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No pain, no gain? The effects of pain-promoting neuropeptides and neurotrophins on fracture healing

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Abstract

Neuropeptides and neurotrophins are key regulators of peripheral nociceptive nerves and contribute to the induction, sensitization, and maintenance of pain. It is now known that these peptides also regulate non-neuronal tissues, including bone. Here, we review the effects of numerous neuropeptides and neurotrophins on fracture healing. The neuropeptides calcitonin-gene related peptide (CGRP), substance P (SP), vasoactive intestinal peptide (VIP), and pituitary adenylate cyclase-activating peptide (PACAP) have varying effects on osteoclastic and osteoblastic activity. Ultimately, CGRP and SP both accelerate fracture healing, while VIP and PACAP seem to negatively impact healing. Unlike the aforementioned neuropeptides, the neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) have more uniform effects. Both factors upregulate osteoblastic activity, osteoclastic activity, and, *in vivo*, stimulate osteogenesis to promote fracture healing. Future research will need to clarify the exact mechanism by which the neuropeptides and neurotrophins influence fracture healing. Specifically, understanding the optimal expression patterns for these proteins in the fracture healing process may lead to therapies that can maximize their bone-healing capabilities and minimize their pain-promoting effects. Finally, further examination of protein-sequestering antibodies and/or small

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molecule agonists and antagonists may lead to new therapies that can decrease the rate of delayed union/nonunion outcomes and fracture-associated pain.

Keywords

Fracture healing; bone regeneration; neuropeptides; nerve growth factors; nociceptors; musculoskeletal pain

1. Introduction

Despite the best efforts of orthopedic traumatologists, other healthcare providers, and patients, about 5–10% of all bone fractures do not successfully heal [1,2]. Impaired bone healing causes a fracture to progress to either a delayed union or nonunion, which becomes more challenging to manage for both the patient and the clinician [1]. Additionally, fractures and reparative surgeries are associated with acute and chronic pain. Both impaired fracture healing and long-term pain result in extensive and prolonged disability to patients, leading to a substantial additional burden on the healthcare system and society as a whole [1–3]. Therefore, an ideal therapeutic strategy for the management of a fracture would need to simultaneously improve the bone healing process and diminish the fracture-associated pain. To date, no such therapy exists.

The perception of pain begins with the activation of peripheral nociceptors and the consequential stimulation of primary sensory afferent neurons. When released locally into sites of injury, various signaling molecules can modulate how peripheral sensory nerves respond to noxious stimuli, thus altering how pain is perceived. Specifically, certain peptides can induce peripheral sensitization, which manifests as primary hyperalgesia. Some peptides may also directly induce pain, augment the release of other pain-sensitizing molecules, stimulate the growth of sensory fibers, or contribute to the development of chronic pain (For review, see [4]). In this review, we have chosen to focus on two classes of peptides that modulate the function of nociceptive nerves: neuropeptides and neurotrophins.

Neuropeptides are a diverse class of signaling molecules that play a crucial role in the nervous system. Classically, they have been defined as small proteinaceous substances (4 to 40+ amino acids long) that are produced by neurons, released at nerve endings upon neuron stimulation, and then act on neural substrates by binding neural receptors [5,6]. Several neuropeptides have been found to localize specifically to peripheral sensory and autonomic nerve fibers, where they play a role in modulating nociceptive, neuropathic, and inflammatory pain. Binding of these neuropeptides either enhances or blocks the effect of other neurotransmitters and/or neuropeptides involved in nociception [4,5,7].

Neurotrophins are a distinct family of peptides that are key regulators of neuronal survival and growth. The family includes the prototypical member, nerve growth factor (NGF), and the related peptides: brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). Numerous studies have identified NGF, and to a certain degree BDNF, as key regulators of pain in the periphery [8,9]. NGF has been shown to not only ensure the survival and sprouting of peripheral nociceptive fibers, but also to sensitize

nociceptive neurons [7] and to induce pain [10]. Through these mechanisms, NGF was identified as a significant contributor to osteoarthritis pain (For review, see [11,12]). These findings eventually led to the first in-human clinical trials testing anti-NGF antibodies as a therapy for osteoarthritis knee pain [13]. BDNF has been found to be constitutively synthesized by small- and medium-sized peptidergic primary afferent sensory neurons [14]. Although the majority of studies have focused on its role as a central sensitizer of pain, there is evidence suggesting it may increase pain in the periphery [8,9,15] and mediate the transition from acute to chronic pain [16].

Both families of proteins have since been discovered to also modulate the functioning of non-neuronal tissue, such as bone. Early denervation studies provided clues that bone could be a target tissue for these proteins. Such studies found that denervation could lead to significantly impaired bone healing with decreased callus density and mechanical strength [17–19]. Thanks to such groundwork studies, and advances in molecular biology, more researchers have become interested in examining the role neuropeptides and neurotrophins play in the regulation of bone healing. Such work could potentially reveal new therapeutic avenues by which both fracture healing could be improved, and fracture-associated pain could be reduced. Therefore, the aim of this review is to identify pain-promoting neuropeptides and neurotrophins, and to then summarize their effects on fracture healing (Fig. 1).

In this review, we highlight neuropeptides and neurotrophins which are known to either induce or sensitize peripheral pain, especially in musculoskeletal disorders. Additionally, we examine proteins that have been localized to peripheral nerves or found to be released locally in sites of injury. These proteins were examined as they are the most likely to both stimulate pain and modulate bone healing during tissue damage, such as that incurred from a fracture or surgical trauma. Specifically, here we examine the following neuropeptides: calcitonin-gene related peptide (CGRP), substance P (SP), vasoactive intestinal peptide (VIP), and pituitary adenylate cyclase-activating peptide (PACAP); and the following neurotrophins: NGF and BDNF.

2. Neuropeptides

2.1. Calcitonin-Gene Related Peptide

CGRP is a 37-amino-acid peptide that is a member of the calcitonin family of peptides, which are derived from alternative splicing of RNA transcripts of the *CALCA* gene. Humans express two forms of CGRP, α-and β-CGRP. They exert similar biological effects but differ in their amino acid composition. CGRP has been found to be distributed throughout various tissues, including the central (CNS) and peripheral nervous systems (PNS), the cardiovascular and respiratory systems, gastrointestinal tract, urogenital tract, and thyroid gland. In the PNS, CGRP co-localizes with SP in peptidergic primary sensory neurons arising from the spinal cord [20]. CGRP's effects are mostly mediated by a high-affinity dimer consisting of calcitonin receptor-like receptor (CRL) and receptor activity-modifying protein 1 (RAMP1) (For review, see [21]). Through this mechanism, CGRP has been identified to facilitate pain transmission and sensitization [22].

CGRP's role in musculoskeletal pain has been characterized best in osteoarthritis (OA). Numerous studies have found that CGRP levels are elevated in the serum, synovial fluid, and synovial tissue from patients with OA. Pain intensity has also been found to be positively associated with serum CGRP levels specifically [23,24]. Unfortunately, CGRP antagonists and antibodies have not found any success in managing OA pain and are instead primarily indicated for migraine treatment [22]. One recent clinical trial reported that the migraine drug galcanezumab, an antibody for CGRP, was no better than placebo for the treatment of mild to moderate OA pain [25]. CGRP's widespread dissemination in fracture tissue also makes it likely to be a contributor to fracture pain. Additionally, it has also been found to be a potent vasodilator [26], to induce angiogenesis [27], and to contribute to inflammation [28]. It is now known that CGRP has numerous non-neuronal tissue targets, including bone.

Studies discovered that CGRP could act similarly to calcitonin by inhibiting osteoclasts and lowering blood calcium both *in vitro* and *in vivo* [29]. In a dose-dependent manner, CGRP inhibited bone resorption stimulated by parathyroid hormone, prostaglandin E2, and 1,25-dihydroxyvitamin D3, but had no effect on basal bone resorption [30]. Still, in comparative studies between CGRP and calcitonin, CGRP was 100-to 1000-fold less potent than calcitonin in preventing bone resorption [31–33]. These results suggested that CGRP was unlikely to be a systemic regulator of bone and plasma calcium but could instead potentially act as a local regulator of bone cell function.

CGRP's regulation of osteoclasts and bone resorption has been further elucidated. A study performed on α-CGRP knockout (KO) mice observed decreased receptor activator of nuclear factor kappa-B ligand (RANKL) expression in the KO mouse calvarium, vertebral bodies, and femurs [34]. Additionally, Wang et al. discovered the presence of complete CGRP receptors (CRL/RAMP1 dimers) on the plasma membrane of bone marrow-derived macrophages (BMMs) and osteoclasts. They also determined that CGRP blocks RANKL-mediated activation of NF-κB in BMMs, thus inhibiting osteoclastogenesis and bone resorption [35,36]. Human CGRP also decreased RANKL expression in primary osteoblasts in a dose-dependent manner, which was reversible by treatment with a CGRP receptor antagonist [37]. Therefore, these studies confirmed that CGRP could control bone resorption via direct regulation of the differentiation and activity of osteoclasts.

In addition to downregulating bone resorption, CGRP upregulates bone formation. *In vitro*, CGRP induces osteogenesis in a dose-dependent manner [38,39] and enhances cAMP levels in multiple osteoblastic cell lines [40]. Interestingly, unlike α-CGRP, β-CGRP has no osteogenic stimulatory effect on rat bone marrow cells *in vitro* [38]. α-CGRP KO mice were found to have reduced bone formation and developed osteopenia. Multiple investigators have found that CGRP can induce various cell types and cultures to differentiate into mature osteoblasts. Examples include primary human osteoblasts and MG-63 osteoblastic cells in co-culture with vascular endothelial cells [41], adipose-derived stem cells in a 3D calcium alginate gel [42], adherent bone marrow derived stromal cells (BMSCs), and MC3T3-E1 osteoblast precursor cells [35]. Just as in BMMs and osteoclasts, complete CGRP receptors have been found on the plasma membrane of BMSCs and osteoblasts. CGRP induces mature osteoblast gene expression, as evidenced by elevated mRNA levels of runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), osteocalcin (OCN), collagen

type I, and activating transcription factor 4 (ATF4) [35,37]. These effects seem to be mediated via the Wnt/β-catenin pathway [43]. These studies thus support CGRP's role in driving the differentiation of precursor cells towards mature osteoblasts and stimulating osteogenesis.

The hypothesis that CGRP was a local regulator of bone cell function gained further support from various immunohistochemical studies examining the sensory innervation of bone. Rat mandibular periosteum was found to be innervated by a network of CGRP-immunoreactive (CGRP+) nerve fibers. Retrograde tracing of these nerves traced their cell bodies back to the ipsilateral trigeminal ganglia. In conjunction with co-staining for SP, CGRP+ nerves were determined to be primary afferent sensory neurons [44]. This result was confirmed in rat long bones; CGRP+ nerves with both vascular and non-vascular endings were identified. Non-vascular endings innervated regions of high osteogenic activity, such as the epiphyseal plate and periosteum [40]. CGRP was also found to be uniformly distributed to bone marrow and periosteal tissues [45]. Additionally, CGRP+ nerves have been associated with the initiation of bone formation, increasing bone mineralization, and cartilage and muscle differentiation in limb bud development [46–48]. Therefore, CGRP+ nerves have been implicated in the local control of bone formation.

Various studies were able to come to a similar conclusion by studying the effects of sensory denervation on bone. Madsen et al. [49] observed that resection of the sciatic and femoral nerves worsened fracture healing; although mineralization was unchanged, callus size increased and strength decreased. Selective denervation of nociceptive Aδ- and C-fibers with capsaicin was also found to dysregulate bone remodeling. Two independent groups discovered that in sensory-denervated rats, bone remodeling was dysregulated during mandibular cortex resorption. Osteoclastic activity and remodeling area both decreased, possibly due to an impairment in osteoclast precursor recruitment [50,51]. However, these results in the sensory-denervated rats were unexpected as removal of CGRP-releasing nerve fibers would be expected to stimulate bone resorption since previous studies have shown CGRP to inhibit osteoclast resorption *in vitro*. Such unexpected results could potentially be explained by loss of other important neuropeptides (especially SP), incomplete sensory denervation, or a role for CGRP+ sympathetic nerve fibers. Indeed, later studies reported that higher doses of capsaicin resulted in increased osteoclast number and surface area, decreased trabecular bone volume, and decreased bone integrity [52,53]. It was also shown that fractures combined with peripheral nerve injury did heal slower [54]. Therefore, CGRP + sensory nerves likely contribute to the maintenance of bone integrity and proper fracture healing.

Interestingly, damage to the CNS seems to have the opposite effects on bone. When fracture was combined with either a spinal cord injury or a cerebral cortex/traumatic brain injury (TBI), fracture healing was accelerated [54–56]. Both injuries were associated with elevated CGRP levels in the dorsal root ganglia (DRG), which contain the cell bodies of primary sensory neurons supplying most of the body. TBIs were also correlated with increased CGRP in the serum, muscle, and brain, especially the hippocampus. Mechanistically, it is thought that TBI raises CGRP levels by inducing injured neural tissue to release it or by stimulating a CNS response to brain injury. CGRP then seeps through the damaged blood

brain barrier to enter the serum [55,56]. Therefore, these investigators have hypothesized acute CNS damage accelerates fracture healing through elevated serum CGRP and additional axoplasmic transport of CGRP to the fracture site via sensory nerves. Changes in fracture healing have unfortunately not been well-studied or reported in the setting of chronic nervous system injury or neurodegenerative disease, such as chronic traumatic encephalopathy, neuropathic osteoarthropathy, cerebral palsy, multiple sclerosis, and spina bifida, to name a few.

Further evidence that CGRP+ nerves contribute to fracture healing comes from animal studies on nerve regeneration after bone trauma. CGRP+ nerve fibers have not been well-identified in the immediate days following fracture, suggesting that they do not begin to proliferate until the inflammatory phase has ended [57]. These fibers reach a peak density in the middle of the bone repair phase [58]; neuronal CGRP can be increased by at least 27-fold in fractures as compared to intact bone after three weeks [59]. One study found that during the repair of devascularized and necrotic endosteal bone after tibial implant placement, actively proliferating CGRP+ nerves were found at the border between new and necrotic bone [60]. In addition, in an elegant study comparing CGRP+ nerve ingrowth between a straight and angulated fracture, Li et al. demonstrated that the greatest levels of neuronal CGRP coincided with the sites of greater bone formation. In the straight fracture, both callus thickness and CGRP innervation were evenly distributed on both anterior and posterior sides of the fracture. In the angulated fracture, callus thickness and CGRP innervation were increased on the concave side, which required more bone formation to correct the deformity. This indicates that CGRP+ nerve ingrowth is induced by both trauma and the demands of deformity correction (such as angulation). Furthermore, CGRP+ nerve fibers were most numerous and actively proliferating during the early active phases of bone healing, but then regressed during later bone remodeling phases. The authors suggested that this may be evidence of an afferent-efferent loop where stimulated mechanoreceptors induce the release of neuropeptides, which can then guide an appropriate bone response [59]. These findings would be consistent with literature reporting that CGRP is required for mediating the bone's response to mechanical loading and cyclic loading fatigue [61,62]. Numerous investigators have further found that CGRP+ nerve fibers innervate the fracture hematoma, periosteum, chondrocytes and neutrophils of the growing fibrous callus, new woven bone, and secondary minor fractures as either free nerve terminals or as networks associated with vasculature [57–59]. Finally, the origin of these CGRP+ nerves appears to be the periosteum. Using a bone conduction chamber to exclude the periosteum from contributing to ingrowing bone, Madsen et al. [63] determined that although some sensory nerves can arise from cortical bone, endosteum, and bone marrow, these nerves are unlikely to contribute to the early bone healing process as they were not present in all samples, even at later time points. Overall, these studies provide strong morphological evidence that CGRP+ sensory nerves from the periosteum proliferate during the bone repair phase to aid in fracture healing.

A few investigators have elucidated CGRP's role in accelerating fracture healing. CGRP was found to induce a concentration-dependent vasodilation in bone arteries, which was hypothesized to contribute to fracture healing by increasing blood flow. This vasodilation was found to be independent of nitric oxide synthase activity (NOS) [64]. However, it was later reported that CGRP expression was positively correlated to the expression and activity

of NOS [65]. NO is a secondary messenger that not only mediates vasodilation, but also aids inflammation, vascularization, and other pathways during fracture healing [66]. Therefore, it is likely that CGRP accelerates fracture healing through other means besides NOS-mediated vasodilation. Zhang et al. reported on the role of local neuronal CGRP in accelerating fracture healing after implantation of an intramedullary nail containing ultrapure magnesium. CGRP substantially increased in both the peripheral femur cortex and the ipsilateral DRG. Activation of the CGRP receptor on periosteum-derived stem cells then initiated the cAMP signaling pathway, leading to phosphorylation of cAMP responsive element binding protein 1 (CREB1), upregulation of the osteogenesis-driving transcription factor Osterix or Sp7, and transcription of osteoblastic genes such as alkaline phosphatase (*ALP*), bone gamma-carboxyglutamate protein/osteocalcin (*BGLAP/OCN*), and osteopontin (*SPP1*). Additionally, surgical removal of the periosteum, sensory nerve denervation, and KO of RAMP1 all ablated these effects, while overexpression of RAMP1 enhanced the magnesium-induced osteogenesis [67]. Implantation of CGRP-containing gelatin microspheres also promoted healing of a bone defect in an osteoporosis model [68]. Finally, twice daily injection of a CGRP inhibitor significantly impaired fracture healing, as evidenced by decreased callus mineralized volume fraction (BV/TV) and increased cartilage area. Additionally, CGRP inhibitors decreased ERK1/2 expression in the fracture area, indicating a downregulation of pathways critical to bone formation and remodeling [69]. These studies provide strong evidence that local CGRP accelerates fracture healing by driving osteogenesis.

Clinically, there are a few studies that have contributed to our understanding of CGRP's role in fracture healing. It has been found that loss of sensory innervation results in impaired fracture healing and poorer bone health. Familial dysautonomia (FD) patients suffer from improper development and poor survival of their sensory and autonomic nerves [70]. This results in diminished local neuropeptide release and serum CGRP levels which may contribute to the increased fracture incidence and bone fragility in FD patients [71,72]. Peripheral neuropathy is also the cause of neuropathic osteoarthropathy, or Charcot foot, in which the bones and joints of the feet and ankles are chronically inflamed, thus leading to progressive degeneration. Loss of neuropeptide signaling, especially CGRP, is suspected as a contributing factor to the pathogenesis [73–75]. Still, the bone changes seen in FD and Charcot foot patients may also be explained by loss of other neuropeptide signaling or other mechanisms. In a histological study of lumbar spondylolysis, one investigator reported that defective healing could partly be explained by lack of sympathetic and sensory innervation [76]. Onuoha et al. [77,78] has reported elevated and maintained serum CGRP and SP levels in patients with fractures relative to non-fractured controls, but was unable to make any comparisons between healing outcomes. Overall, the majority of what is now known about CGRP's effect on bone and fracture healing has come from *in vitro* and animal *in vivo* studies.

2.2. Substance P

SP is an 11-amino-acid peptide of the tachykinin family. Along with CGRP, SP can be found in peptidergic sensory fibers. Upon preferential activation of its receptor neurokinin 1 receptor (NK1R), SP facilitates nociceptive signaling through primary sensory afferent

nerves to the CNS. SP is also produced by and acts on non-neuronal cells. Other functions of SP include potent arterial vasodilation, inflammation, and regulation of non-neuronal tissue [20,79].

SP has long been thought to be involved in numerous pathologies causing musculoskeletal pain. For example, SP+ nerve fibers have been found to be distributed throughout the knee, retinaculum, synovium, fat pad, and bone in anterior knee pain syndrome [80]. SP concentration has been found to be higher in chronic tendon pathologies and to increase hypercellularity and angiogenesis in tendinopathy models [81]. Patients with painful hip joint OA have increased density of SP+ nerve fibers [24], while patients with painless failed total hip arthroplasties completely lack SP+ nerve fibers [23]. However, NK1R antagonists have failed to show any benefit in a variety of clinical pain states, including OA, in clinical trials, despite promising animal studies [82,83].

In bone tissue, SP+ nerves have been discovered in many of the same locations as CGRP+ nerves, such as the periosteum, bone marrow, epiphyseal growth plate, subchondral bone, ligaments, and the synovium [84]. And like CGRP+ nerves, some SP+ nerves were also found to arise from the cortical bone, endosteum, and bone marrow. But again, SP+ nerves from these locations did not seem to be active participants in early bone repair [63]. In addition, SP levels were elevated post-femoral neck fracture [77]. SP+ nerve fiber density was found to increase and peak at approximately two weeks post-trauma and revert to normal after the 24th day [57]. These results indicate that SP has some association with fracture healing.

In vitro experiments have characterized the effects of SP on osteoclasts. Multiple groups have confirmed that NK1R is expressed on the plasma membrane of both BMMs [85] and differentiated osteoclasts [86,87]. Activation of this receptor by SP increases both osteoclastogenesis and osteoclastic activity [88].

Interestingly, SP seems to function as a potentiator of osteoclastogenesis. Although SP activates NF-κB in BMMs, differentiation was found to only occur in the presence of RANKL and macrophage colony stimulating factor [85,87].

SP has been associated with osteocytes as well. Fukuda et al. [89] found that HK-1, a tachykinin found to inhibit SP, is expressed by osteocytes. This study also found that HK-1 positive osteocytes were increased on the compressive side after application of an orthodontic force and decreased on the tensile side [89]. Osteocytes were also found to express SP and NK1R [86,90]. Autocrine function was demonstrated through in situ hybridization which showed SP mRNA in osteocytes. Furthermore, SP transcription was increased in response to exercise-induced enhancement of mechanical loading [90]. This indicates that load-induced bone formation may be modulated in part through SP expressed by osteocytes.

SP has also been found to have a dose-dependent osteogenic stimulating effect *in vitro* [91], suggesting it could regulate osteoblast function. Since then, NK1R expression has been confirmed on osteoblasts [86], [92], preosteoblastic MC3T3-E1 cells [93], and BMSCs [94]. SP has been shown to activate the Wnt/β-catenin pathway in osteoblast precursor cells, thus

stimulating the expression of various osteoblastic genes, such as RUNX2, ALP, OCN, and collagen type I, in BMSCs [87,93,94]. In BMSCs, SP additionally seems to stimulate expression of vascular endothelial growth factor, expression of bone morphogenetic protein-2, and migration [94]. These effects of SP on bone regulation could impact fracture healing.

Poor fracture healing in ovariectomized mice was found to be associated with a significant decrease in SP [95]. He et al. [96] found that following osteotomy, denervated mandibular bone had increased fibrous callus formation with decreased mature/woven bone and peak SP levels around four weeks. On the other hand, Rusanen et al. [97] found peak levels of SP+ nerve fibers at two weeks post fracture. This seems to indicate a delayed response of SP in response to fracture healing, which may be pivotal in developing fracture healing treatment modalities.

To clarify the paradoxical *in vitro* observation that SP increases both bone formation and resorption, Li et al. [98] analyzed the occurrence of SP+ nerve fibers during the repair of straight versus angulated fractures over time. SP+ nerve fibers first appeared in the fracture hematoma closely associated with chondrocytes, suggesting a role in the inflammatory phase and fibrocartilage callus formation. The number of these fibers then peaked in areas of maximal bone formation, indicating neurogenic SP promotes osteogenesis. However, in later timepoints, SP+ nerve fibers were found to be absent in all fracture sites except for the convex side of the angulated fractures. After repair of the angulated fracture, the convex side undergoes substantial bone remodeling to correct the angulated defect; therefore, the presence of SP+ nerve fibers suggests a role in bone resorption. This seems to indicate that SP+ nerve fibers contribute to bone formation first, and then regulate bone resorption during remodeling later. Li et al. [98] hypothesize that the amount of loading on the bone is related to the change in SP's effects, but this remains unclear.

Identification of how SP modulates fracture healing is also of great interest in the development of appropriate and effective treatments. SP induces a transient vasorelaxation in bone arteries. Specifically, it was found that there was desensitization of arteries upon continued exposure to SP [64]. Vendégh et al. [99], however, found no significant effect to bone marrow microvascular resistance from CGRP or SP. Interestingly, increased angiogenesis in calvarial defect sites was noted with use of SP/dexamethasone-encapsulated PLGA scaffolds [100]. SP was also found to mobilize CD29+ stromal cells from various connective tissues including the bone marrow. This was correlated with accelerated wound healing in rabbits [101]. Furthermore, SP was found to have anti-inflammatory effects following spinal cord injury by inducing IL-10 and M2 macrophages and suppressing inducible NOS and tumor necrosis factor-α [102]. All of these effects likely impact SP-mediated fracture healing.

The development of treatments with SP for repair of compromised bone is currently under investigation. Recently, gelatin microspheres containing SP have been utilized to promote osteogenesis in a dose-dependent manner [103]. In addition, a polylactic acid/β-tri-calcium phosphate (PLA/β-TCP) scaffold composite combined with a self-assembling peptide and a

SP peptide motif (KLD12/KLD12-SP) was used to repair flat bone defects without cell transplantation, resulting in a more rapid bone healing response compared to controls [104].

2.3. Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Peptide

VIP is a 28 amino acid cleavage product of pre-pro-VIP [105]. PACAP is also derived from pre-pro-VIP. PACAP has two forms: PACAP 27 and 38. VIP is located in post-ganglionic sympathetic nerves in bone, parasympathetic nerves, and primary sensory neurons [50,106,107]. Developmentally, nerves initially display noradrenergic properties, but mature innervation contains VIP and is cholinergic [108]. Periosteum has also been found to be a candidate for cholinergic sympathetic innervation [107]. Interestingly, VIP+ nerves were located in a manner similar to CGRP and SP-related fibers [109]. VIP+ nerves are often located in the epiphysis and periosteum of bone [107,110]. PACAP receptors appear to be mostly sensory in origin [111].

VIP has been found to have contradictory effects on joint pain in OA models. Some reports have stated that VIP has anti-inflammatory effects and decreases cytokines that contribute to joint pain and destruction, while other studies have found that VIP inhibition can help control OA pain. No clinical studies have been done with VIP or its antagonists to further clarify its role in OA joint pain [112]. PACAP, however, has been shown to have anti-inflammatory and chondroprotective effects in preclinical OA models [113,114].

Three different subtypes of VIP receptors have been identified: $VPAC_1$, $VPAC_2$, and PAC_1 (also designated VIP-1R, VIP-2R, PACAP-R) [115]. All of these receptors are G-coupled protein receptors. VIP activates bone resorption through a G_s receptor cAMP-mediated second messenger pathway [116]. Paradoxically, VIP and PACAP also decreased the stimulatory effects of vitamin D_3 on RANKL and RANK expression and reversed the inhibitory effects of vitamin D_3 on osteoprotegerin (OPG) expression (a RANK antagonist).

This would directly inhibit bone resorption [117]. VIP may also take part in bone resorption by stimulating prostaglandin E2 [118]. VIP binding sites have been identified on osteoblasts and osteoclasts [119]. $VPAC_2$, but not $VPAC_1$ or PAC_1, was detected in undifferentiated murine osteoblasts. $VPAC_1$ was shown to be induced after differentiation of osteoblasts [120]. $VPAC_1$, but not $VPAC_{2\ n}$or PAC_1, were found in human periosteum-derived osteoblasts. On osteoclasts, $VPAC_1$ and PAC_1, but not $VPAC_2$, have been identified [121]. Bone resorption effects in mice appear to be mediated by $VPAC_2$ receptor [122]. VIP was also found to increase differentiation for osteoblasts via the $VPAC_2$ receptor [120].

VIP was found to transiently increase bone formation and then delay bone resorption [123]. Similar to SP [98], PACAP was shown to have a higher binding affinity and induce a larger cAMP response in mouse calvarial osteoblasts compared to VIP [120]. VIP causes cytoplasmic contraction and decreased motility of osteoclasts. Further, VIP was shown to cause an increase in the number of resorption pits [123]. Thus, VIP's stimulation of bone resorption effects appear to dominate its impact of bone formation. That said, ALP, which is associated with bone formation, was found to be activated by VIP [124]. Schirrmacher and Bingman [125] studied the effect of electric coupling of osteoblast-like cells *in vitro*, but

they did not find a significant effect. Indeed, only 30–40% of cells were found to be responsive to VIP.

VIP may also affect fracture healing indirectly; VIP induces a concentration-dependent vasorelaxation in bone arteries [64]. VIP has also been shown to promote stromal cell IL-6 production, which in turn stimulates bone resorption [126].

3. Neurotrophins

3.1. Nerve Growth Factor

NGF is an important regulator of sensory and sympathetic neuron survival, growth, proliferation, and plasticity (For reviews, see [127,128]). The biologically active form of NGF is derived via self-cleavage of a much larger, multimolecular complex called 7S NGF [129]. In a healthy adult human, NGF is minimally expressed and seems to be absent in numerous cell types. However, inflammatory conditions have been associated with increased NGF production from cells such as keratinocytes [130], mast cells [131], and macrophages [132], (For review, see [133]).

In addition to its neurotrophic capabilities, NGF has been identified as a critical player in wound healing that regulates the functioning and survival of other non-neuronal cells (For review, see [134]). Relevant to bone, *in vitro* experiments have found that NGF can prevent apoptosis [135] and induce differentiation in cultured osteoblastic cell lines [136,137]. In combination with porous biphasic calcium phosphate ceramics, which promote osteoconduction and concentrate proteins at its surface, NGF promoted osteoblast growth and differentiation via activation of the BMP2/Runx2 signaling pathway [138]. NGF can also induce osteoclastogenesis in a RANKL-independent manner [139]. Therefore, via regulation of osteogenesis and bone resorption, NGF seems to directly modulate the repair and remodeling of bone during fracture healing. However, a recent study by Tomlinson et al. [140] suggested a model where mechanical loading stimulates NGF expression in osteoblasts, which then activates its high-affinity receptor tropomyosin receptor kinase A (TrkA) on periosteal sensory nerves. Active NGF-TrkA signaling then causes load-induced nerve sprouting, release of osteogenic cues from the sensory nerves, and enhanced bone formation via upregulation of Wnt/β-catenin signaling in both osteocytes and osteoblasts [140]. Furthermore, Wnt/β-catenin signaling was not required for load-induced NGF expression in osteoblasts and NGF expression was not found in osteocytes in either loaded or unloaded limbs. This suggests that osteoblasts and osteocytes respond to load in different, but perhaps overlapping, mechanisms. Additionally, Wnt/β-catenin activation in osteocytes may be downstream of NGF-TrkA signaling [140]. These findings may also suggest a role for CGRP and SP as critical players in load-induced bone formation, as they both induce osteoblastic differentiation of BMSCs via Wnt/β-catenin signaling. Still, there are certainly other neural factors that may be responsible. In summary, NGF may also be an indirect modulator of fracture healing by the recruitment of sensory nerves.

The expression of both NGF and TrkA has been identified in fractures. Compared to unfractured ribs in which NGF was only found in periosteal mesenchymal osteoprogenitor cells, NGF was found in fracture callus periosteal osteoprogenitor cells, marrow stromal

cells, osteoblasts, young osteocytes, and new capillary endothelial cells. Six weeks after injury, once the ribs were healed, NGF staining was confined again to the periosteal cells [141]. Asaumi et al. [142] reported that NGF and TrkA were localized to almost all bone-forming cells in fractured ribs. NGF mRNA expression was also elevated throughout the healing process and peaked two days after the fracture [142]. Another study also confirmed the expression of NGF in bone-regenerating cells. Specifically, NGF was expressed in osteoblast-like cells contacting osteoid tissues, fibroblast-like cells surrounding osteoblast-like cells, and chondrocytes within granulation tissue [143]. These studies suggest that in normal bone, NGF expression is limited to the periosteum, which may explain the rich sensory innervation of the periosteum. However, during fracture healing, NGF expression peaks early and then remains elevated until the bone is healed, suggesting an integral role in bone repair. Additionally, it is closely localized to bone-forming cells and vasculature. Therefore, NGF may promote fracture healing via direct stimulation of osteogenic cells and indirect recruitment of sensory nerves carrying osteogenic factors. Indeed, NGF expression has been found to induce nerve sprouting and local, mechanical hyperalgesia [143].

Multiple investigators have studied the effects of exogenous NGF on fracture healing. Continuous administration of NGF to fracture sites improved the rate and effectiveness of bone repair. In general, mechanical strength was increased and transverse cross-sectional area of the repair site was decreased with NGF treatment as compared to fractures treated with saline [144]. In models of mandibular distraction osteogenesis, NGF has repeatedly promoted new bone formation and callus maturation. NGF increased callus maximum load, bone volume density, and mineral apposition rate, especially when NGF was delivered in a collagen/nano-hydroxyapatite/kappa-carrageenan gel [145–147]. NGF delivered on biphasic calcium phosphate ceramic improved the healing of rat calvarial defects by improving new bone formation [138]. Sang et al. [148] utilized NGF-transgenic mice to elucidate the mechanism by which NGF promotes fracture healing. In the callus of NGF-transgenic mice, they observed increased TRAP staining, TRAP mRNA, *COL2A1* mRNA, and SOX9 mRNA as compared to wild-type mice. Taken together, these data suggest that NGF promoted callus formation by increasing osteoclast formation and accelerating cartilage differentiation [148].

Due to NGF's well-established role in pain, the use of NGF-sequestering antibodies to reduce musculoskeletal pain has received much attention, especially in the OA community. Several other groups have also investigated whether anti-NGF could reduce fracture pain without impairing bone healing [149–152]. Anti-NGF therapy was found to successfully reduce fracture pain without having an effect on callus formation, fracture site bridging, and callus mechanical strength [149–151]. Another study reported that mice receiving anti-NGF treatment had increased callus sizes. However, these mice were also more physically active, most likely due to anti-NGF inhibition of peripheral sensitization [152]. Together, these studies support the use of anti-NGF antibodies as a therapy to reduce fracture pain. Unfortunately, in 2010, the U.S. Food and Drug Administration halted all clinical trials involving anti-NGF antibodies due to reports of rapid joint degeneration (For review, see [153]). This illustrates the importance of investigating possible side effects/consequences of new therapies.

Small molecule inhibitors of the Trk family receptors have also been explored as an alternative to anti-NGF therapy. Ghilardi et al. [154] demonstrated that twice-daily administration of a Trk inhibitor significantly reduced fracture pain-related behaviors without significant inhibition of fracture healing. However, Trk inhibitors have not yet reached clinical trials due to their lack of specificity [153]. One study has also examined the opposite question: how does activation of the TrkA receptor influence fracture healing? Johnstone et al. [155] administered gambogic amide (GA), a nonpeptide NGF-mimetic, to mice with bilateral fibular fractures daily for two weeks. GA treatment decreased the size of the callus and increased its mechanical strength without detrimental side effects. *In vitro* experiments found that GA treatment of osteogenic mesenchymal progenitor cells increased their expression of the mature osteoblastic markers ALP and OCN and stimulated mineralization [155]. These results are consistent with how NGF influences bone tissue healing and point to a potential therapeutic role of TrkA agonists in stimulating fracture healing.

Serum NGF has also been implicated in the improved bone healing seen in the context of TBI. In a simultaneous TBI-fracture rat model, enhanced early callus formation was associated with increased serum NGF, Wnt-3a, and ACTH [68]. However, it cannot be concluded that NGF is the main factor responsible for the improved bone healing. It is possible that this effect is also mediated by Wnt-3a, ACTH, an unmeasured protein such as CGRP or SP, or even a combination of factors.

Clinical studies on NGF have largely been limited to patients with simultaneous brain injury and bone fracture. Patients with both injuries, fracture only, and TBI only all had increasing levels of serum NGF up to two weeks after their trauma. However, serum NGF levels were significantly higher in patients with both injuries [156]. Another study examining patients with simultaneous clavicle fracture and TBI also reported elevated serum NGF levels. This was then found to be correlated with a shorter fracture healing time and elevated expression of CD31 (a marker of endothelial cells), NGF, and VEGF in the callus tissue [157]. Therefore, TBI may induce the CNS to release NGF into the serum and accelerate fracture healing by promoting VEGF-mediated angiogenesis.

There are also a few clinical case reports that discuss prolonged fracture healing and hypertrophic callus formation in patients diagnosed with hereditary sensory and autonomic neuropathy type IV (HSAN-IV), which is caused by a mutation in the gene that encodes TrkA. In addition to lacking the C- and Aδ- fibers required for somatic pain perception, these patients also suffer from anhidrosis, intellectual disability, and self-harm [158–160]. The changes in fracture healing seem to be consistent with what would be expected based on the NGF literature; it may even suggest the potential complications of excessive pharmacologic TrkA blockade for managing fracture pain. The complete absence of sensory nerves at the fracture site may also be responsible. However, other factors that may delay healing, such as poor patient compliance with post-operative instructions and frequent infections, must also be considered.

A recent review also detailed important insights learned from HSAN-V patients, who carry a homozygous NGF^{R100W} mutation. Similar to HSAN-VI patients, these patients lack somatic

pain perception, but have no mental disability. Further examination of the mutated NGF indicates that it binds TrkA normally but has decreased affinity for the low-affinity NGF receptor $p75^{NTR}$. It has also lost its ability to induce pain. These studies indicate that NGF^{R100W} has uncoupled nociceptive and trophic functioning [161]. This protein may prove to be useful for improving fracture healing and inhibiting fracture pain via the TrkA pathways previously discussed. Indeed, there is an orthopedic case report of six HSAN-V patients who had uneventful fracture healing, indicating a notable difference from HSAN-IV patients [162].

3.2. Brain-Derived Neurotrophic Factor

BDNF is another member of the neurotrophin family that has been localized to a subpopulation of DRG neurons. Peripheral inflammation leads to an upregulation of BDNF mRNA and protein in TrkA+ sensory neurons via an NGF-dependent mechanism. BDNF is then transported and released at the spinal cord, where it mediates central sensitization to pain (For review, see [8]).

Both BDNF and its receptor, TrkB, have been localized to fracture tissues during the inflammatory and early bone formation phases, concentrated in both endothelial and osteoblastic cells. BDNF could only be found in granulation tissue at the margins of woven bone and were absent in both chondrocytes and mature bone [142,163], suggesting the BDNF-TrkB pathway regulates early stages of fracture healing.

Osteocyte-like cell line OmGFP66 was found to express NGF and BDNF [164]. Osteocytes were also found to strongly express positive immunoreaction of TrkB, which binds exogenous BDNF. BDNF also promoted osteosclerosis after cortical osteotomy in an *in vivo* experiment [165]. This indicates that osteocytes may also modulate bone healing via BDNF.

Like NGF, BDNF has been found to positively regulate new bone formation by inducing osteoblast proliferation, differentiation, and mineralization [137]. It has also been found to increase the secretion of RANKL by human BMSCs, contributing to osteoclastogenesis [166]. Since BDNF acts similarly to NGF, it can be hypothesized to have similar promoting effects on fracture healing. Indeed, in a mouse fracture model, it was found that a pasty bone cement/mesoporous bioactive glass composite containing BDNF promoted the healing of a femoral metaphyseal fracture by increasing bone formation [167].

4. Conclusions

Neuropeptides and neurotrophins have been well-studied for their effects on neurons. A number of these proteins have further been implicated as important players in the onset, maintenance, and sensitization of peripheral pain. These proteins include: CGRP, SP, VIP, PACAP, NGF, and BDNF. Increasing evidence is now indicating that these proteins also have direct effects on osteoblasts, osteoclasts, their respective progenitor cells, and osteocytes, suggesting that neuropeptides and neurotrophins can also directly impact fracture healing.

These neuropeptides have varied effects on bone. CGRP and SP seem to have differential regulation of osteoclasts. CGRP downregulates osteoclastogenesis and osteoclastic activity via the blockade of RANKL signaling. SP, on the other hand, functions to potentiate osteoclastogenesis and osteoclastic activity in the presence of RANKL and macrophage colony stimulating factor. However, both proteins also appear to upregulate osteoblast activity and new bone formation. Ultimately, *in vivo* studies suggest that CGRP and SP both accelerate fracture healing. Functionally, this would be logical, since CGRP and SP are frequently co-localized in the same peptidergic nociceptive fibers and are likely released into injured tissues together. Therefore, it may be possible that these two neuropeptides can act synergistically to improve the healing response. With regard to VIP and PACAP, evidence suggests that these neuropeptides likely have a negative impact on fracture healing. They appear to preferentially upregulate bone resorption, while only transiently increasing bone formation primary through G-protein coupled receptor cAMP elevation. However, additional studies will be necessary to confirm the effects of VIP and PACAP on fracture healing.

The neurotrophins NGF and BDNF are more uniform in their effects. Evidence indicates that they both upregulate osteoblast differentiation, growth, and activity. They also drive osteoclastogenesis, albeit by different mechanisms. NGF and BDNF both seem to promote fracture healing by stimulating osteogenesis and possibly by regulating bone remodeling. Additionally, NGF's role in fracture healing may be similar to its role in load-induced bone formation. High NGF expression in the healing callus promotes the survival, ectopic sprouting, and sensitization of TrkA sensory and sympathetic nerves, which can contain CGRP and SP. These nerves then respond to the abnormal mechanical and signals in fractured bone by releasing neuropeptides that can promote osteogenesis and remodeling. In successfully healed bone, NGF expression then disappears and again becomes limited to the periosteum. But in nonunion or otherwise poorly healed bone, NGF expression may still persist, thus driving excess sensory nerve sprouting and the generation of chronic pain. Ectopic nerve sprouting has previously been shown to be associated with chronic pain in unhealed bones [168].

Although substantial progress has been made in terms of identifying the role of neuropeptides and neurotrophins in fracture healing, more work remains to be done. The existence of an NGF-neuropeptide-bone signaling axis that regulates bone formation needs to be validated in both normal mechanical loading of bone and in fracture healing. This will aid in better understanding the bone healing process and the drivers of acute and chronic fracture pain. Additionally, doing so may shed insight on the pathophysiology contributing to osteoporotic and fracture healing changes in patients who are missing a component of this axis. This may include patients with disrupted NGF signaling (HSAN-IV and -V), absent innervation (diabetic neuropathy, Charcot foot), and decreased ability of the bone to respond to osteogenic factors (elderly patients with degenerated osteocyte lacunocanicular systems – for review, see [164]).

Another question that needs to still be clarified is at what time are these proteins most effective in promoting fracture healing? It would be useful to understand when the healing-promoting effects of these proteins could be maximized and when the pain-promoting effects could be minimized. Some evidence suggests that CGRP, SP, NGF, and BDNF are

most important for fracture healing in the early to middle bone repair phase [57–59,142,143,163]. Therefore, it may be possible to safely reduce fracture-associated pain by antagonizing these proteins and their pathways later in the healing process. This question is especially timely with the advent of recent research on sensitization of bone nociceptors, as well as ectopic nerve sprouting into normally poorly innervated bone, and their contributions to fracture-associated pain (For review, see [169]). Furthermore, studies with new small molecule agonists and antagonists may reveal methods of selectively activating healing-promoting pathways in targeted bone cells without inducing pain in sensory nerve fibers.

Additionally, it remains unclear what drives the upregulation of NGF expression in fractured bone. Mechanical loading directly stimulates NGF expression in osteoblasts [140], so disrupted mechanical forces in fractured bone may be the stimulus. However, NGF expression has also been found to be produced by various cells during inflammation. Understanding the stimulus for NGF upregulation may be critical in identifying why unhealed bone leads to chronic pain. Another question is which of NGF's effects on fracture healing is the more significant one: the direct bone effects or the recruitment of neuropeptide-containing sensory nerves? Perhaps the early bone healing stage is promoted by NGF, and then later fine-tuning of the bone is mediated by CGRP and SP according to selective mechanoreceptor activation. Alternatively, CGRP and SP may be the main drivers and NGF serves to bring more of these neuropeptides to the injury site. Further elucidation of this process will aid in understanding previous studies that administered exogenous NGF to fractures and may guide towards novel therapeutic ideas. Additionally, although BDNF is known to play a role in central sensitization, it is unclear if it mediates peripheral sensitization during fracture repair.

Overall, it is likely that fracture-associated pain cannot be completely eliminated based on the healing effects of the discussed neuropeptides and neurotrophins. However, future investigations in this area may lead to new therapeutic developments that can effectively improve fracture healing and mitigate associated acute and chronic pain. Such developments could contribute to reducing pain medication usage, decreasing the opiate addiction crisis, and improving patient quality of life.

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Highlights

- Poor fracture healing and chronic pain cause significant disability.
- Neuropeptides and neurotrophins promote bone pain and bone healing.
- These proteins may be targetable clinically, but limited success so far.
- Further work may yield novel methods of healing fractures and decreasing pain.

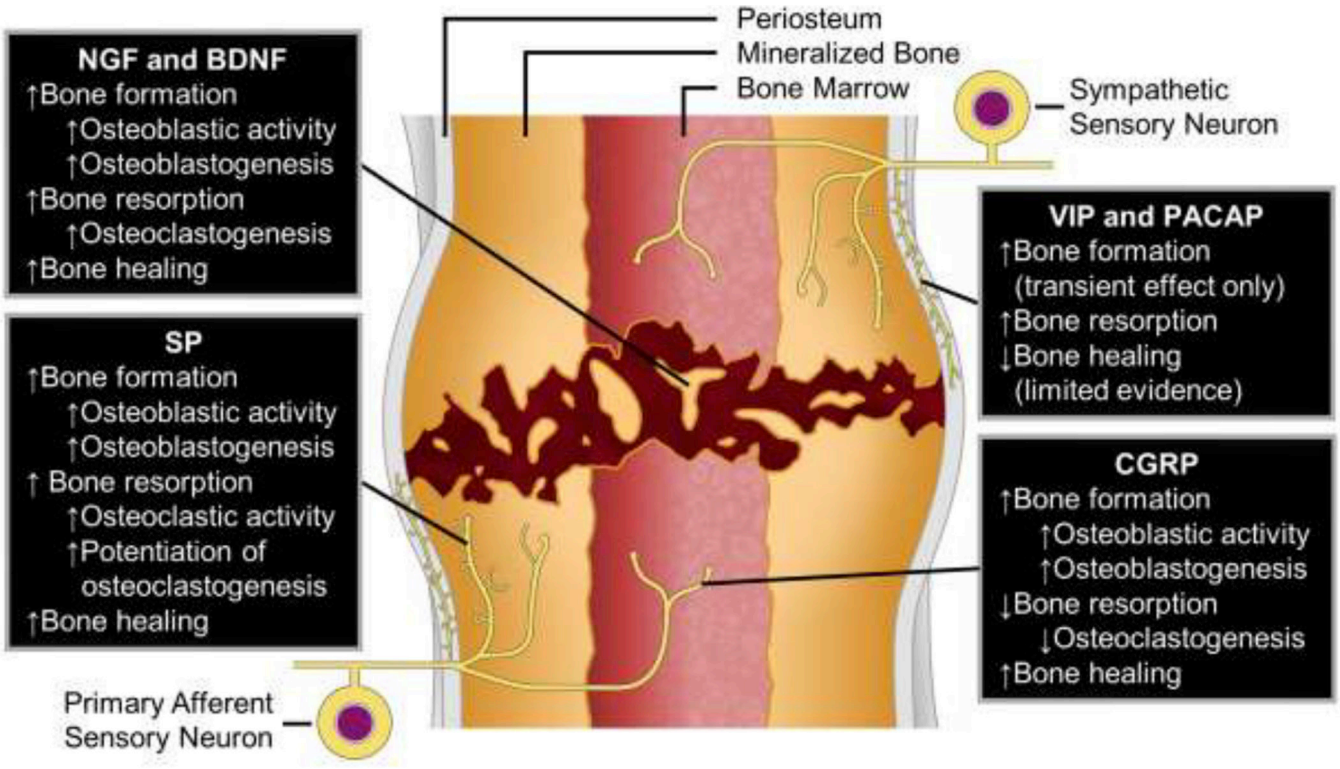

Figure 1. The effects of neuropeptides and neurotrophins on bone healing.

This schematic illustrates the distribution of sensory nerve fibers in the periosteum, mineralized bone, and bone marrow. Following bone injury, various neuropeptides and neurotrophins are upregulated and released at the injury site. These proteins include calcitonin-gene related protein (CGRP), substance P (SP), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF). In addition to their pain-promoting activities, these proteins are also direct modulators of bone formation and bone resorption, thus influencing the bone healing process.