of bone cells express protein components necessary for neuronal-like glutamatergic signaling and implicated glutamate as having a role in mechanically induced bone remodeling. Initial functional studies concentrated on the role of glutamate signaling in bone resorption and provided compelling evidence to suggest that glutamate signaling through functional NMDA type ionotropic glutamate receptors (iGluRs) is a prerequisite for *in vitro* osteoclastogenesis. Originally, effects of iGluR antagonists seen in co-cultures were attributed to antagonists acting directly on osteoclast precursors. However, in the light of recent osteoblast studies it now seems likely that the observed effects on osteoclastogenesis are an indirect effect of modulating the function of pre-osteoblast present within these cultures. The presence of iGluRs in osteoblasts suggests a role for them in bone formation and this paper reviews and discusses the emerging data relating to the role of glutamate signaling in osteoblasts. A number of recently published studies have shown that osteoblasts not only express a wide number of 'pre-synaptic' glutamatergic proteins but also possess the ability to both regulate glutamate release and actively recycle extracellular glutamate. The functionality of osteoblastic 'post-synaptic' glutamatergic components has also been shown as both primary and clonal osteoblasts express electrophysiologically active iGluRs, metabotropic type glutamate receptors (mGluRs) along with a variety of glutamate receptor associated signaling proteins. There is, however, little published data regarding the actual role of glutamatergic signaling in osteoblastic bone formation. *In vivo* and *in vitro* studies performed provide evidence that glutamatergic signaling is a necessity for normal osteoblast function. In a number of different models of *in vitro* bone formation, the addition of non-competitive antagonists of iGluRs prevents the formation of mineralized bone, moreover antagonizing some sub-types of iGluR mediates the differentiation of pre-osteoblasts. iGluR antagonists modulate osteoblast function in a manner that correlates with the previously reported data regarding *in vitro* osteoclastogenesis. Interestingly iGluR mediated glutamate signaling appears to function differently in osteoblasts derived from flat and long bones. This implies the components of osteoblastic glutamatergic signaling may be adapted *in vivo* possibly to reflect the differential function of osteoblasts in those regions of the skeleton.

Keywords: Osteoblast, Glutamate, NMDA, AMPA, Bone Formation

The idea that glutamate acted as a signaling molecule within the CNS was met with initial scepticism, but over the past forty years the role of the glutamatergic synapse has been demonstrated unequivocally. Emerging as a result of a number of recent studies is a role for receptor-mediated glutamatergic signaling in many non-neuronal tissues including bone, skin and pancreas[1]. To date some of the strongest evidence for receptor mediated glutamate signaling existing outside of the CNS has been provided by work performed in bone cells. Numerous pre- and post-synaptic components of neuronal glutamate signaling have been identified in cells of the osteoblast lineage[2] (Fig. 1).

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Glutamate receptors

Before discussing the role of glutamate receptors in osteoblasts, it is first necessary to describe the process by which neuronal glutamate acts as a signaling molecule: as the glutamatergic proteins currently identified in bone suggest that glutamate is released and recycled in a similar mechanism by osteoblasts. Glutamate signaling in the CNS occurs in a tightly regulated cycle (Fig. 1). Briefly, in response to a stimulus, the pre-synaptic cell releases

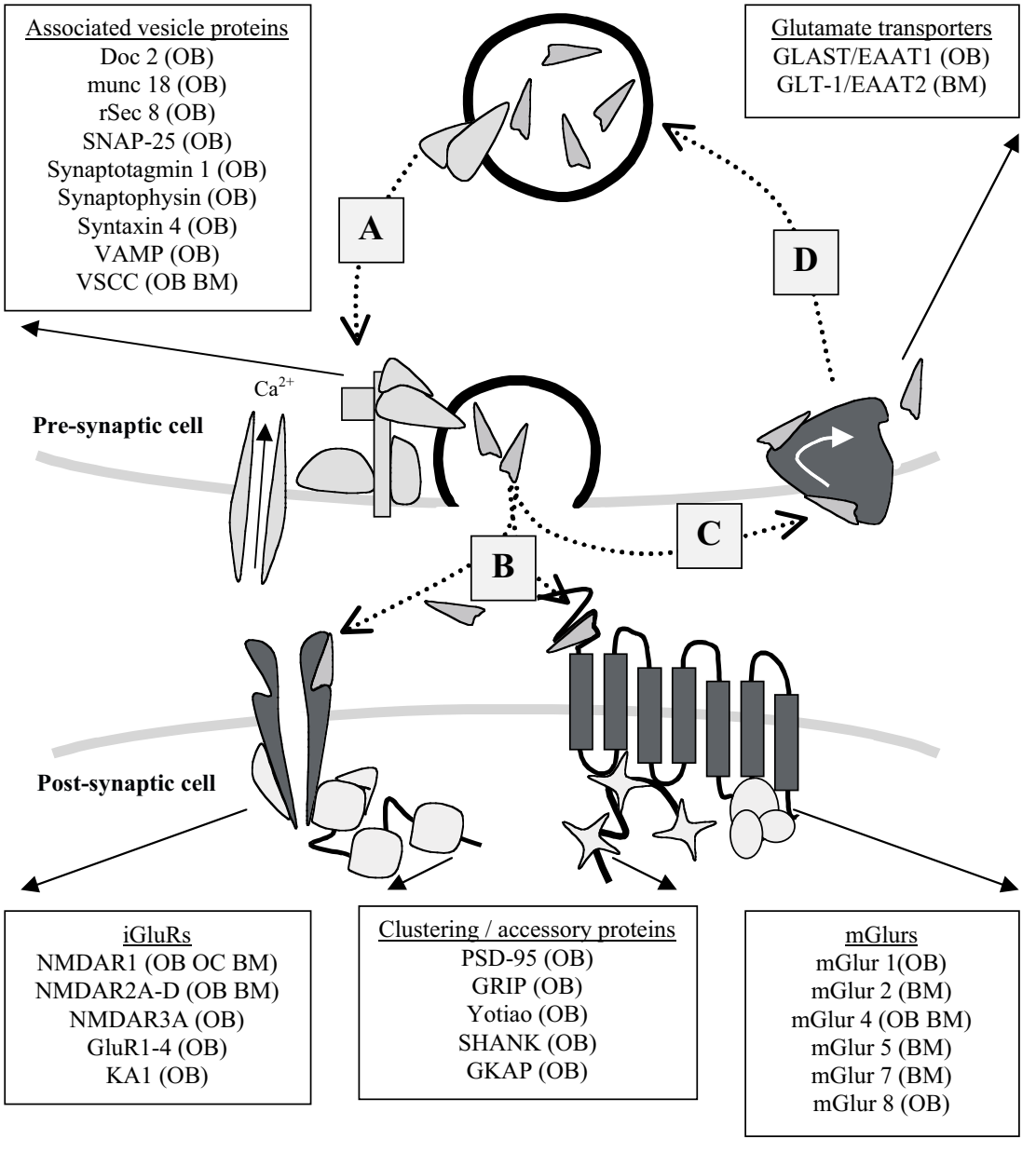

Figure 1. A schematic of the protein components found in a glutamatergic synapse and those identified within bone cells. Within pre-synaptic neurones glutamate is packaged into intracellular vesicles which are then subsequently directed to and tethered at the pre-synaptic membrane (**A**). In response to specific stimuli glutamate is released from the pre-synaptic cell into the synaptic cleft wherein it binds to and activates both iGluR and mGlur (**B**). Glutamate is removed from within the synaptic cleft by glutamate transporters terminating this episode of glutamatergic signaling (**C**) and permitting the recycling of glutamate molecules and the regeneration of the glutamatergic signal (**D**). Glutamatergic proteins identified in osteoblasts (OB), osteoclasts (OC) and bone marrow (BM) are listed in accompanying boxes.[2, 9, 10-12, 14, 20, 21, 23]

glutamate from primed intracellular vesicles into the synaptic cleft. This released glutamate acts on receptors present on the post-synaptic cell, whilst pre-synaptic transporters actively remove the glutamate from the cleft causing a cessation of the signal.

Neuronal glutamate receptors fall into one of two classes, the Metabotropic type (mGlur) are large 7 transmembrane G-protein linked receptors whereas the Ionotropic type (iGluR) are multimeric glutamate gated ion channels[3]. Members of these families of glutamate receptors are further classified according to consequences of activation and both kinetic and pharmacological properties[3,4] (Fig. 2). iGluRs will be the mainstay of this review as the majority of published data regarding glutamatergic signaling in bone is based on observations centred around the function of osteoblastic and osteoclastic iGluRs.

The iGluR family of glutamate receptors are further sub-divided into three groups according to their differential kinetic and responsiveness to the artificial ligands N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole

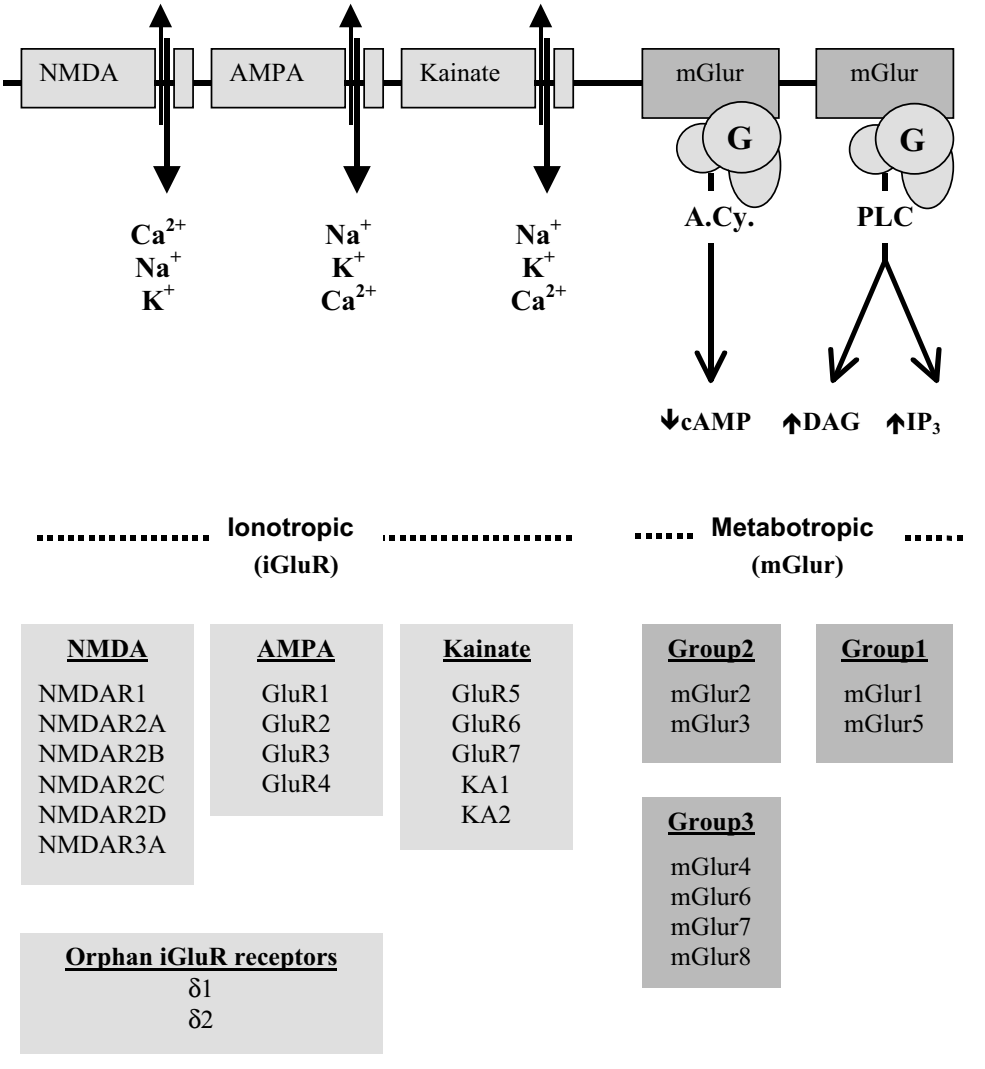

Figure 2. Members of the glutamate receptor family. Glutamate receptors are classified as either ligand gated ion channels or G-protein linked receptors (iGluR or mGlur, respectively). The iGluR family is split into 3 sub-groups according to the kinetics of activation and the selectivity of the ion channel (most favoured listed first and least last). Below each sub-group of iGluR are the sub-units that combine to form these receptors. Although classified as iGluR sub-units there is little evidence that orphan receptors form functional iGluR, hence they are only included in this diagram for completeness The mGlurs are further sub-classified by the intracellular pathways triggered upon activation.[3,4]

propionic acid (AMPA) and Kainate. There is however some confusion regarding the nomenclature of iGluRs. The 3 sub types of iGluR (NMDA, AMPA and Kainate) exist and function as independent receptors within the CNS. Despite this, AMPA and Kainate type iGluRs display very similar properties of activation. This is further complicated by the relative paucity of pharmacological compounds that can differentiate reliably between AMPA and Kainate type iGluRs. Because of this the AMPA and Kainate type iGluRs are often grouped together as "non-NMDA" type iGluRs and in some of the studies on bone ionotropic receptors agents used are not capable of discriminating beyond this.

A number of problems are presented when studying non-neuronal glutamate receptor function *in vivo*, the most telling being that the majority of the commercially available glutamate receptor antagonists are specifically designed to readily cross the blood brain barrier and modulate neurological function. A common consequence of these neurological effects are that animals treated with glutamate receptor antagonists often display abnormal behaviour. For example, the classical NMDA type iGluR antagonist PCP (or angel dust) when administered to rats rather unsurprisingly induces a catatonic state. Thus, the associated behavioural effects of iGluR antagonists often hamper *in vivo* studies of osteoblastic (and osteoclastic) iGluR function. Hence, the majority of the information gleaned about osteoblastic glutamatergic signaling has been obtained from *in vitro* studies.

Glutamate signaling in osteoblasts

As eluded to earlier, an episode of glutamate signaling can be divided into three key areas; pre-synaptic release of

glutamate, post-synaptic consequence of extracellular glutamate and pre-synaptic recovery of extracellular glutamate. Unsurprisingly, within the CNS these events are controlled by a number of key specialised proteins, many of which are also detected within cells of the osteoblast lineage.

Pre-synaptic glutamatergic proteins identified in osteoblasts

Research published by our laboratory show that osteoblasts express the pre-synaptic protein machinery necessary for both regulated glutamate release and extracellular recovery[5-8] (Fig. 1).

Recent studies have shown that primary and clonal osteoblasts contain glutamate filled vesicles similar to those seen in glutamatergic neurones, additional kinetic studies have show that this glutamate is spontaneously released into the extracellular media[9]. Further kinetic studies have shown similarities between the way in which osteoblasts appear to actively recover this extracellular glutamate and the recovery of the neurotransmitter glutamate within the CNS[9,10]. These data provide compelling evidence of the functionality of "pre-synaptic" glutamatergic proteins identified previously in osteoblasts.

Post-synaptic glutamatergic proteins identified in osteoblasts

To date the majority of data published on osteoblastic post-synaptic glutamatergic proteins has concentrated on osteoblastic iGluRs although two independent laboratories have recently cloned and sequenced a number of mGluRs from osteoblasts[11,12]. Osteoblastic mGluRs appear to be electrophysiologically functional[12] but there is currently little data regarding their physiological role in osteoblasts. Hence the physiological role of osteoblastic iGluRs will be the mainstay of this paper.

As mentioned earliernumerous post-synaptic glutamatergic proteins have been identified in both primary osteoblasts and osteosarcoma cell lines (Fig. 1). These include a number of NMDA, AMPA and Kainate type iGluR sub-units necessary for the formation of NMDA, AMPA and Kainate type iGluR. Importantly, it has also been possible to identify and clone from osteoblasts a number of proteins essential for the formation and functionality of neuronal iGluR receptor complexes such as PSD-95, GRIP, Yotiao and SHANK[13,14].

A number of elegant electrophysiological studies have shown that osteoblastic NMDA type iGluRs display very similar functional characteristics as those seen in neuronal systems[12,15]. Using the patch-clamp technique Laketic-Ljubojevic et al.[15] have shown in osteoblasts and osteocytes that a rapid increase in membrane currents is seen following the application of L-glutamate or NMDA. This membrane depolarisation was significantly blocked by co-application of the NMDA type iGluR antagonists MK801, Magnesium and R-CPP in a manner similar to those seen in neurones.

Further studies by our laboratory show that the activity of the transcription factor AP-1 is modulated by activation of osteoblastic iGluRs again in a similar manner to that which is seen within neurones[16]. These data provide compelling evidence for the existence of functional osteoblastic iGluR, which is again suggestive that osteoblasts utilize glutamate as a signaling molecule.

Osteoblastic iGluRs function in bone formation

Evidence for the necessity of glutamate signaling in bone formation is for the most part based on *in vitro* studies. A number of studies performed using both calvarial and long bone derived osteoblasts have shown that non-competitive blockade of either NMDA or NON-NMDA type osteoblastic iGluRs results to inhibit bone formation *in vitro* (KR Dobson et al. and AF Taylor et al. 2002, both currently in preparation).

Interestingly, there are different observed effects of inhibiting glutamatergic signaling in osteoblasts derived from different skeletal sites. In the CFU-F assay total inhibition of glutamatergic signaling mediated through NMDA type iGluR results to convert cells within the culture from a pre-osteoblastic to a pre-adipocytic phenotype, as measured by a decrease in collagen type 1, osteopontin and osteocalcin expression and a reciprocal increase in PREF-1 and C/EBP-α expression. Whereas in calvarial derived systems inhibition of NMDA type iGluR results to prevent only the mineralisation of bone nodules and not the maturation of the osteoblastic phenotype. For example, these cultures express similar levels of collagen type 1, osteopontin and osteocalcin as controls.

Within all of the *in vitro* models of bone formation utilized by our laboratory to assess the role of glutamatergic signaling in bone formation one thing has become apparent, that NMDA and non-NMDA type iGluRs appear to perform different functions within osteoblasts of the same lineage. Whilst non-competitively inhibiting glutamatergic signaling through either type of iGluR results to prevent *in vitro* bone formation the manner and resulting outcome of this inhibition is highly dependent on which type of iGluR is inhibited. With in CFU-F system inhibition of non-NMDA type iGluR prevents the adherence of osteoblast precursors, whereas, as mentioned above, inhibiting NMDA type iGluR results to switch osteoblastic cultures to adipocytic ones (KR Dobson et al. 2001, in preparation). Within calvarial derived osteoblasts this appears to be reversed, inhibiting NMDA type iGluR has no phenotypic effect on calvarial derived pre-osteoblasts; within the same culture system inhibition of non-NMDA type iGluRs induces such levels of adipogenesis that mature adipocytes expressing aP2, C/EBP-a, lipoprotein lypase and PPARg are detectable (AF Taylor et al. 2002, in preparation). Interestingly within these cultures there is compelling evidence that low level inhibition of osteoblastic

non-NMDA type iGluRs actually increase bone formation *in vitro* (AF Taylor et al. 2002, in preparation). The mechanism by which glutamate's osteotropic effect is mediated remains as yet unknown but is an interesting observation, implicating that glutamatergic signals mediate both osteoblastic activity and differentiation.

The necessity of glutamatergic signaling in bone formation is further evident when cultures of calvarial derived osteoblasts are treated with L-trans-2, 4-PDC [trans-4-Carboxy-L-proline/L-trans-Pyrrolidine-2,4-dicarboxylic], a selective antagonist of glutamate transporters including GLAST. These cultures again fail to produce bone *in vitro* and also appear to shift from an osteoblastic to a more adipogenic phenotype (GJ Spencer et al. 2002, in preparation).

In a recently published paper Gray et al.[17] suggest that glutamatergic signaling plays little or no important role in either bone formation or resorption. Evidence for such a conclusion is drawn from a number of *in vitro* studies using competitive antagonists of NMDA type iGluR and the skeletal phenotyping of transgenic mice lacking the glutamate transporter GLAST. Although their results pose a number of interesting questions the authors fail to consider a number of key facts before drawing their scathing conclusions, these and other shortcomings are the subject of a publication by Skerry et al.[18].

Osteoblastic iGluRs function in bone resorption

A number of laboratories have utilized a number of different assays to show that both osteoclast activity and maturation is sensitive to iGluR antagonists[19-21]. These studies are the basis of Dr Chenu's paper on "Regulation of bone resorption by osteoclastic glutamate receptors" and are to be discussed separately. Further osteoclast and osteoblast studies performed by our laboratory have however meant that initial conclusions drawn from Peet et al.'s paper[20] in which the co-culture model of *in vitro* osteoclastogenesis was utilized may need to be reassessed.

Studies from our laboratory using the co-culture system of maturing osteoclastic precursors by simultaneously culturing them with pre-osteoblasts have shown that application of non-competitive NMDA type iGluR antagonists inhibits *in vitro* osteoclastogenesis. In light of the recent studies described above which implicate glutamate signaling as a prerequisite of osteoblast function, it is no longer possible to be certain that the effects seen within the co-culture model are due to iGluR antagonists acting directly on osteoclast precursors. Instead, the effects of NMDA type iGluR antagonists observed by Peet et al.[20] may be due to modulating the osteoblastic activity/phenotype of pre-osteoblast present within these cultures which has the secondary effect of inhibiting *in vitro* osteoclastogenesis.

To support this theory there are striking similarities between the profile of inhibition seen when osteoclasts generated in co-cultures are treated with iGluR antagonists and the inhibition profile of osteoblasts treated with the same iGluR antagonists. A second striking similarity is that low level inhibition of non-NMDA type iGluRs increases *in vitro* osteoclastogenesis in co-cultures in a similar manner to the observed effects on *in vitro* bone formation[21] (AF Taylor et al. 2001, in preparation). Although there is as yet little direct evidence to confirm that iGluR antagonists act directly on pre-osteoblasts present within the co-culture system to modulate *in vitro* osteoclastogenesis further studies investigating expression of osteotropic factors, such as RANK-L, may better clarify this situation.

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