

'Pre-synaptic' vesicular glutamate release mechanisms in osteoblasts

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Abstract

Identification of intercellular signalling pathways in bone represents an important therapeutic target for drug development in the treatment of clinical conditions such as osteoporosis. One such intercellular signalling pathway in bone appears to be mediated by the excitatory amino acid glutamate, exhibiting remarkable similarities to synaptic neurotransmission. Bone cells (osteoblasts and osteoclasts) express functional glutamate receptors that are electrophysiologically and pharmacologically similar to those expressed in the CNS and there is evidence for their involvement in both bone formation and bone resorption. However, to date the cellular source of glutamate for the activation of these specific glutamatergic receptors in bone has remained unclear. This review provides a synopsis of our current understanding of these 'pre-synaptic' signalling mechanisms, presenting compelling evidence that osteoblasts possess the molecular capability to direct regulated vesicular glutamate release in response to osteotropic regulatory inputs. In addition, we discuss mechanisms other than 'pre-synaptic' glutamatergic mechanisms that could account for the source of glutamate for receptor activation in osteoblasts. Finally, convincing evidence reporting physiologically released glutamate in varied osteoblasts and osteoblastic cell lines is discussed. The overwhelming conclusion of this review is that by defining both the characteristics and regulatory control of this process, highlighting both similarities and differences between the CNS and bone may provide compelling evidence for the role of glutamate in bone cell function and physiology.

Keywords: Osteoblast, CNS, Glutamate, Vesicle, SNARE, Exocytosis

Introduction

Bone cells (osteocytes, osteoblasts and osteoclasts) are intimately involved in communication with each other. Osteoblasts receive the majority of local and systemic signals, which they transmit to the other cells in bone. In contrast, osteocytes are thought to be responsive to strain-generated signals with their regulatory information passed on to osteoblasts. Regulated intercellular signalling between bone cells regulates their respective activities to produce appropriate responses to external stimuli and ensure coupled remodelling in the maintenance of bone mass.

Extensive evidence has shown that with striking similarity to synaptic neurotransmission, bone cells communicate using the excitatory amino acid glutamate¹⁻¹⁵. To summarize

current understanding, bone cells have been shown to express functional glutamate receptors that are both electrophysiologically and pharmacologically similar to those expressed at glutamatergic synapses in the central nervous system. Additionally, glutamatergic signalling mediated through the action of glutamate on specific receptors in osteoblasts has been implicated both in the regulation of bone formation¹⁶ and bone resorption^{11,13}. Osteoblasts have also been shown to express functional glutamate transporters that take up glutamate in a regulated manner consistent with a role in the highly targeted and focal glutamate mediated intercellular signalling pathway^{6,17,18}. Until recently however, the cellular source of glutamate for the activation of osteoblastic receptor signalling components remained unclear and these latest developments in our understanding of glutamatergic signalling in osteoblasts are the primary focus of this review.

Role of glutamate within the CNS

In the central nervous system (CNS), the amino acid glu-

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Neurotransmitter	Biosynthesis	Vesicular Uptake	Storage vesicle	Reuptake	Degradation
Glutamate	Glutaminase	Vesicular Glutamate transporter (BNPI/VGLUT1)	Synaptic vesicles	Excitatory amino acid transporters (EAATs)	Glutamine synthetase
GABA	Glutamic acid decarboxylase	Vesicular GABA transporter (VGAT)	Synaptic vesicles (GAT)	GABA transporter	GABA transaminase (GAT)

Table 1. Comparison of the glutamatergic and GABAergic neurotransmitter cycles. Transmitter identity depends on the expression of specific biosynthetic, storage, reuptake and degradation machinery.

tamate is the major excitatory neurotransmitter and is believed to be involved in most aspects of normal brain function including cognition, memory and learning^{19,20}. It also plays major roles in the development of the central nervous system through regulation of synapse induction and cessation as well as neuronal cell migration, differentiation and death^{21,22}.

Glutamatergic synapses are highly specialised zones of contact between two neurones that contain the full complement of functional ‘molecular machinery’ that is capable of transducing a signal from the ‘pre-synaptic’ neurone to the signal receiving ‘post-synaptic’ cell through the excitatory amino acid glutamate. This ‘molecular machinery’ includes all the pre-synaptic protein components required for the generation and loading of glutamate transmitter containing vesicles as well as the trafficking, docking and fusion proteins that mediate regulated vesicular release or exocytosis. Also integral are glutamate transporters, responsible for both generation and cessation of signal through the recycling of transmitter. Finally, the precise alignment of the pre-synaptic ‘active zone’ (the region of plasma membrane specialized for vesicle docking) with the synaptic cleft (extracellular space) and the post-synaptically located target glutamate receptors at the ‘post-synaptic density’ (the region of plasma membrane that contains clusters of neurotransmitter receptors and ion channels at high density) makes up the synaptic signalling complex (Figure 1).

In the CNS, post-synaptic glutamate receptor activation is reliant on elevated extracellular glutamate levels as a direct result of pre-synaptic neurotransmitter release. In this system, prolonged alterations in release leads to severe mental illnesses such as schizophrenia, highlighting the importance of this process in normal brain function. Similarly to the CNS, modulation of extracellular glutamate levels through both release and reuptake will have direct consequences on post-synaptic receptor activation in bone cells and hence the downstream properties of glutamatergic transmission within this system. Therefore, defining these mechanisms may have critical bearings on our understanding of bone cell function

and physiology and also represent an emerging therapeutic target.

‘Pre-synaptic’ vesicular glutamate release in the CNS

The molecular mechanisms that ensure regulated vesicular glutamate exocytosis in the CNS are extensively described in the literature as based on a specialised membrane trafficking cycle termed the synaptic vesicle cycle (SV cycle). Essentially, the SV cycle represents a series of specific protein-protein interactions that direct intracellular synaptic vesicle traffic, ensuring both the fidelity of vesicle-membrane recognition and targeted exocytosis at the active zone. Exocytosis as a process is finally and critically dependent on the activation of voltage dependent calcium channels, which results in calcium influx and consequently the driving of vesicle fusion. The currently held views on these mechanisms are the focus of discussion below.

The synaptic vesicle (SV) cycle

In comparison with other cellular transport pathways, the SV cycle exhibits some remarkably unique characteristics such as fast excitation-exocytosis coupling, ability to respond to prolonged high-frequency activity and being efficiently responsive to changes in microenvironmental stimuli^{23,24}. The key features of the SV cycle and the individual roles of the protein mediators and regulators are critical in ensuring these responses.

The primary sequence of events directs and regulates the synthesis and loading of the low molecular mass neurotransmitter glutamate into synaptic vesicles and is encompassed as a sub-cycle within the SV cycle termed the neurotransmitter cycle. This step confers transmitter identity to the cycle and is largely dependent on the expression of specific biosynthetic, storage, reuptake and degradation machinery. Table 1 compares these specific components within glutamatergic

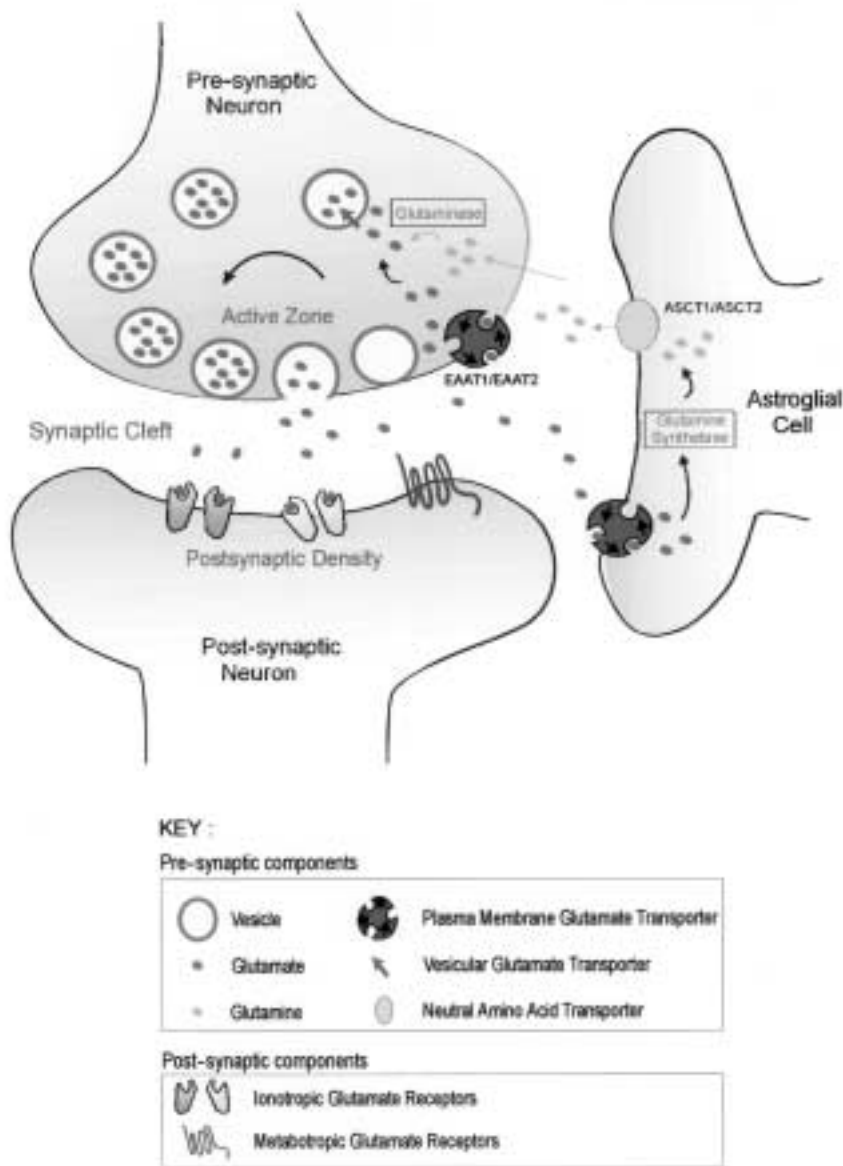


Figure 1. The glutamatergic synapse: a highly specialized zone of contact between two neurons. The synaptic signalling complex comprises the alignment of the pre-synaptic ‘active zone’ (the region of plasma membrane specialized for vesicle docking) with the synaptic cleft (extracellular space) and the post-synaptically located target glutamate receptors at the ‘post-synaptic density’ (the region of plasma membrane that contains clusters of neurotransmitter receptors and ion channels at high density). This functional complex is capable of transducing a signal from the ‘pre-synaptic’ neurone to the signal receiving ‘post-synaptic’ cell through the excitatory amino acid glutamate.

and GABAergic networks highlighting the high degree of molecular precision required within the two discrete neuronal circuits.

The individual molecular stages underlying the SV cycle in the CNS, resulting in regulated glutamate exocytosis at the pre-synaptic nerve terminal are both discussed in detail and illustrated diagrammatically below (Figure 2).

(1) SV neurotransmitter loading

SVs are relatively small, clear organelles (~50-100nm), their membranes accommodating only a limited number of proteins and phospholipids²⁴. The structures of most SV proteins are known and a great deal of effort has been expended in determining their specific roles.

Till recently the vesicular transporter of glutamate evaded molecular identification but has since been identified as the brain-specific Na⁺-dependent inorganic phosphate co-transporter, (BNPI)²⁵⁻²⁹. This BNPI co-transporter is driven by an electrochemical proton gradient across the vesicle membrane, concentrating and repackaging glutamate synthesized from astroglial derived glutamine or recycled locally by plasma membrane glutamate transporters (also see Figure 1).

(2) Generation of functionally discrete SV pools

Once loaded with neurotransmitter, SVs are thought to enter three functionally discrete vesicle pools, defined as (1) the readily releasable pool (RRP), (2) the reserve pool (RV) and (3) the resting pool (RP).

The maintenance of discrete synaptic vesicle pools is thought to enhance the responsiveness to diverse signals, both short term through exocytosis stimulated by the extraordinary speed of calcium action (to which the RRP pool is responsive) and longer term during extensive stimulation observed in synaptic plasticity (in addition to the RRP pool both RV and RP pools also respond)^{23,30}.

(3) Transport and targeting of SVs to the 'active zone'

Prior to recruitment for docking and fusion, SVs in the reserve pool (RV) are organized in clusters linked reversibly to the cytoskeleton immediately adjacent to the 'active zone'. It is thought that this organization is mediated by reversible molecular links between the SVs and actin filaments within the cytoskeleton. Actin filaments do not contact SVs directly, but are connected to these vesicles by the SV protein synapsin, which is a target for phosphorylation. Five isoforms are thought to exist, Synapsin Ia, Ib, IIa, IIb, and III, with isoforms I and II phosphorylated by Ca²⁺/calmodulin-dependent protein kinase I (CaMKI) and protein kinase A (PKA). In addition, synapsin I is phosphorylated by mitogen-activated protein kinase (MAPK) and CaMKII. A rise in intracellular calcium levels, leads to the phosphorylation of the CaMKI, PKA and CaMKII sites and the dephosphorylation of the MAPK sites of synapsin I; a process central to the action of the SV cycle^{31,32}.

It is vitally important that exocytotic vesicles are targeted to the appropriate region of the plasma membrane. There is some evidence that actin filaments may also serve as a track for the movement of SVs to the 'active zone' on the plasma membrane, even though there is little evidence for a cortical actin network in the pre-synaptic nerve terminals of neurons³³. Other cytoskeletal elements postulated to be involved in vesicle transport include microtubules^{33,34}.

The action of a large protein complex termed the sec6/8 exocyst complex and its interactions with the cytoskeletal elements described above has also been implicated in SV targeting³³. However, this complex is expressed predominantly in developing synapses and perhaps serves as an early organiser of 'active zone' components that then assume a later role in vesicle targeting³⁵.

(4) SV tethering to the pre-synaptic plasma membrane

A family of low molecular weight GTPases that belongs to the ras oncogene superfamily, termed Rabs, have been implicated in reversible SV attachment to the plasma membrane at the 'active zone', a process known as tethering³⁶. Based both on their proposed roles and specific location on SV membranes, Rabs have been postulated to bear key responsibility in maintaining the fidelity of membrane interactions. Whilst playing this key role in ensuring appropriate localization of SVs at the active zone, Rabs have also been proposed to function at a late step in fusion as a constitutive negative regulator of fusion^{36,37}. Various isoforms of Rab3 are associated with secretory vesicles in a variety of cell types but the Rab3A and Rab3C isoforms are thought to be primarily expressed in neurons³⁸.

(5) SV docking

Regulated SV docking is vital in ensuring the spatial regulation of exocytosis and efficient excitation-exocytosis coupling. The last few years has seen a re-evaluation of existing theories which suggested that docking was the result of interactions between a vesicular or v-SNARE (Soluble NSF Attachment REceptor) protein VAMP and the target membrane t-SNARE proteins syntaxin and SNAP25 (synaptosomal-associated protein, 25kDas) (see⁶). Instead, new evidence supports a role for these proteins not necessarily in specific docking of SVs to the active zones, but rather as representative of the true fusion proteins (discussed below).

At the present time, a number of *in vitro* experiments have identified potential interactions between SV proteins and pre-synaptic membrane proteins that may represent true docking events. These candidates include some downstream fusion proteins such as the calcium sensor in fusion, the SV protein synaptotagmin, which interacts directly with several pre-synaptic membrane proteins such as syntaxin, SNAP-25 as well as directly to calcium channels²³. Other potential candidates exist but for the purposes of this review these will not be discussed further as there remains considerable debate surrounding these mechanisms.

(6) SV priming

The next stage of the SV cycle involves vesicle priming. 'Priming' refers to the conversion of the docked vesicles into a form poised on the brink of exocytosis, largely believed to be a metastable hemifusion state.

Central to this process is the formation of a core exocytotic SNARE protein complex as encompassed by the SNARE (Soluble N-ethylmaleimide-sensitive factor [NSF] Attachment protein [SNAP] REceptor) hypothesis. This hypothesis describes the recognition and interaction between the vesicle associated membrane protein (VAMP/Synaptobrevin) and the tSNAREs syntaxin and SNAP-25 (synaptosomal-associated protein, 25kDa) on the plasma membrane of pre-synaptic cells.

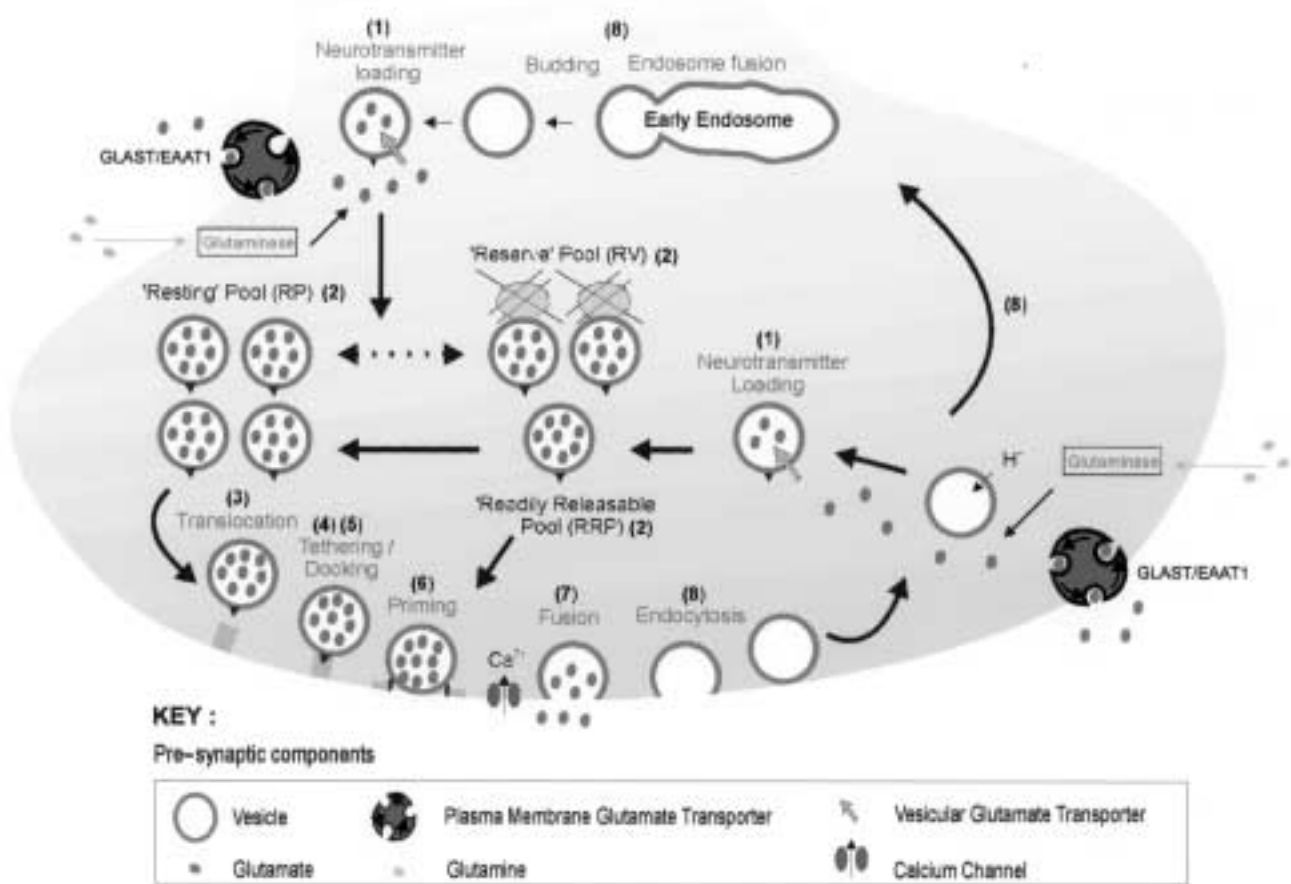


Figure 2. The Synaptic Vesicle (SV) cycle represents the series of specific protein-protein interactions that direct intracellular synaptic vesicle traffic to ensure the fidelity of vesicle-membrane recognition and targeted exocytosis at the active zone. Series (1) - (8) represent the individual molecular stages underlying the SV cycle and these are discussed in some detail in the text.

This results in the formation of a ternary core exocytotic complex. These proteins interact with each other in a parallel 4-helix bundle, which pushes the vesicle and the plasma membrane into close contact so that they are ready or 'primed' to fuse.

Multiple accessory proteins regulate these interactions, for example at steady state intracellular environment, the SV protein synaptophysin binds to VAMP and Munc-18 binds to syntaxin to disable the formation of the core exocytotic 'SNARE' complex so the process is not constitutively active. DOC2 and Mint proteins on the other hand are vesicular adapter proteins that regulate Munc-18/Syntaxin interactions³⁹⁻⁴².

(7) SV fusion

In response to an action potential reaching a pre-synaptic nerve terminal, depolarization leads to calcium entry through voltage-dependent calcium channels. In response to this, fast neurotransmitter release occurs just 150ms after pre-synaptic stimulation. The time delay between calcium entry and exocytosis is only 60ms highlighting the speed and efficacy of the response to incoming stimuli⁴³. This efficient

coupling of electrical activity with neurotransmitter release is achieved by the spatial proximity of the neuronal exocytotic machinery to calcium channels (~10nm). This is critical as calcium concentrations in the order of hundreds of micromolar are needed to produce physiological rates of neurotransmission.

Also, the calcium receptor for exocytosis is able to bind calcium rapidly and with relatively low affinity. This receptor, the SV protein synaptotagmin, is integral to the fusion core complex (through its interaction with syntaxin). It is thought that upon calcium binding, it induces a very rapid electrostatic and/or conformational change in the complex that destabilizes the hemifusion 'primed' state. This results in fusion of the SV and the plasma membrane, leading to exocytosis of glutamate into the intercellular space^{23,44-46}. Following membrane fusion, the SNARE complex is dissociated through the action of α -SNAP and the ATPase NSF, allowing the proteins to recycle⁴⁷.

(8) SV retrieval and recycling

Two modes for retrieval of fused vesicles are generally

accepted to be operational. Vesicles are either retrieved through clathrin coated-vesicle assembly after complete collapse into the pre-synaptic membrane (full fusion) or alternatively, they are quickly recycled by a direct reversal of the exocytotic process without intermixing with the pre-synaptic membrane ('kiss and run'). It is thought that the molecular make up, location and specific 'competence' for undergoing 'kiss and run' determine which mode of SV retrieval is operational⁴⁷⁻⁴⁹.

Finally, there is conflicting evidence as to whether mandatory SV recycling via endosomal intermediates occurs, or whether more direct recycling mechanisms involving the regeneration of synaptic vesicles from clathrin coated vesicles without intermediate fusion and budding steps operates. At this present moment the precise mechanism has not been elucidated.

Table 2 summarizes the predominant localizations and roles of these SV and related proteins in the Ca²⁺ regulated vesicular glutamate release in the CNS.

Evidence for 'Pre-synaptic' glutamate release mechanisms in osteoblasts

Though glutamatergic exocytotic mechanisms in the CNS are generally well established, proposed modes for osteoblastic glutamate release have not been the focus of extensive study. However, recent work in our laboratory has provided us with considerable insight into these mechanisms highlighting both similarities and differences between the CNS and bone that provide compelling evidence for the role of glutamate in bone cell function and physiology^{1,2}. This work is reviewed in some detail below.

Data from our laboratory has identified both specific structural and proteinaceous components in osteoblasts that perform essential roles during regulated vesicular glutamate exocytosis by pre-synaptic neurones. This suggests a similar 'pre-synaptic' cellular source for glutamate in osteoblasts for post-synaptic receptor activation. This extensive framework was previously believed to be confined to the CNS but our studies concur with more recent evidence suggesting a wider distribution for this focal and targeted signalling mechanism other than in the CNS^{50,51}.

We report in this review for the first time, the presence of small clear, electron translucent vesicles in osteoblasts, morphologically similar to neuronal glutamate filled synaptic vesicles (typically 50-100nm in diameter) undergoing what appear to be docking, fusion and exocytotic events^{52,53} (Figure 3). Vesicle congregations were observed adjacent to specific localities of the plasma membrane in osteoblasts where vesicles were docking and fusing, mirroring key features of the SV cycle. Also, the localization of these vesicles was most prominent at the periphery of adjacent cells, indicative of a signalling role. Interestingly, there appeared to be bi-directional communication mediated through these vesicles at different cellular sites between juxtaposed cells. With these preliminary observations, however, it is hard to

reconcile whether this is a phenomenon associated with a cell line trait or whether this represents a truly physiological response.

There exists evidence for glutamate filled 'synaptic-vesicle' like structures outside of the central nervous system and so it is not unlikely that these structures in bone cells also contain glutamate. Counterparts of synaptic vesicles have previously been found in mammalian pinealocytes; endocrine cells that synthesize and secrete melatonin. These organelles described as synaptic-like microvesicles are morphologically similar to neuronal synaptic vesicles but distinct from secretory granules and have been shown to express markers of glutamatergic synaptic vesicles such as the nerve terminal marker, synaptophysin. Similarly to neurones, they accumulate glutamate and secrete it through regulated exocytosis but use it either as a paracrine - or autocrine-like chemical neurotransmitter in a receptor-mediated manner resulting in the inhibition of melatonin synthesis^{50,54}. Recently, immunoelectronmicroscopy with antibodies against the synaptic vesicle marker synaptophysin, revealed a large number of synaptophysin-positive clear vesicles also in pancreatic islet cells⁵¹.

Published data have also shown that the core exocytotic 'SNARE' proteins expressed in osteoblastic cells are identical to those identified and characterized in neuronal cells as effectors of regulated vesicular glutamate exocytosis. Identification of both the v-SNARE isoform VAMP-1 and the t-SNAREs SNAP-25 and Syntaxins 1, 4 and 6 in osteoblasts confirmed the components required to form the ternary core exocytotic complex exist in osteoblasts¹. Of the eight known VAMP isoforms (VAMP 1-8), VAMP-1 and VAMP-2 are thought to be exclusively associated with trafficking events that involve regulated exocytosis and are found localized to SVs in neurons⁴². The other VAMP isoforms appear to function in both constitutive and regulated trafficking pathways in a variety of systems. Neurones also express multiple t-SNAREs, conferring a certain degree of promiscuity in v-SNARE-t-SNARE interactions. Of the Syntaxins (1-18), any one of Syntaxins 1, 4 and 6 are expressed at the plasma membrane in regulated vesicular glutamate exocytosis, although Syntaxin 1 is found most commonly associated within the SNARE complex in the highly regulated release of the neurotransmitter glutamate⁴². There appear however to be no clear implications in the CNS on the intrinsic physio-chemical properties of the core complex with differential syntaxin isoform expression. Differential expression of these tSNAREs in osteoblasts indicates vSNARE-tSNARE promiscuity or functional redundancy may also operate in this system and suggest that similarly to neurons the organization of the exocytotic pathway relies on other mechanisms distinct to core complex formation to generate specificity⁵⁵⁻⁵⁶. Significantly, *in vivo* immunohistochemistry showed that the final component of the trimeric SNARE complex in neurons, SNAP-25, was found localized on osteoblasts lining the periosteal surface of rat neonatal tibia, indicative of an osteoblast specific exo-

Classification (based on role)	Proteins	Predominant localization	Function in Ca²⁺ regulated vesicular glutamate localization release
SNARE complex	VAMP Syntaxin SNAP-25	Synaptic vesicles Plasma membrane Plasma membrane	Trimeric core complex components that assemble to form the exocytotic fusion machinery
Accessory proteins (SNARE complex interacting proteins)	Munc-18 Mint rSec8 DOC2	Plasma membrane Plasma membrane Cytosol/Plasma membrane Synaptic vesicles	Regulates the formation of core complex during vesicle docking by interacting with the tSNARE syntaxin Multimodular adapter Munc-18-1 isoform interacting proteins Associates with regulatory protein Rab3A and tSNARE syntaxin Vesicular adapter protein regulating munc-18-syntaxin binding and so vesicle docking
Regulatory proteins	Synaptophysin Rab3A Synaptotagmin I Synapsin I	Synaptic vesicles Synaptic vesicles Synaptic vesicles Synaptic vesicles	Binds v-SNARE VAMP to regulate core complex formation Tethering synaptic vesicles to the plasma membrane whilst downstream limiting fusion machinery as function of Ca ²⁺ Ca ²⁺ sensor in fusion. Binds Ca ²⁺ leading to a conformational change in SNARE complex resulting in resolution of fusion
Endocytotic proteins	Rabaptin 5	Cytoplasm	Influence vesicle mobility to maintain reserve vesicle pools Interacts with Rab5 to regulate endocytotic membrane fusion
Voltage-operated Ca ²⁺ channels	N- and P/Q-type Ca ²⁺ channels	Plasma membrane	Associate and form complexes with the ternary core complex proteins and the vesicular Ca ²⁺ binding protein, synaptotagmin I, thus transmitting voltage signal to SNARE complexes

Table 2. Summary of the predominant localizations and roles of these SV and related proteins in the Ca²⁺ regulated vesicular glutamate release in the CNS.

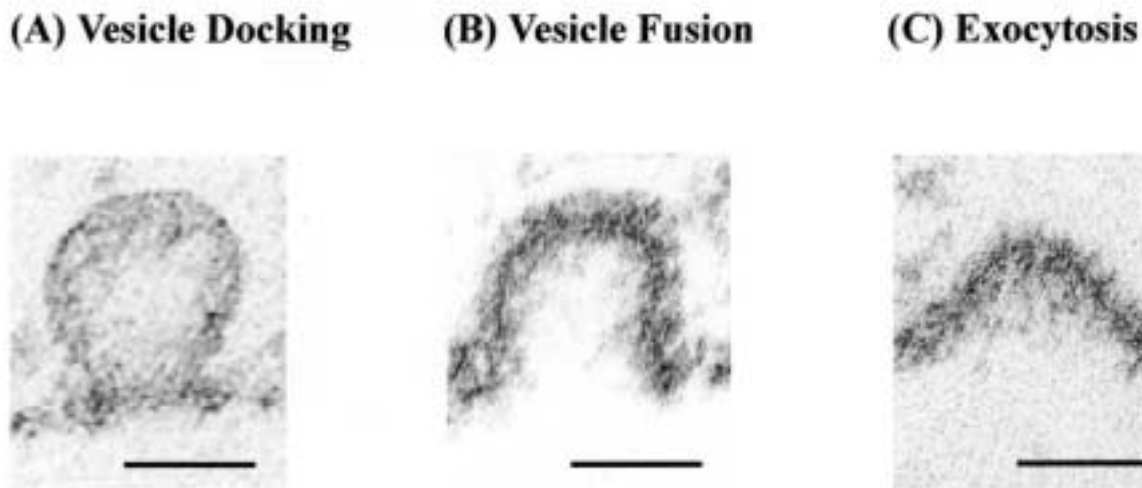


Figure 3. High power (x80k) electron micrograph images of MG63 osteoblastic cells identifying electron translucent vesicles (~80nm in diameter) (A) docking, (B) fusing and (C) exocytosing with the plasma membrane. Scale bars: (A), (B), (C) (50nm).

cytotic expression profile within the bone microenvironment (unpublished observations).

Furthermore, the identification of multiple accessory proteins involved in targeted vesicular glutamate exocytosis (Munc-18-1, Mints 1 and 2, DOC2, rSec8, Complexin 2, Synapsin 1 and Synaptophysin) as well as endocytosis (Rabaptin 5), established that much of the related machinery was also present¹. Though collectively termed accessory proteins, these proteins are essential components of neurotransmitter release throughout the brain. For example, gene deletion of the Munc-18-1 isoform in the CNS (also expressed by osteoblasts) results in complete paralysis of mice at birth. Munc-18-1 expression is considered to be neuron-specific and it is significant that osteoblasts express not only this protein but also the ‘neurospecific’ multimodular adapter Munc-18-1 interacting proteins Mint 1 and 239-42.

In addition, we have also shown that osteoblasts express the key regulatory proteins involved in the Ca²⁺ dependent regulation of vesicular glutamate exocytosis in the CNS, namely Rab3A and synaptotagmin I (the isoform characteristically specialized for fast Ca²⁺ dependent exocytosis)¹. Significantly, Western blot analysis of whole cell lysates generated from rat calvarial osteoblasts undergoing differentiation in osteogenic media (days 0-20), identified a differentiation dependent upregulation of both key regulatory proteins Rab3A and synaptotagmin (Figure 4). These data would appear to suggest that specific alterations in the expression profiles of fundamental vesicular exocytotic proteins occur during osteoblast differentiation and implicate a role for regulated glutamatergic responses during osteoblast development.

It is important however to recognize that constitutive and regulated exocytotic pathways share many similarities in

their molecular effector components. For example, the SNARE family of proteins have been implicated as the conserved core protein machinery for all intracellular membrane fusion events. However, the remarkable efficiency and sophistication of neurotransmitter release relies on the participation of “synapse-specific” proteins rendering exocytosis in neurons highly regulated, temporally efficient, and resistant to exhaustion. It is highly significant therefore that we identified these components, such as synaptotagmin I, Munc-18-1, Mint 1 and 2, synapsin 1 and synaptophysin, to name a few, in bone cells. These proteins transform standard membrane trafficking processes into the highly regulated, fast, calcium dependent exocytosis seen during vesicular glutamate release in the CNS³³.

Also, no feature more clearly defines the physiological function of a secretory cell or the phenotype of a neuron than the identity of the transmitter it releases. Thus, the molecular components that are represented within the neurotransmitter cycle (Table 1): the specific biosynthetic, storage, reuptake and degradation machinery pose critical evidence in supporting the glutamatergic secretory identity of osteoblasts. This review has earlier provided evidence supporting the presence of synaptic vesicles outside of the neuronal and endocrinal systems but significant literature exists that provides further evidence for biosynthetic (glutaminase)⁵⁸, reuptake (excitatory amino acid transporters (EAATs))^{6,17-18} and degradation machinery (glutamine synthetase) in osteoblasts. Evidence for uptake and packaging of glutamate into synaptic vesicles by the ‘brain-specific’ sodium-dependent inorganic phosphate co-transporter (BNPI/VGLUT) as present in the CNS, or even a bone specific homolog of this protein would underscore the ‘glutamatergic phenotype’ of osteoblastic cells highlighting the

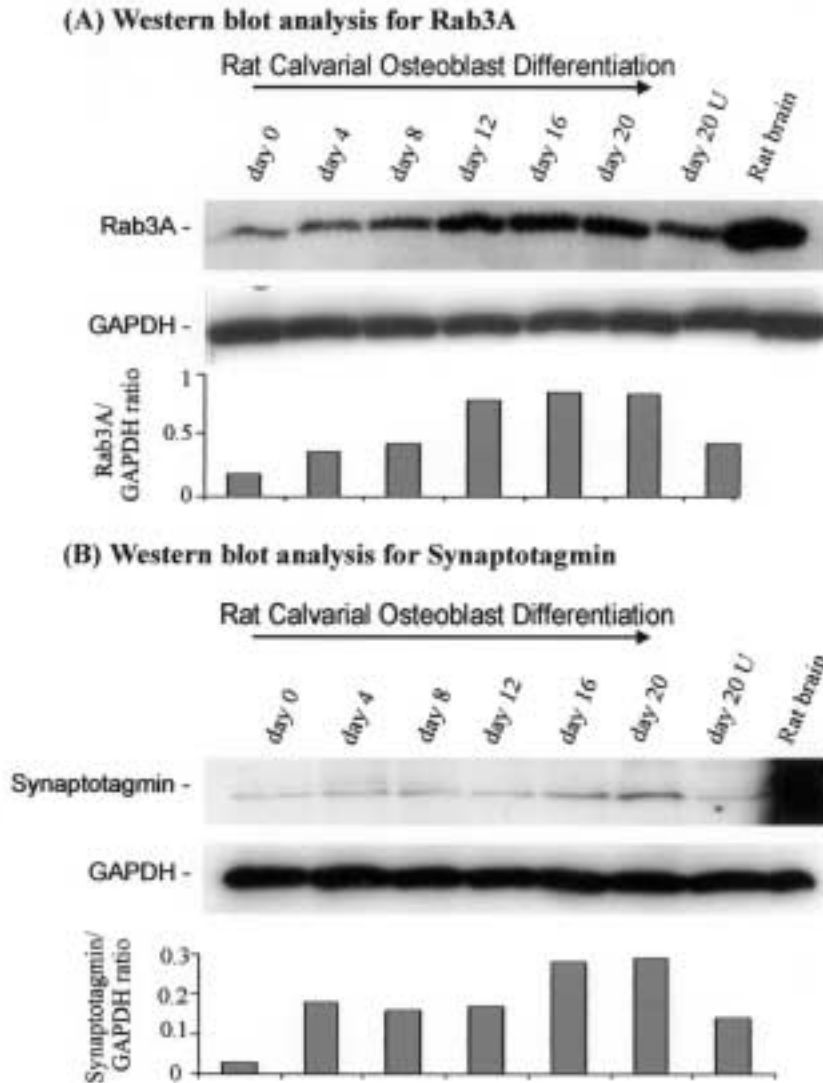


Figure 4. Western blot analysis for (A) the key regulatory proteins Rab3A and (B) Synaptotagmin in rat calvarial osteoblasts undergoing differentiation (days 0-20) in osteogenic conditions compared with control non-differentiating rat calvarial osteoblasts (day 20 U). Rat brain was used as a positive control for antibody. The middle panels show GAPDH protein expression as controls for loading. The lower panels show densitometry based elucidations of Rab3A or Synaptotagmin/GAPDH protein expression ratios over the culture period.

conservation in specific mechanisms for both intracellular vesicular packaging and glutamatergic signalling between the CNS and bone. Preliminary evidence suggests that these mechanisms are indeed conserved between brain and bone, the significance of which is reflected by recent work in the CNS which has shown that no other component is needed to make a neuron store glutamate and release it by exocytosis upon stimulation^{57,59}. This mode for transmitter selectivity in glutamate-releasing neurons sets them apart from other neurons containing different neurotransmitters and so provides compelling evidence for a similar mode in osteoblasts^{25,26-28,60}. Modulation of vesicular glutamate

uptake in osteoblasts could exert a profound influence on the magnitude of glutamatergic signalling by regulating the quantity of neurotransmitter available for release. Hence, this represents a highly specific target for drug development through the precise control of osteoblastic glutamatergic circuitry (such as through therapeutic targeting to bisphosphonate action).

However, the work described so far does not describe the physiologically relevant regulatory inputs that could guide intercellular communication in bone through glutamate. Regulated vesicular glutamate exocytosis in the CNS results through the activation of pre-synaptic voltage-gated or sen-

sitive Ca²⁺ channels (VSCC) leading to Ca²⁺ influx⁶¹⁻⁶³. In this system these Ca²⁺ channels form complexes with the ternary core complex proteins (syntaxin, SNAP-25 and VAMP) and the calcium binding protein, synaptotagmin I, with calcium influx resulting in neurotransmitter release. Of the multiple types of voltage gated Ca²⁺ channels expressed in the CNS, (L-N-P-Q-R and T-type channels), N- and P/Q-type Ca²⁺ channels predominate in pre-synaptic nerve termini at glutamatergic active zones⁶². Osteoblasts too would require this precise functional linkage ensuring a mechanism for targeting synaptic vesicles to sites of Ca²⁺ entry. There is substantial evidence that osteoblastic cells express a range of VSCCs, traditionally associated with excitable cells; L-type, T-type and mechanosensitive^{64,65}. More significantly however, recent evidence suggests that osteoblasts also express N- and P/Q-type Ca²⁺ channels conferring a mechanism for the association and forming of complexes with the ternary core complex proteins and vesicular calcium binding proteins at osteoblastic glutamatergic active zones⁶⁶. Ca²⁺ channels in osteoblasts have been shown to be able to respond to key osteotropic influences such as hormonal, cytokine and mechanical stimulation with Ca²⁺ signals occurring as early events in processes associated with bone cell differentiation and bone remodelling. Hence, these osteotropic influences could modulate the exocytotic glutamatergic signalling activity in osteoblasts so directing intercellular communication in bone during these processes.

Intercellular signalling by glutamate within the bone microenvironment through mechanisms similar to the CNS could be reliant on the formation of adhesive junctions between osteoblasts and their target cells, similarly to synapses between neurons. This would preserve both the signal-to-noise ratio for released transmitter to act on specific 'post-synaptic' receptors whilst ensuring specificity in directed intercellular communication. There is significant evidence suggesting that osteoblasts communicate with opposing cells in the bone milieu, with functional communication existing not only between cells of the osteoblast lineage but also osteoclasts, osteocytes and marrow cells⁶⁷⁻⁷². Osteoblasts express a variety of adhesion molecules on their surface with their putative significance in cell-cell interactions the subject of extensive work^{67,71,73-74}. The resultant junctional coupling could contribute to the coordinated and specific responses of these cellular networks to extracellular signals mediated through glutamate.

In our opinion, the evidence reviewed above represents the most likely way that glutamatergic signalling operates in bone; via regulated pre-synaptic osteoblastic glutamate release. Another published study has presented evidence for glutamate-containing nerves in bone using an immunohistochemical approach¹². It is highly improbable however that these peripheral nerve fibres represent the physiological source of glutamate in 'post-synaptic' receptor activation in bone. Few osteoblasts are innervated directly and owing to the highly motile nature of osteoclasts during bone remodelling, it is unlikely that direct influences could be exerted

through glutamatergic signalling in directing bone cell activity. We would favour local as opposed to remote influences on cell activity in bone, accounting for this exquisite precision and site specificity of remodelling events. Interestingly, Huggett et al.⁶ have hypothesised that the GLAST/EAAT-1 transcript (GLAST-1a) cloned in whole rat bone has an opposite orientation to conventional GLAST, implicating structure-induced reversal of transport as a likely mechanism for glutamate release. The absence of truncated GLAST/EAAT-1 (GLAST-1a) however would render structure-induced reversal of glutamate transport unlikely. The relative importance of this transporter if functional as a candidate for an alternative mechanism of glutamate release remains to be seen.

To conclude, it is clear that osteoblasts are the integral source of glutamate in the bone microenvironment and the molecular mechanisms that operate in the CNS to ensure regulated vesicular glutamate exocytosis are also utilized by osteoblasts in this process. This is the first evidence for glutamatergic exocytotic mechanisms for post-synaptic receptor activation outside of the CNS and endocrine systems, the latter of which has only recently come to light. The myriad of osteotropic regulatory inputs that could act through glutamatergic signalling underscores the potential importance of this signalling mechanism in bone.

Osteoblasts secrete glutamate at levels similar to those in the CNS capable of activating post-synaptic receptors

The data reviewed in the preceding text provides considerable insight into possible 'pre-synaptic' mechanisms responsible for osteoblastic glutamatergic signal propagation without providing direct evidence supporting the physiological release of glutamate within the bone microenvironment. A recent paper by Genever and Skerry² provides convincing evidence that osteoblasts do indeed release glutamate at similar or even greater levels than those released by depolarized neurones at glutamatergic synapses. Interestingly, though similarly to neurones glutamate release in osteoblasts was susceptible to depolarization, negatively regulation by voltage-dependent calcium entry was observed rather than positive. There also appeared to be some heterogeneity both with glutamate release levels and with susceptibility to depolarization between different osteoblastic cell types; possibly a reflection of polarization and differentiation states of the different cells. This latter suggestion was supported by studies in this paper showing both elevated glutamate release and susceptibility to depolarization in MC3T3-E1 cells that had attained a more differentiated osteoblastic phenotype in osteogenic conditions compared with undifferentiated cells grown in the absence of osteogenic stimuli. This finding would also corroborate with Figure 4 as both data suggest that the glutamatergic phenotype is more prevalent in differentiated cells in comparison

with undifferentiated cells. The mechanistic differences between the osteoblastic and CNS systems that are responsible for this significant contrast in release characteristics is unclear at this time.

Significantly, the authors also report studies in this report in which using the glutamate release inhibitor riluzole, they observe at low concentrations (1-10 μ M) both inhibition of glutamate release and significant inhibition of alkaline phosphatase in MC3T3-E1 cells without any effect on cell viability. At concentrations of 25 μ M and above however, (concentrations that are neuroprotective within the CNS) the osteoblastic cells appear to undergo morphological and biochemical characteristics associated with apoptosis. The authors propose a role as an osteoblast survival factor for glutamate, hence the continual requirement for glutamate release.

It seems then that there is still much to add to our current understanding of glutamate release mechanisms in osteoblasts but certainly the distinct characteristics of this process in osteoblasts highlight significant differences in pre-synaptic signalling between the CNS and bone and represent exciting and compelling evidence for the specificity of the intrinsic role of glutamate in bone cell function and physiology.

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