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Review article

INNERVATION OF BONES: WHY IT SHOULD NOT BE NEGLECTED?

INERVACIJA KOSTIJU: ZAŠTO JE NE TREBA ZANEMARITI?

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Abstract

Bones encompass a diverse network of sensory, sympathetic and even parasympathetic nerve fibers. While there is still insufficient understanding of the exact roles of these fibers in the skeleton, there is increasing evidence that they serve both afferent and efferent functions. Apart from pain transmission, some of their functions are regulation of bone remodeling, skeletal growth and fracture healing. That indicates that further research on bone innervation may shed more light on the main topics of bone biology, such as bone fragility in aged and osteoporotic individuals, alterations in fracture healing in various conditions, bone cancer pain, etc. This review article will present main morphological and functional characteristics of bone innervation.

Keywords:
bone,
nerve fibers,
bone remodeling,
pain transmission

Sažetak

Kosti sadrže raznovrsnu mrežu senzornih, simpatičkih, pa čak i parasimpatičkih nervnih vlakana. Iako tačne uloge ovih vlakana nisu još uvek sasvim jasne, sve je više dokaza da ova vlakna imaju i aferentne i eferentne uloge. Osim prenošenja bolnih nadražaja, neke od uloga ovih vlakana su i regulacija koštanog remodelovanja, koštanog rasta i zarastanja preloma. Ovo pokazuje da dalja istraživanja inervacije kostiju mogu doprineti rasvetljavanju glavnih istraživačkih pitanja u oblasti koštane biologije, kao što su fragilnost kosti kod starijih osoba i u osteoporozi, promene u zarastanju preloma u različitim stanjima, kancerski bol i dr. Ovaj pregledni članak prikazuje osnovne morfološke i funkcionalne karakteristike koštane inervacije.

Ključne reči:
kost,
nervna vlakna,
koštano remodelovanje,
transmisija bola

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Introduction: Bone innervation as a neglected topic

Nervous system is an important regulatory system in the body, controlling a number of body functions and ensuring responses to internal and external stimuli. Bones are capable of reacting both to external stimuli (mechanical loading) and internal demands (hormonal and metabolic), providing the body with mechanical stability during motion and stance, as well as acting as a depot for calcium and phosphorus.

Standard anatomy textbooks (1,2) describe innervation of bones only marginally (e.g. Hilton's law), while standard physiology textbooks (3,4) provide no information on bone innervation. Considering that mechanical and hormonal factors are regarded as the main regulators of bone homeostasis (5,6), the topic of innervation of bones usually does not seem as an essential one. Hence, there is a general lack of understanding of the roles of nerve fibers in the skeleton. Nevertheless, as severe bone pain becomes more prevalent due to increasing frequency of malignant tumors and bone metastases (7), specific therapeutic approaches targeting bone pain transmission are needed (8). Therefore, the topic of bone innervation now attracts an increasing attention in research studies, and researchers realize that nerve fibers may be of extreme importance for many processes in the skeleton, not just for pain transmission.

This review paper will present the main morphological and functional characteristics of bone innervation, with the emphasis on how such a relatively neglected topic can give missing answers to some of the key questions of bone biology.

Bone-related nerve fibers are sensory and sympathetic fibers

So far, the studies in bones identified fibers of various diameters and different myelination status, but with the exception of thick myelinated fibers (9). After early opinions that all fibers are solely autonomic, it was shown that bones have both sensory and sympathetic fibers (10,11). Based on the diameter, impulse conduction velocity and presence or absence of a myelin sheath, these fibers correspond to A-delta and C type of fibers (12). The fibers found in bone tissue can be further classified into subpopulations based on the markers that they express and that allow their visualization by the methods of immunohistochemistry or immunofluorescence (10,11,13) (**Table 1**).

Previous studies confirmed that sympathetic fibers express tyrosine hydroxylase (TH), which is a rate limiting enzyme in the process of synthesis of noradrenaline, as the main sympathetic neurotransmitter (10,14). Beside TH, sympathetic fibers often express neuropeptide Y (NPY) and some of them contain vasoactive intestinal peptide (VIP) (10,14). All these fibers are postganglionic and they reach the bones via the peripheral nerves that also supply sensory fibers to the bone (9) or via perivascular meshes.

On the other hand, the subpopulations of sensory fibers express calcitonin gene related peptide (CGRP), substance P (SP), isolectin B4 (IB4) and neurofilament H (NFH or NF200, RT-97) (10,13) (**Table 1**). They are peripheral processes of pseudounipolar neurons, with the neural cell bodies located in the sensory ganglia (15,16).

More recent study showed that TrkA receptor (TrkA = Tropomyosin receptor kinase A; neurotrophic tyrosine kinase receptor type 1) is expressed by the majority of myelinated/unmyelinated sensory and sympathetic nerve fibers that innervate the periosteum, bone marrow and mineralized bone, in contrast to few sensory fibers supplying the skin, which allows specific skeletal analgesia with NGF/TrkA inhibitors (17,18).

Bone related nerve fibers reach all bone compartments

Considering strong pain that occurs after a fracture or traumatic injury of the periosteal surface, it is usually considered that periosteum is the most innervated tissue

Table 1. Morphological, functional and immunohistochemical characteristics of subpopulations of nerve fibers inervating bone.

Functional type of fibers	Morphology	Marker/s	Localization in bones
Sensory fibers	Mostly myelinated (A-delta)	Neurofilament H, 200 kDa (RT-97) (NF200)	Periosteum, bone marrow and mineralized bone
	Mostly unmyelinated (mostly C fibers)	*Peptidergic C fibers:* Calcitonin gene-related peptide (CGRP); Substance P (SP)	Periosteum, bone marrow and mineralized bone
	Unmyelinated (C fibers)	*Non-peptidergic C fibers:* Isolectin B4 (IB4); Purinergic P2X3 receptor	Only at muscle attachment sites
Postganglionic sympathetic fibers	Unmyelinated (C fibers)	Tyrosine hydroxilase (TH); Neuropeptide Y (NPY); Vasoactive intestinal peptide (VIP)	Periosteum, bone marrow and mineralized bone

in bone. However, previous studies in mouse femur showed a rich network of sensory and sympathetic fibers in the bone marrow, mineralized bone and periosteum (13,17). While indeed high numbers of fibers per area were found in the periosteum, the highest number of fibers was found in the bone marrow compartment, considering its greater volume (13). Mineralized bone (cortical bone) compartment also showed nerve fibers spreading through many of the Haversian and Volkmann's canals (10,13). There was also inter-site variation in the density of neural fibers within a long bone, so that the region of the diaphysis showed the lowest number of fibers, whereas the metaphyseal regions had the richest nerve supply (13). Most of the fibers are associated with blood vessels in bone; nevertheless, those unassociated with blood vessels and free nerve endings were also found (13,19). While mouse long bones showed peculiar distribution of fibers among the main anatomical parts of the bone, calvaria and mandible did not demonstrate outstanding regional differences (13).

Most information about localization of nerve fibers in bone comes from the studies in animals, while human data are rather scarce. In humans, it was shown in lumbar and first sacral vertebra that dense network of fibers mostly concentrates in the central zone of the vertebra, and that both the endplate and the body of the vertebra are densely innervated, as visualized using immunohistochemistry staining against a ubiquitous neural marker PGP 9.5 (20). Additional staining for CGRP, in the lumbar vertebral body, showed that most of the fibers within the vertebral body were CGRP-positive, indicating their role in nociception and explaining bone pain even in the cases where periosteum is not damaged (21).

Bone related nerve fibers have both afferent and efferent roles in the skeleton

Although the exact functions of these fibers are still unclear, it is striking that there are experimental data that they serve both afferent and efferent roles (13). They certainly have important functions in transmission of bone pain, but also play a role in bone remodeling, osteogenic differentiation during skeletal growth, as well as in bone repair and fracture healing (22).

Markers expressed by most of the sensory neurons are consistent with a role in nociception (16). The fact that they are localized not only in the periosteum, but also in the bone marrow and mineralized bone compartments, may explain the origin of skeletal pain even in the lesions that do not affect the periosteum. In addition to nociception, periosteal fibers respond to mechanical, chemical, and thermal stimuli to the periosteum (for a review see (16)). In particular, considering that CGRP+ and NF200+ sensory fibers form a dense mesh in the periosteum of the mouse femur, it was suggested that they are strategically organized to detect mechanical distortion of the periosteum and underlying mineralized bone (23). The fibers innervating the bone marrow are also able to detect various sensory modalities, given that it was shown that whole nerve is stimulated by increasing intra-osseous pressure, chemical stimulation or temperature changes of the bone marrow (16). In particular, they can recognize changes in local pH, where local acidification due to osteoclastic and bone-colonizing cancer cells' release of protons is detected via acid-sensing nociceptors expressed on sensory neurons (transient receptor potential channel-vanilloid subfamily member 1 - TRPV1, and the acid-sensing ion channel 3 - ASIC3), thus contributing to bone cancer pain (24).

Nevertheless, a number of other important functions of these fibers were acknowledged in the experimental studies. It is evident that sensory and sympathetic neurotransmitters and neuropeptides have trophic effects that are critical for joint and bone homeostasis (25). For instance, using capsaicin in an experimental study to selectively destroy unmyelinated sensory neurons in rats, led to depletion of substance P and CGRP in bone and caused significant loss of trabecular bone, suggesting that capsaicin-sensitive sensory nerves contribute to trabecular bone integrity (26,27). Obviously, CGRP and SP that are released from the peripheral terminals of sensory neurons are important local mediators ensuring maintenance of normal bone balance. This is probably mediated via specific receptors on bone cells, where CGRP stimulates osteoblasts while inhibiting osteoclast differentiation and/or function (22,26,28,29). Unlike CGRP that shows bone anabolic behavior, substance P can increase bone formation when present in high concentrations; otherwise, it increases bone resorption (30,31). The TrkA-expressing sensory nerves innervating long bones stimulate load-induced bone formation through the Wnt/β-catenin pathway (32), and it was shown in experimental studies in mice that NGF-TrkA signaling in skeletal sensory nerves mediates bone formation in response to mechanical loading (33).

Sympathetic fibers are mostly related to the blood vessels (23) and likely control blood flow in bone through vasoconstriction. Like CGRP-positive sensory fibers, VIP-positive sympathetic fibers play a role in suppressing bone resorption through RANKL/OPG pathway, similar to mechanical loading, as shown in cell culture experiments (34,35). Nevertheless, the role of sympathetic system in bone remodeling is still contradictory (25), considering that destruction of sympathetic neurons by guanethidine was found to reduce the differentiation and activity of osteoclasts (36). Furthermore, there is evidence that sympathetic system increases bone resorption when subjected to microgravity conditions, i.e., that beta blockers may be used to prevent bone loss (37). However, more studies are needed until beta blockers could be targeted as an osteoporosis prevention drug (22).

A recent study in mouse femur showed that aging leads to a reduced number of nerve fibers in bone, particularly decline in sympathetic fibers (19). Further research is needed to understand whether aging and various disease processes in humans affect the density of bone related nerve fibers, and whether neural alterations may be related to the observed increase in bone fragility in various diseases, as well as altered fracture healing and bone pain.

Both sensory and sympathetic fibers establish synapse-like contact with bone cells which display receptors for the substances released from the neural fibers

The observed effects of neurotransmitters and neuropeptides on bone metabolism suggest the relationship between the nerve fibers and bone cells (**Figure 1**). Considering that intercellular communication is difficult to analyze in hard tissue in situ, there is not much direct evidence of the type of the connection between the fibers and bone cells. It was shown on rat's long bone by electron microscopy that nerve fibers running along blood vessels are located in vicinity of hematopoietic cells and bone cells (11). Some of these nerve fibers clearly showed "local dilatations in contact with medullary cells and bone cells that were immunolabeled for synaptophysin, a nerve terminal marker" (11). A more detailed assessment was possible in cell co-culture of sensory neurons and osteoblasts, showing that they established a close synapse-like contact (38-40). Moreover, cell culture experiments showed that osteoblasts and sensory neurons communicate bidirectionally: peripheral neurite terminals release glutamate and substance P by exocytosis (efferent signal to osteoblasts) and osteoblasts release adenosine triphosphate - ATP (afferent signal to neurites) (39,40). It is interesting that mechanical stimulation of osteoblasts in cell culture was able to activate the neurite of the co-cultured sensory neurons, which was based on the release of ATP from osteoblasts and its binding on the purinergic receptors on the neurite (40). In that way, sensory neurons can further transmit the information of mechanical loading, which may be a part of the regulatory loop between the central nervous system and bone that controls bone homeostasis.

There is also a direct communication between sympathetic neurons and bone cells (osteoblasts and osteoclasts), where osteoblastic and osteoclastic activation by sympathetic neurons in vitro is mediated, at least partly, by noradrenaline acting through α1-adrenergic receptors on bone cells (41,42). The already mentioned contradicting effects of sympathetic neurons on bone may partly originate from differential effects of noradrenaline on different types of adrenergic receptors (**Figure 2**). For example, osteoblasts are activated by stimulation of α1-adrenergic receptors, and inhibited by acting on β2-adrenergic receptors (43-45). Osteoclastogenesis is suppressed via α2-adrenergic receptors, and stimulated via α1- and β2-adrenergic receptors (42,46-48). In the situation when different adrenergic receptor types are expressed by the same cell, the concentration of noradrenaline is likely a decisive factor determining the preferred receptors and corresponding effects (22).

It was suggested that various neuropeptides or neurotransmitters released from the skeletal nerve fibers have paracrine effects on the neighboring bone cells (49),

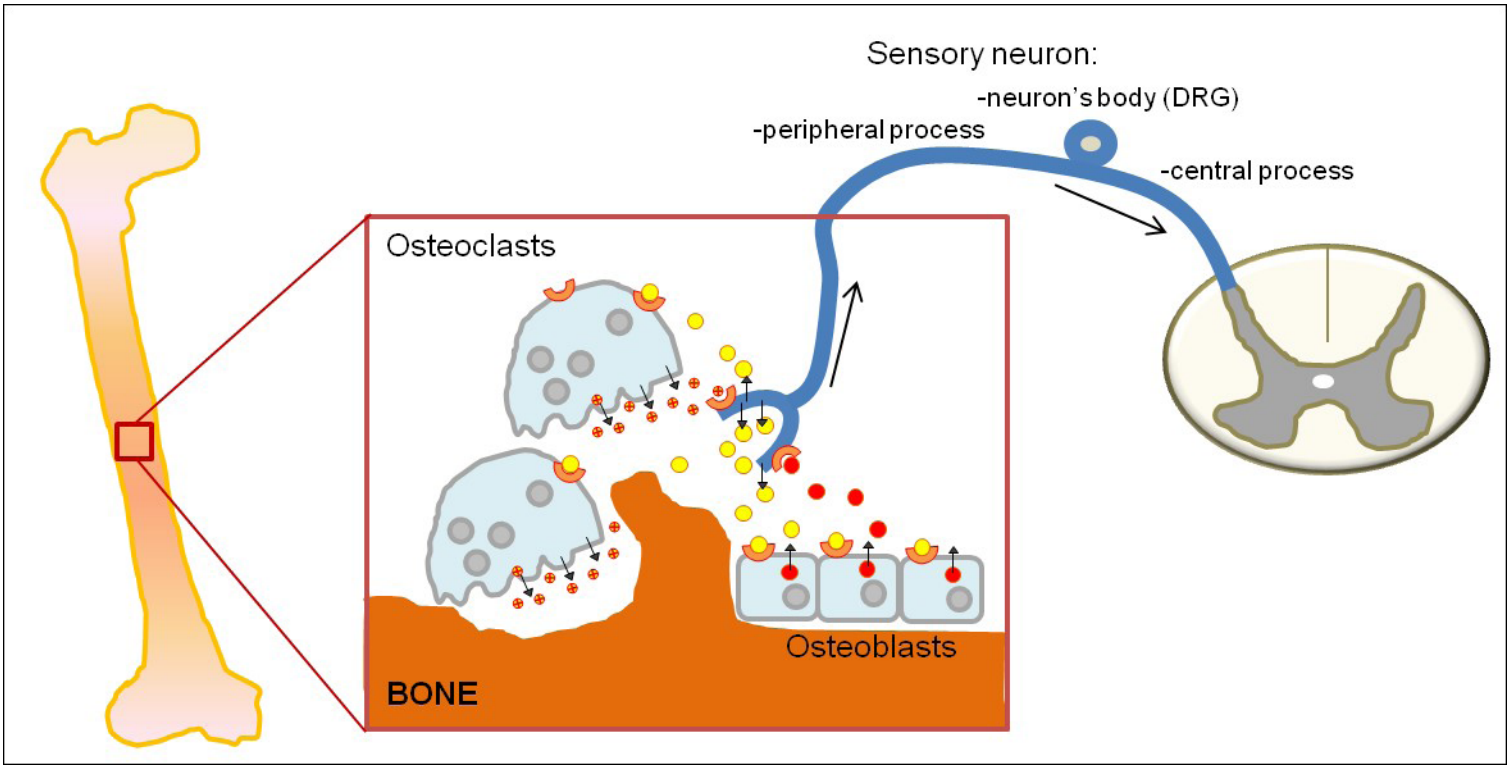

Figure 1. Schematic example of bidirectional communication between sensory neurons and bone cells. Note that peripheral terminals of sensory nerve fibers (blue) release neuropeptides (yellow vesicles: e.g. CGRP, SP, etc.) that bind to the receptors on bone cells and affect their activity. On the other hand, osteoblasts release adenosine triphosphate (red vesicles) and osteoclasts release protons (small dots) that activate the corresponding receptors on the peripheral nerve terminals. The sensory nerve carries the electric impulse to the spinal cord. (DRG-dorsal root ganglion).

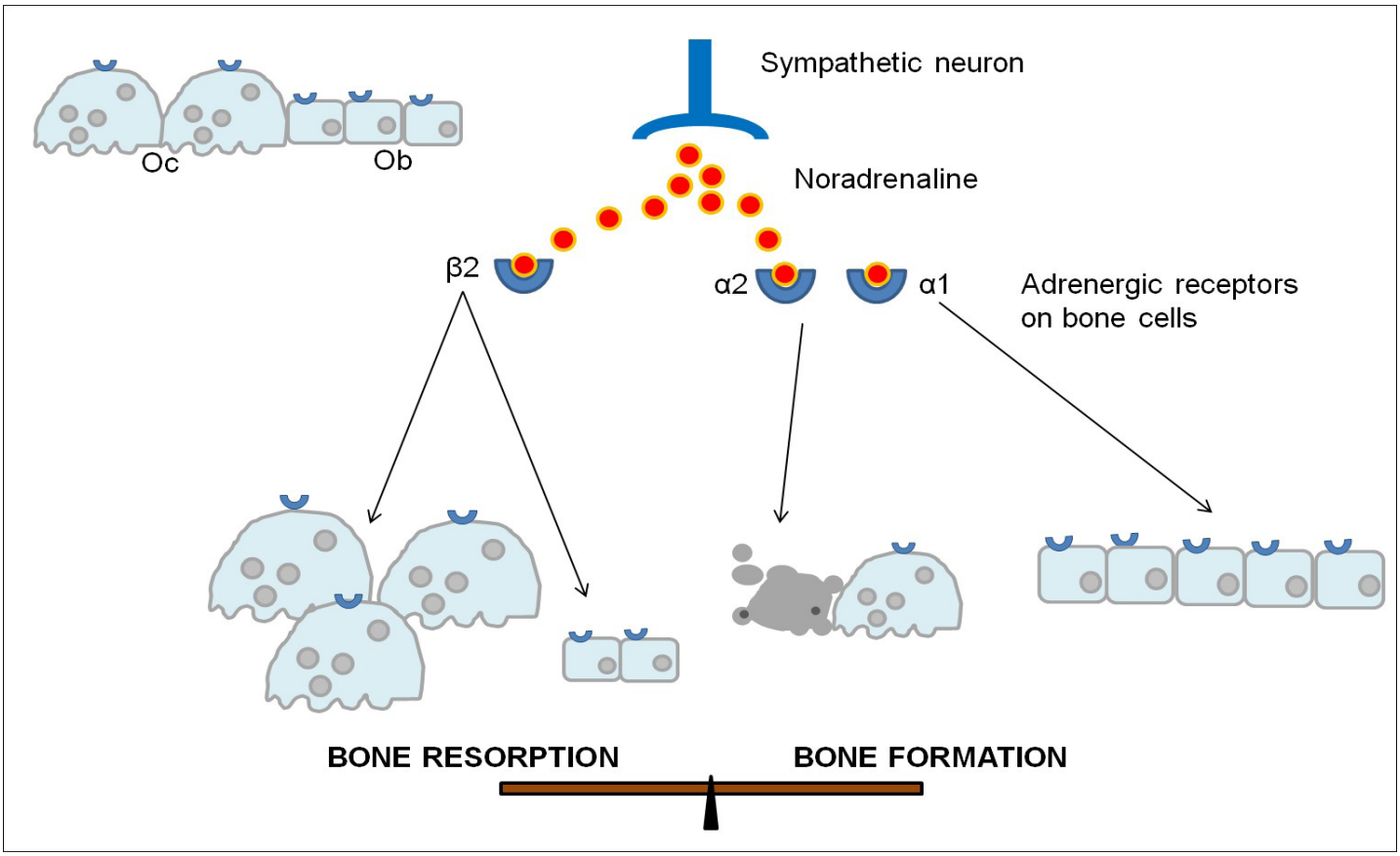

Figure 2. Effects of the sympathetic neurons on the main processes in bone. Sympathetic neurons release noradrenaline that binds to adrenergic receptors on bone cells. Depending on the type of adrenergic receptor, the sympathetic effects vary considerably. While activating β2 receptors leads to a shift to bone resorption (increased number and activity of osteoclasts, and decreased osteoblastic number and activity), activation of α1 and α2 receptors favors bone formation (reduced osteoclasts number, increased number and activity of osteoblasts). (Oc-osteoclasts, Ob-osteoblasts).

considering that treatment of osteoblasts with SP, CGRP, VIP, NPY or TH in vitro increased osteoblasts viability, induced alkaline phosphatase activity and osteocalcin production (49). In addition, glutamate signaling was shown to promote differentiation and activation of osteoblast cell lineage (50). Indeed, osteoblasts and osteoclasts have receptors for these soluble factors (**Table 2**), but more research is needed to understand the relevance of each neuropeptide and receptor type for bone remodeling activities.

All studies considered the effects of neuropeptides and neurotransmitters on osteoblasts (bone forming cells) and osteoclasts (bone resorbing cells), and we do not know whether osteocytes that are the most numerous bone cell type also express receptors for these neuropeptides and neurotransmitters. Considering the strategic distribution of osteocytes through the mineralized bone matrix, their

Table 2. Neuropeptide and neurotransmitter receptors on bone cells

Receptor	Abbreviation	Ligand	Locations
Neurokinin 1 receptor	NK1R	Substance P	Osteoblasts, osteoclasts and preosteoclasts, bone marrow stromal cells
CL receptor/RAMP	CLR	CGRP	Osteoblasts, osteoclasts, bone marrow stromal cells
β٢ adrenergic receptor	β٢-AR	Noradrenaline	Osteoblasts, osteoclasts
α١ adrenergic receptor	α١-AR	Noradrenaline	Osteoblasts, osteoclasts
α٢ adrenergic receptor	α٢-AR	Noradrenaline	Osteoblasts, osteoclasts and preosteoclasts
VIP receptors	VIP-1, VIP-2	VIP	Osteoblasts, osteoclasts
Glutamate receptors (various classes)	GluR	Glutamate	Osteoblasts, osteoclasts
ACh receptors (various classes)	ACh receptors	Acetylcholine	Osteoblasts, osteoclasts

neuron-like shape and interconnectivity of osteocytic dendrites, they are nowadays considered as the main sensors of mechanical loading, as well as orchestrators of bone remodeling/repair (6). The abundant data from our group showed that aging and osteoporosis are associated with a decline in number, viability and connectivity of osteocytes, resulting in altered mechanosensing ability of bone and delayed and/or deficient bone remodeling (6,51-56). It would be of particular interest to investigate whether osteocytes communicate with nerve fibers and/or whether they respond to main neuropeptides.

Presence of acetylcholine (ACh) receptors on osteoblasts (57) raises the question whether bone also contains parasympathetic fibers. It has recently been shown that mouse's femoral metaphysis had some nerve fibers expressing VAChT (vesicular ACh transporter) (58) which is believed to be a marker of parasympathetic cholinergic fibers (58,59). Moreover, retrograde propagation of immunoreactive pseudo rabies virus from the femoral metaphysis to the sacral parasympathetic center of the spinal cord confirmed the parasympathetic origin of these fibers (58). It was shown that cholinergic signaling in bone specifically stimulates osteoclasts' apoptosis, but also can increase osteoblasts' number (58), resulting overall in positive bone balance. However, more research is needed to demonstrate whether different bones have parasympathetic fibers and whether that also occurs in humans.

Conclusion

Having in mind all the previous considerations, we can conclude that bone houses a diverse network of sensory, sympathetic and even parasympathetic neural fibers that may have specific functions related to the bone metabolism. Specific subpopulations of fibers can be visualized under the microscope after immunostaining for specific markers, such as CGRP, substance P, tyrosine hydroxylase, etc. Further research will identify whether aging and various disease processes in humans affect the density of these fibers in the bone tissue and whether that relates to increased bone fragility, altered fracture healing and bone pain.

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