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Review

Do Bone Cells Behave Like a Neuronal Network?

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Abstract. Bone cells are organized into an interconnected network, which extends from the osteocytes within bone to the osteoblasts and lining cells on the bone surfaces. There is experimental evidence suggesting that bone tissue exhibits basic properties of short- and long-term memory. An analogy might be made between the bone cell network and neuronal systems. For instance, recent studies suggest that the neurotransmitter glutamate may play a role in cell-to-cell communication among bone cells. Glutamate is a key neurotransmitter involved in learning and memory in reflex loops and the hippocampus. The simplest forms of memory include habituation (desensitization) and sensitization. It is argued that bone cells exhibit habituation to repeated mechanical stimuli and sensitization to mechanical loading by parathyroid hormone (PTH). Acquired long-term memory of a mechanical loading environment may influence the responsiveness of bone tissue to external stimuli. For instance, bone tissue from the skull shows markedly different responses to several stimuli, e.g., mechanical loading, disuse, and PTH, compared with long bones. We speculate that the history of weight bearing imparts long-term cellular memory to the bone cell network that modulates the cellular response to a wide variety of stimuli.

There is a growing body of evidence suggesting that the interconnected network of bone cells that includes osteoblasts, osteocytes, and lining cells may in fact have higher levels of organization and function. Experimental findings discussed below suggest that bone cells have basic properties of habituation, sensitization, and long-term memory first described in neuronal systems. The property of habituation in bone was described previously as "cellular accommodation" [1] and there is evidence that some hormones, e.g., parathyroid hormone (PTH), can sensitize bone cells to biomechanical stimuli [2]. The discovery of glutamate signaling pathways in bone [3–8] suggest, that the molecular pathways key to memory and learning in neuronal systems might be employed by bone cells. Perhaps the bone cell network mimics a simple neuronal system, and thus is capable of learning and memory.

It has been proposed that bone cells accommodate to their physical surroundings and mechanical loading environment, allowing them to adjust their sensitivity to mechanical loads or strains [1]. The principle of cellular accommodation proposes that cells in weight-bearing bones are 'programmed' differently than cells from non-loaded skeletal sites. As Currey noted, "If bone cells remodel according to some universal rule about what is an effective strain, then it would be highly likely that the strain appropriate for the tibia would be completely inappropriate for the top of the skull and the stapes, which are not loaded to high stresses, but which must not be removed by osteoclasis" [9].

Indeed, osteoblasts extracted from the skull (calvaria) or long bones (ulna) exhibit different sensitivities to mechanical loading—calvarial cells are much less sensitive than ulnar cells [10]. This observation suggests that bone cells retain information about their skeletal site of origin when placed under culture conditions. Likewise, the skull is less sensitive to the anabolic effect of PTH fragment (1–34) than are long bones [11], suggesting that mechanical loading history may influence bone tissue's responsiveness to hormones.

In human volunteers subjected to 17 weeks of bed rest, bone loss was greatest in the distal bones of the leg (i.e., the calcaneus). There was less bone lost in the lumbar spine and hip and no significant bone loss was found in the ribs or arm bones [12, 13]. Surprisingly, there was a significant gain in bone mass in the head (Fig. 1). Here a seemingly homogeneous state of disuse throughout the body produced site-specific skeletal responses. These data suggest that the response of bone tissue to an external stimulus is determined by the bone cells' perceived change in their local environment. Bed

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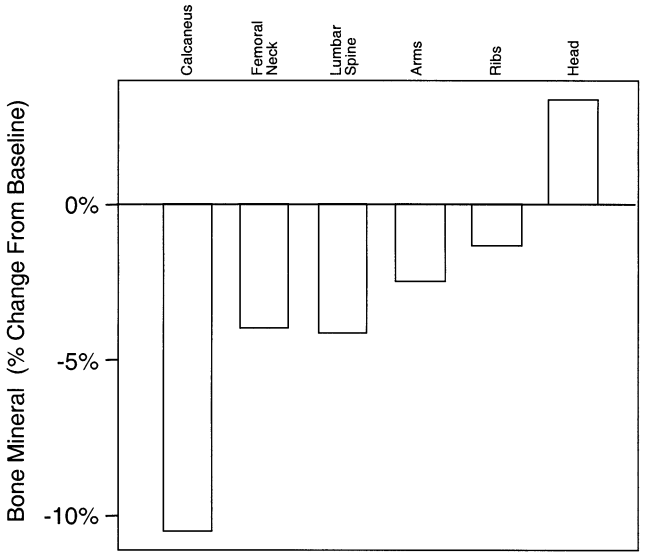

Fig. 1. Volunteers subjected to bed rest for 17 weeks lost a statistically significant amount of bone mass in their lower extremities. Bone loss was not significant in the arm bones or ribs, and there was a stastistically significant gain in bone mass within the head [12, 13]. Reprinted from "Site-specific skeletal effects of exercise: Importance of interstitial fluid pressure." In Bone, Vol. 24, p 162, Copyright 1999, with permission from Elsevier Science.

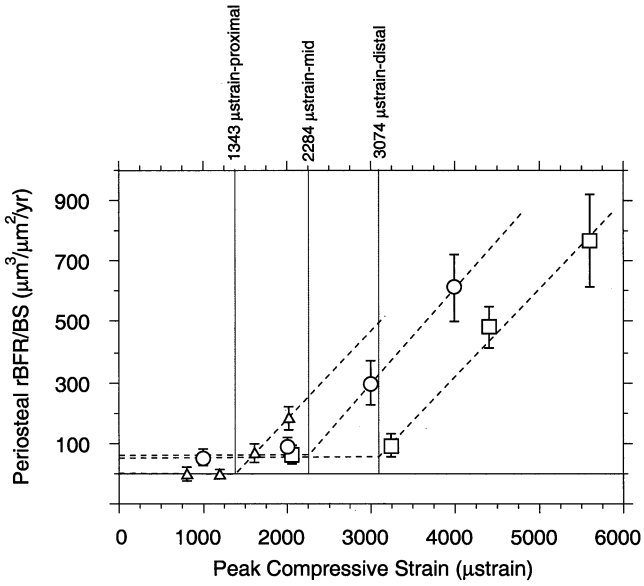

Fig. 2. Periosteal responses in the rat ulna after application of axial loading. The bone formation rate (BFR/BS) on the periosteal surface was increased when applied strains surpassed a threshold. The threshold strain value varied considerably at different sites along the ulna. The threshold was highest in the distal ulna (squares), less at the ulnar midshaft (circles), and lowest in the proximal ulna (triangles). Adapted from [14].

rest causes a fluid shift in the body that decreases extracellular fluid pressures in the feet and legs and increases fluid pressure in the head. It appears that bone cells grow accustomed to (learn from) the every-day pattern of fluid pressure, i.e., bone cells near the feet become adapted to higher fluid pressure and bone cells in the head to lower pressure. Only when the customary pattern is broken by prolonged bed rest are catabolic or anabolic processes initiated.

Recently, we found that the osteogenic response of the rat ulna to mechanical loading varied with proximal-distal location and that this variation followed a clear pattern: The distal-most region required more strain before bone formation was initiated (Fig. 2). Our measurements showed that the distal ulna was subjected to higher strain during axial loading, suggesting that the bone tissue habitually receiving higher mechanical strains had become less responsive to loading [14]. Thus, mechanical loading history appears to affect cellular mechanosensitivity.

Memory and Learning in Neuronal Systems and Bone

Memory, the outcome of learning, has several forms. The simplest form of learning in sensory-motor reflex loops is habituation (desensitization)—the cellular system learns, after repeated exposure, to ignore a persistent signal. Habituation involves the decrease in cell-to-cell signal transduction in response to a repeated or habitual signal. Another form of cellular learning is sensitization—the enhancement of cellular response to a stimulus. Sensitization is more complex than habituation and involves the strengthening of a stimulus applied to one pathway by a stimulus coming through another pathway. In simple neuronal systems, habituation and sensitization can last for a few minutes or for many days, depending upon the duration and repetition of the stimulus. These simple forms of short- and long-term memory arise from different molecular mechanisms. Short-term memory results from changes in neurotransmitter release or through enhancement of biochemical pathways, while long-term memory requires gene transcription and structural changes, e.g., an increase or decrease in cellular connectivity [15].

Habituation

Habituation is the diminishment of responsiveness to a repetitive stimulus in neuronal systems. Bone tissue demonstrates habituation in its response to repetitive mechanical stimuli. Bone cells desensitize rapidly to repetitive mechanical loads and after about 40 loading cycles the responsiveness of the tissue is mostly depleted (Fig. 3) [16, 17]. In simple neuronal systems (reflex loops), glutamate is the transmitter from the sensory neuron. The motor neuron has N-methyl-D-aspartic acid (NMDA) glutamate receptors. Habituation occurs when glutamate release from the sensory neuron is diminished [15]. Recently, Skerry's group identified a neuronal glutamate/aspartate transporter (GLAST) in bone tissue and functional NMDA glutamate receptors

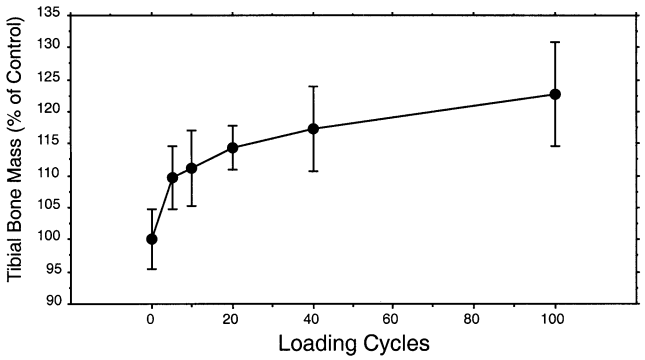

Fig. 3. Umemura et al. [17] trained rats to jump various numbers of times per day. They found that jumping increased tibial bone mass, but increasing numbers of jumps gave diminishing returns. It appeared that the bone became desensitized to mechanical loading after about 40 loading cycles.

in bone cells [3, 4]. The glutamate transporter moves glutamate into cellular vesicles and thus is necessary to 'reload' glutamate after it has been released from the cell. Interestingly, Mason et al. [3] showed that the expression of the glutamate transporter was decreased shortly after mechanical loading of bone tissue. In addition to NMDA glutamate receptors, metabotropic glutamate receptors have been identified in bone cells [5, 6], molecular machinery for glutamate exocytosis has been identified in osteoblasts [7], and there is new evidence of innervation in bone tissue with glutamate-containing fibers [8].

These studies demonstrate the possible role of glutamate signaling among bone cells, or from nerves to bone cells. However, glutamate signaling typically occurs across synapses and no synaptic structures have yet been identified among bone cells or linking nerves to bone cells, thus raising questions about the importance of glutamate signaling in bone.

There are other plausible molecular mechanisms by which bone could be desensitized to prolonged mechanical loading. For instance, osteoblast-like cells can communicate through autocrine activity of secreted adenosine triphosphate (ATP) on $P2Y_2$ purinergic receptors [18]. A local mechanical stimulus initiates intercellular calcium signaling mediated by ATP receptors and secreted ATP rapidly desensitizes these receptors to a mechanical stimulus [18]. In addition, bone cells reorganize their cytoskeleton in response to a mechanical stimulus [19]. Cytoskeletal reorganization in turn changes a cell's mechanosensitivity, possibly decreasing cellular responsiveness.

Finally, it is possible that habituation in bone cells occurs via desensitization or internalization of G protein-coupled receptors. Desensitization can occur rapidly due to G protein uncoupling after phosphorylation [20]. Additionally, a prolonged stimulus can cause internalization of the G protein-coupled receptor within the cell [21]. G proteins appear to play a role in bone cell mechanotransduction [22] as do prostaglandins [23] that bind to G protein-coupled prostanoid receptors [24].

Sensitization

Sensitization of a reflex is a heterosynaptic process; the enhancement of synaptic strength is induced by modulatory interneurons activated by stimulation [15]. The suggested mechanism for presynaptic sensitization involves a neurotransmitter, e.g., serotonin, released from the presynaptic interneuron binding to G protein coupled receptors, and increasing adenylyl cyclase activity or activating phospholipase C (PLC). Signal transduction can occur via the cAMP-dependent protein kinase A (PKA) or protein kinase C (PKC) pathways. PKA subsequently has three key actions: (1) phosphorylation of K^+ channels occurs, thereby decreasing the outward potassium current (this prolongs the action potential and increases the influx of Ca^{2+}, thus augmenting transmitter release); (2) the release of glutamate from the sensory neuron is enhanced; and (3) L-type Ca^{2+} channels are opened. PKC acts on pathways 2 and 3 [15].

It is unclear whether structures similar to interneurons exist in the bone cell network, yet there is new evidence for a role of the interneuron neurotransmitter serotonin in bone biology. Serotonin or 5-hydroxytryptamine (5-HT) receptors have been identified in osteocytes, osteoblasts, and osteoblast precursors within the periosteum [25, 26]. Agonists for the $5\text{-}HT_{2B}$ serotonin receptor isoform stimulate proliferation of periosteal fibroblasts and presumably osteoblast precursors in the periosteum [25]. In addition, there is some preliminary evidence suggesting a role for serotonin in osteoblast mechanotransduction [25]. Osteoblasts express a serotonin transporter and are capable of serotonin uptake [26]. Importantly, serotonin potentiates the PTH-induced increase in AP-1 activity in osteoblast-like cells [26], suggesting sensitization.

In addition to the possibility of sensitization of bone cells by neurotransmitters, there is some evidence for sensitization of bone cells by peptides, most notably PTH. PTH-(1–34) activates both PKA and PKC pathways through at least two G protein coupled receptors. PTH-(1–34) modulates the mechanically sensitive calcium channel (MSCC), which was previously identified in osteoblasts [27], increasing calcium entry into the cell in response to mechanical stimuli [28, 29]. PTH-(1–34) also decreases outward potassium currents in osteoblasts [30], which prolongs the influx of Ca^{2+}. Moreover, PTH-(1–34) enhances the response of osteoblasts to mechanical stimuli by increasing Ca entry through the L-type Ca^{2+} channel [2]. Each of these effects of PTH-(l-34) sensitizes osteoblasts to mechanical signals (Fig. 4). There is evidence that PTH-mediated sensitization of bone cells is crucial for mechanically induced bone formation, as

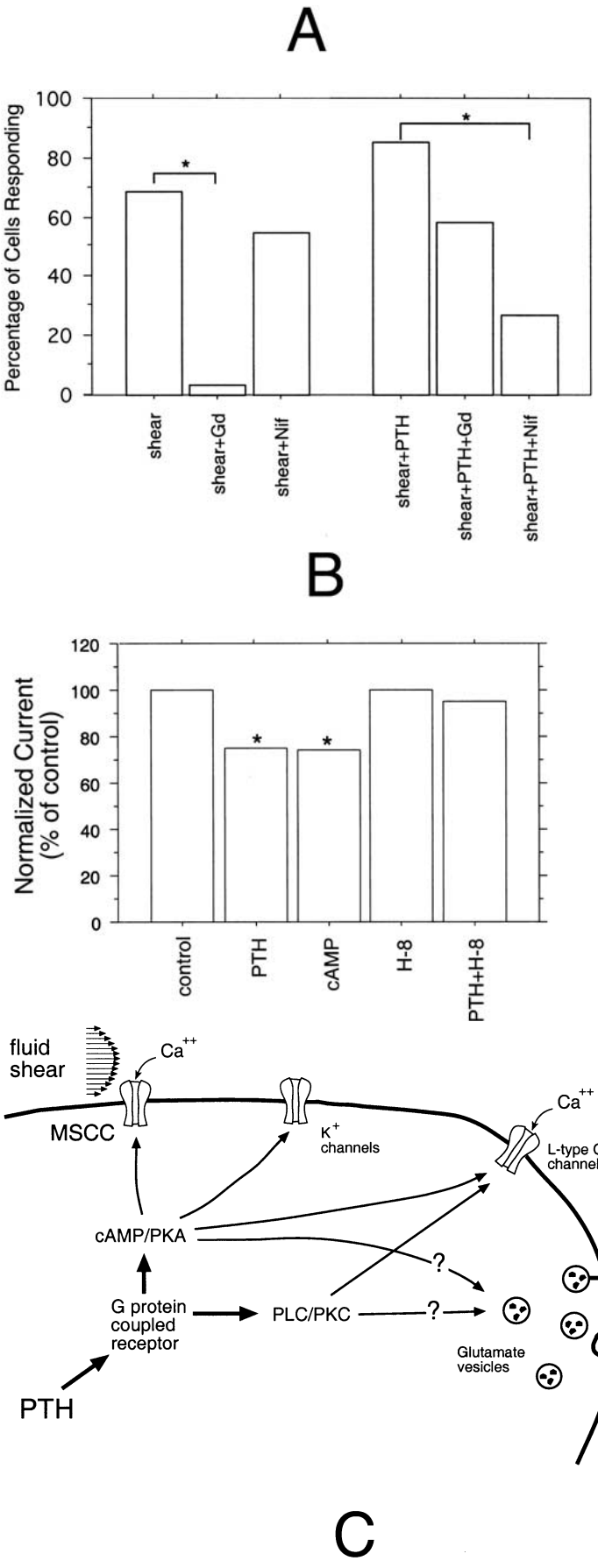

Fig. 4. Sensitization pathways in bone cells. (**A**) Fluid shear stress causes the majority of cultured osteoblasts to respond with increased intracellular calcium. Blocking the mechanically sensitive calcium channel (MSCC) with gadolinium (Gd) significantly inhibited the number of osteoblasts responding to fluid shear stress ($P < 0.001$ indicated by asterisk). The addition of PTH-(1–34) sensitized the cells to fluid shear, resulting in an enhanced intracellular calcium response (not shown), compared with fluid shear alone. Blocking the L-type calcium channel with nifedipine (Nif) completely blocked the PTH-(1–34) sensitization of cell (i.e., shear + Nif was not different from shear + PTH + Nif) [2]. (**B**) PTH-(1–34) reduces outward K^+ currents in osteoblasts, as does cyclic AMP. PKA inhibition with H-8 does not affect basal outward currents, but prevented PTH-(1–34) from inhibiting these currents [30]. *(Significant differences). (**C**) Like serotonin in the *Aplysia* reflex loop, PTH-(1–34) sensitizes bone cells through at least three biochemical pathways. PTH-(1–34) binds to two G protein-coupled receptors, which activate the cAMP/PKA and PLC/PKC pathways. The downstream effects of PTH-(1–34) include: (1) decreased potassium current through K^+ channels, (2) increased calcium current through L-type Ca^{2+} channels, and (3) increased calcium current through MSCCs. Based upon findings in neuronal systems, one might speculate that PTH enhances the release of glutamate from the bone cell, but this signaling pathway has not been studied.

surgical removal of the parathyroid glands from rats eliminates skeletal responsiveness to mechanical loads [31]. The combined effects of PTH tend to prolong osteoblast depolarization in a similar manner as serotonin-induced sensitization of neurons. In neurons, prolonged depolarization leads to enhanced glutamate release. However, at present, the effects of PTH (or serotonin) on glutamate signaling among osteoblasts has not been studied, so it remains unclear whether sensitization analogous to neuronal systems occurs in bone cells.

Effects of Spaced Training

In learning psychology it has been consistently shown that spaced training is more effective than massed training for producing long-term memory [15]. In the *Aplysia* reflex loop, a single session of 40 repeated stimuli causes short-term habituation lasting a few hours. However, four such sessions of 10 repeated stimuli, each separated by a day, produces long-term memory lasting for over a week [32]. An analogous experiment was recently completed in our lab [33]. The right ulnae of rats were subjected to mechanical loading in the form of 360 cycles once per day, or 90 cycles given in four bouts each separated by 3 hours. After 16 weeks, the latter loading regimen increased the bone mineral content of the ulna by 12% whereas the former loading regimen caused only a 7% increase (Fig. 5). This result demonstrates that partitioning exercise into multiple sessions is much more effective for 'training' bone to be stronger. This approach works because bone becomes habituated (desensitized) to prolonged mechanical loading and inserting 'rest' periods between loading bouts allows bone tissue to recover its mechanosensitivity.

Cellular Communication and Memory

Doty [34] established the existence of gap junctions between bone cells over 20 years ago. Gap junctions serve as conduits between the cytoplasm of two cells. They consist of two hemichannels called connexons, which in turn are made up of six subunits called connexins. The major functional connexin in bone cells is connexin 43 [35]. Gap junctions in bone facilitate mechanotransduction [36]. Consequently, increasing or decreasing cell coupling (by connexin 43 expression) may be one mechanism by which bone's mechanosensitivity might be modulated. Gap junctions require close contact between two adjacent cells—adjoining cell membranes are 3.5 nm apart. Chemical synapses required for neurotransmitter signaling typically require a distance of 20–40 nm between communicating cells. Unlike gap junctions, chemical synapses have not been identified in bone cells, yet the molecular machinery for neurotransmitter signaling is present [3–7]. One might assume that for

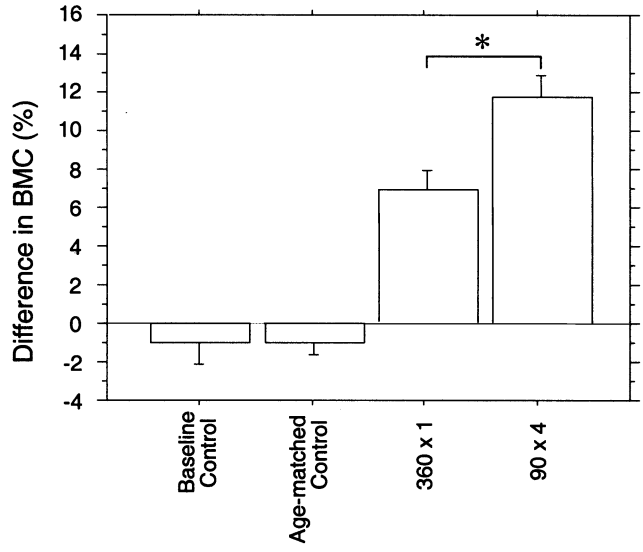

Fig. 5. Differences in bone mineral content (BMC) between right and left ulnae of rats that were trained for 16 weeks by applying axial loads to the right ulna. Training involved a single loading bout of 360 loads or 4 loading bouts of 90 loads, each separated by 3 hours. The latter training program was significantly ($P < 0.05$) more effective for building BMC in the ulna. Also shown are right-left differences in baseline and age-matched control rats (these were not significantly different from zero) [33]. Reprinted from "Shorter, more frequent mechanical loading sessions enhance bone mass", by Robling AG, Hinant FM, Burr DB, and Turner CH, In Med. Sci. Sports Exer., Copyright 2002, reprinted with permission of Lippincott Williams & Wilkins.

neurotransmitter signaling to be important in bone, chemical synapses between bone cells should exist. Scanning electron microscopy studies of osteocytes and osteoblasts *in vivo* demonstrate connections between osteocyte processes and the osteoblast cell body [37] (Fig. 6). Such connections could possibly form chemical synapses. Yet definitive evidence of chemical synapses, synaptic clefts for instance, between bone cells has not been demonstrated. Alternatively, it has been suggested that glutamate might act in bone somewhat like a cytokine, not requiring chemical synapses for signal transduction [38]. However, should this be the case, signaling by glutamate would be far less efficient than synaptic signaling.

As in neurons, bone cells may incorporate long-term memory through structural changes in the bone cell network, i.e., by an increase or decrease in the intercellular connections resulting in altered cellular communication. Unlike neurons, however, these structural changes may be 'set in stone' as the osteocytes become surrounded by mineralized matrix. Thus, any memory incorporated into the osteocyte network would be fixed until remodeled. Only at such time that matrix is resorbed by osteoclasts and reformed could the bone cell network, and memory, be restructured.

Osteocytes do not possess a functional glutamate receptor [39], so it is unlikely that they utilize glutamate

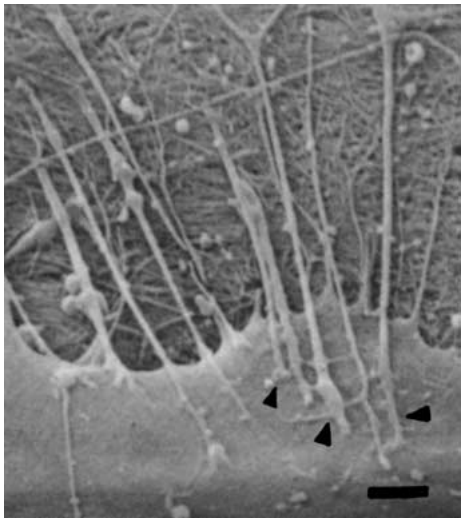

Fig. 6. An osteoblast showing cell processes extending from the cell body and penetrating the fibrillar matrix. Some processes appear to originate from osteocytes within the matrix and terminate on the cell body (arrowheads) [37]. Reprinted from "From bone lining cell to osteocyte–an SEM study", by Menton DN, Simmons DJ, Chang S-L, Orr BY, in Anat. Rec., Vol. 209, p. 30, Copyright 1984. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

for signaling with other osteocytes. Osteocytes do express GLAST, suggesting that they have the capacity to sequester and secrete glutamate [3]. Glutamate signaling might occur between osteocytes and osteoblasts or osteoclasts, since both cells have glutamate receptors. Furthermore, glutamate could serve as a signaling molecule among mature osteoblasts or between osteoblasts and osteoclasts [38]. Thus glutamate signaling is probably most important at "the point of attack", i.e., on surfaces with active osteoblasts or osteoclasts.

Among the possible scenarios of cell-to-cell communication in bone, one cannot rule out the importance of central control of bone cell responses. Recent studies show that leptin exerts influence on bone biology through a neuroendocrine pathway. Leptin acts on the hypothalamus and seemingly initiates control of bone formation through a neuronal pathway [40]. Likewise, mice lacking neuropeptide Y2 receptors have elevated bone volume, and ablation of the Y2 receptor gene specifically in the arcuate nucleus of the hypothalamus results in increased bone mass [41]. These results suggest that signals originating in the hypothalamus influence bone formation, possibly through a neuronal pathway. Bone tissue is known to be innervated by fibers containing substance P, vasoactive intestinal peptide, calcitonin gene-related peptide, and glutamate [8, 42–44]. During long bone development, both sensory and sympathetic nerve fibers are observed in intimate contact with bone marrow cells, osteoblasts, and osteoclasts. These fibers do not form typical synapses in the nerve endings [8], so it is unclear how they transmit or receive signals from bone cells. Nevertheless, studies have shown that denervation can affect bone remodeling [45] and mechanically induced osteogenesis [46]. In addition, vasoactive intestinal peptide has been shown to inhibit bone resorption *in vitro* [47].

Biological Effects of Cellular Memory in Bone

The idea of cellular memory has some similarities with another prominent model of bone biology, Frost's mechanostat [48]. The central idea of the mechanostat is that systemic influences (hormones, drugs, etc.) are translated into local bone biological effects through cellular machinery associated with mechanical loading responses. The theory predicts, for instance, that an anabolic drug will have greater effect in bony regions subjected to more intense mechanical stimulus. Thus, one molecular mechanism of the mechanostat might be sensitization, as described in Figure 4. However, the prospect of cellular memory implies biological possibilities well beyond the mechanostat, which is modeled after a linear system resembling a thermostat. There is growing evidence that bone tissue responses to mechanical loading are, in fact, nonlinear, suggesting that the system should be modeled using nonlinear mathematics, e.g., neural networks. The experimental evidence also suggests that the bone cell network might act as a learning system incorporating memory such that its responses to external stimuli vary depending upon previous loading history, i.e., the system is nonlinear or "plastic". Furthermore, emerging evidence showing control of bone biology by the central nervous system opens the possibility that bone biology cannot be reduced to a series of local autocrine/paracrine interactions, as suggested by the mechanostat. Central neuronal control could provide one mechanism for cellular memory in bone.

The molecular mechanisms of bone cell plasticity could be similar to those observed in neuronal systems although other pathways are possible. One interesting pathway involves calmodulin, which translocates from the cytoplasm to the nucleus and causes the phosphorylation of cAMP-response element binding (CREB) [49]. Interestingly L-type Ca^{2+} channels and NMDA receptors are able to cause the mobilization of calmodulin, whereas N- and P/Q-type Ca^{2+} channels are not, Calmodulin also acts as a Ca sensor that can autoregulate L-type Ca^{2+} channels [50]. The calmodulin pathway is particularly intriguing considering the established role of the L-type Ca^{2+} channel in PTH sensitization of osteoblasts (Fig. 4). Evidence supporting the neuronal analogy was reported recently by Spencer et al. [51] who showed that mechanical loading of osteoblast-like cells stimulated autophosphorylation of activated CAM kinase II, an event associated with learning in neuronal systems.

In neuronal systems, application of repeated stimuli results in diminished glutamate release and habituation

[15]. Thus, by analogy one might speculate that glutamate signaling is key to bone tissue desensitization after repeated mechanical loading. This hypothesis could be tested using mice deficient in the gene for the GLAST (glutamate transporter). These mice develop normal skeletons indicating that GLAST is not necessary for skeletal development [52]. However, if glutamate signaling involving GLAST is important in bone desensitization to loading, GLAST-deficient mice will not respond better to spaced training compared with massed training. Hence, an experiment similar to that described in Figure 5 using GLAST-deficient mice compared with normal mice would provide insights into the role of GLAST (and glutamate) in bone memory. Alternatively, habituation of bone cell responses to mechanical loading may involve desensitization of G protein-coupled receptors. Mice deficient in G protein-coupled receptor kinase 5 exhibit behavioral supersensitivity upon challenge due to a deficit in receptor desensitization [53]. Studies of the response of these mice to skeletal loading could provide information about the role of G protein desensitization in habituation of bone tissue exposed to mechanical loading.

Learning, i.e., the accumulation of memories, imparts individuality. Consequently, the incorporation of memories of mechanical loading events during growth and development might strongly influence bone biology. Consider two identical twins, one who grew up on earth and the other who grew up in the weightless environment of a space station. Assume the two are reunited on earth after many years and their bone biology is evaluated. Through the cellular memory mechanism, the time spent in space could have lasting effects on the bone cell responsiveness to a wide variety of external stimuli. Investigation of these hypothetical twins is in fact possible. Inbred mice or rats (twins) could be raised in simulated weightlessness (e.g., tail suspension) or under normal weightbearing conditions, and their skeletal responses to external stimuli measured. This experiment would be an excellent test of the hypothesis that bone incorporates long-term memory of loading history.

In conclusion, the idea of cellular memory implies that the bone cell network is plastic, i.e., its biologic responsiveness is continuously changing in response to its environment. There are a number of promising cellular and molecular pathways that might impart cellular memory in bone, yet none has been confirmed experimentally. Little is known about how bone cells behave as a network. It is intriguing to view bone as a neuronal network, i.e., a learning system. Further studies are needed to confirm this viewpoint.

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