9-2011

Effects of Neurotrophic Factors on Osteoblast Growth and Differentiation

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Faculty of Graduate Studies

Effects of Neurotrophic Factors on Osteoblast Growth and Differentiation

by

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A Dissertation submitted in partial satisfaction of
the requirements for the degree of
Doctor of Philosophy in Anatomy

September 2011

Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ACKNOWLEDGEMENTS

I would like to thank my committee members for their guidance, knowledge, support, and patience. To Dr. Linkhart, Dr. Farley, Dr. Nava, and Dr. Wright—you are mentors in science, teaching, career, and life.

Thank you to the Loma Linda University Department of Anatomy and JL Pettis Veteran's Affairs Hospital Musculoskeletal Disease Center and NIH Training Grant "Research Training in Biomedical Studies of Bone."

To my family and friends, thank you for supporting me through this journey. Special thanks to my mother for showing me the importance of strength and perseverance. This is dedicated to my father, who instilled in me the fascination, appreciation, and love of science and spirit.

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ABBREVIATIONS

CGRP	Calcitonin gene-related peptide
SP	Substance P
MC3T3-E1	Murine osteoblastic cell line
RT-PCR	Real-time polymerase chain reaction
CSR	Calcitonin Splice Regulator
IGF-I	Insulin-like growth factor
PPT-I	Preprotachykinin I
PPT-II	Preprotachykinin II
NK-1R	Neurokinin-1 recepeptor
IL1β	Interleukin 1 beta
COX	Cyclooxygenase
DMEM	Delbucco's Modified Eagle Medium
α-MEM	Alpha Minimum Essential Medium
BSA	Bovine serum albumin
PBS	Phosphate buffered saline
ALP	Alkaline phosphatase
NGF	Nerve Growth Factor
CPC	Cetylpyridinium chloride
RNA	Ribonucleic acid
NSAIDS	Non-steroidal anti-inflammatory drugs

ABSTRACT OF THE DISSERTATION

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Colleen M. Moran

Doctor of Philosophy, Graduate Program in Anatomy
Loma Linda University, September 2011
Thomas A. Linkhart, Chairperson

Recent evidence suggests that bone metabolism may be influenced by the innervation of skeletal tissues. Innervation of skeletal tissues might directly influence bone volume by the release or secretion of osteogenic growth factors in the form of neuropeptides. These neuropeptides could act locally on osteoblasts to increase bone formation and/or mineralization. Since calcitonin gene-related peptide (CGRP) and Substance P (SP) are the most abundant neuropeptides present in sensory nerves in bone, the current studies were intended to test the hypothesis that these two neuropeptides may have direct effects on osteoblast growth, differentiation, and mineralization. Replicate cultures of murine calvarial osteoblasts were grown with and without CGRP and/or SP, across a range of physiologic to pharmacologic doses. Cell growth was assessed by changes in cell layer protein. Differentiation was assessed by changes in cellular alkaline phosphatase levels, and mineralization was measured by

alizarin red staining. Although we did not observe effects of CGRP or SP on cell growth, we did observe effects on differentiation—a 48 hour exposure to CGRP induced a dose-dependent increase in alkaline phosphatase levels in the calvarial osteoblasts ($p<0.001$). Alkaline phosphatase activity was also increased in a dose-dependent manner following treatment with SP ($r = 0.979$, $p<0.001$). The murine cell line MC3T3-E1 was used to determine the effect of CGRP and SP on osteoblastic mineralization. After 7–21 days of continuous treatment with CGRP and SP in mineralization media, cells were fixed and mineralization was assessed by staining with alizarin red. The results of those studies showed that both CGRP and SP increased the rate and the extent of mineralization ($p<0.001$) and the effects of both neuropeptides together were greater than either alone ($p<0.001$). The effects of CGRP, SP, and the combination of peptides on osteoblast gene expression, as assessed by RT-PCR, have further revealed that exposure to CGRP and/or SP increased the mRNA levels for the Cox 2 protein, suggesting a possible role for prostaglandins as determinants of the osteogenic action(s) of CGRP and SP.

Together, these findings are consistent with the hypothesis that neuropeptides released from neurons in bone may be involved in the local regulation of bone volume, by effects on osteoblast growth, differentiation, and mineralization.

CHAPTER ONE

INTRODUCTION

Numerous clinical and experimental studies indicate that the nervous system influences the regulation of bone growth, remodeling, and repair (Chiego and Singh 1981; Singh, Herskovits et al. 1982; Aro, Eerola et al. 1985; Hill and Elde 1991; Hukkanen, Konttinen et al. 1993; Konttinen, Imai et al. 1996; Imai and Matsusue 2002; Togari 2002; Lerner 2006; Elefteriou 2008; Lerner and Persson 2008; Marenzana and Chenu 2008; McGregor, Poulton et al. 2010). There have been many functional studies, which have indicated a role for the nervous system in bone volume regulation; for example, during embryonic development (Frenkel, Guerra et al. 1990), in fracture healing (Aro, Eerola et al. 1985; Asaumi, Nakanishi et al. 2000; Koewler, Freeman et al. 2007; Bei, Lin et al. 2009), in peri-implant bone remodeling (Buma, Lamerigts et al. 1996; Daimaruya, Nagasaka et al. 2001; Gorustovich and Guglielmotti 2001; Abarca, Van Steenberghe et al. 2006), in the skeletal response to denervation (Garces and Santandreu 1988; Hill and Elde 1991; Duan, Inoue et al. 1993; Cardozo, Qin et al. 2010; Ding, Arai et al. 2010), and in the

pathologic process of heterotopic bone formation (van Susante, Buma et al. 1996; Ekelund and Erlinge 1997).

Several lines of evidence suggest that intact innervation is essential for proper bone growth and maintenance: bone deprived of neural input demonstrates significantly altered growth, repair, and remodeling (Smith, Wolff et al. 1958; Hulth and Olerud 1965; Quilis and Gonzalez 1974; Frymoyer and Pope 1977; Aro, Kallioniemi et al. 1981; Chiego and Singh 1981; Aro 1985; Aro, Eerola et al. 1985; Hill and Elde 1991; Hukkanen, Konttinen et al. 1993; Nordsletten, Skjeldal et al. 1994; Madsen, Wang et al. 1996; Zeng, Jee et al. 1996; Lerner 2006); brain damage and spinal cord injuries affect bone growth as indicated by an increased incidence of excessive callus growth and fracture nonunion (Glenn, Miner et al. 1973; Spencer 1987; Garland 1988; Renfree, Banovac et al. 1994; Banovac, Williams et al. 2004; Ivanhoe and Hartman 2004). Paradoxically, pathological bone growth, known as heterotopic ossification, occurs in 20%-40% of people following spinal cord injury (Garland 1988; Renfree, Banovac et al. 1994; Kluger, Kochs et al. 2000; van Kuijk, Geurts et al. 2002; Banovac, Williams et al. 2004). Presumably, these effects are dependent on the innervation bone receives from both sensory and autonomic nerves, and may also involve the skeletal response to different levels of muscle contractile activity and resultant mechanical

loading (Harrigan and Hamilton 1993; Barros, Muramoto et al. 2007). It is also possible that the effects of denervation on the skeleton may reflect disruptions in the localized secretion of neuropeptides released by those neurons (Konttinen, Imai et al. 1996; Villa, Melzi et al. 2000; Schinke, Liese et al. 2004; Offley, Guo et al. 2005; Lerner 2006; Lerner and Persson 2008; Naot and Cornish 2008; Han, Zhang et al. 2010; McGregor, Poulton et al. 2010; Xu, Kauther et al. 2010). Neuropeptides found in bone include calcitonin gene-related peptide, SP, neuropeptide Y, vasoactive intestinal peptide, nerve growth factor, neurotrophin-3, brain-derived neurotrophic factor, and neurokinin A (Hill and Elde 1991; Lundy and Linden 2004; Offley, Guo et al. 2005; Allison, Baldock et al. 2007; Lerner and Persson 2008; Naot and Cornish 2008). The neuropeptides produced most commonly by sensory nerves in bone are calcitonin gene-related peptide (CGRP) and Substance P (SP).

Three key studies strongly support the hypothesis that adverse effects of denervation are mediated to a large extent by the reduced secretion of neurotrophic factors from nerves innervating bone rather than merely by skeletal unloading and decreased mechanical stimulation. Madsen et al. (Madsen, Wang et al. 1996) created internally stabilized tibia fractures in rats, then performed sciatic neurectomy or sham surgery in the operated leg. The operated legs of

both groups were also immobilized with an external cast so both would be subject to loss of mechanical loading. In nerve intact rats the fractured tibias gained 50% of initial strength while fractured tibias of the neurectomy group gained only 20% of initial strength, even though callus size determined radiographically was larger than in the nerve intact group. Kingery et al. (Offley, Guo et al. 2005) found that SP levels in rat tibias fell by 50% after sciatic neurectomy, and that bone loss in both denervated and contralateral control tibia was increased by 2 week treatment with a SP antagonist LY303870. Offley et al. (Offley, Guo et al. 2005) reported that subcutaneous injection of SP antagonist capsaicin induced a 57% loss of unmyelinated peripheral sensory nerves without affecting motor nerves. This resulted in reduced bone density in the tibia and femur, with no effects on body weight, physical activity pattern, or muscle mass. Bone loss was associated with reduced bone formation rate. The chemical neurectomy by capsaicin produced a pathology similar to that observed in familial dysautonomia, an autosomal recessive disease characterized by loss of unmyelinated peripheral nerves, low bone mass, and increased fracture risk (Maayan, Ofek et al. 1985). These results together with observations that neurotrophic factors such as CGRP and SP affect osteoblasts suggest that peripheral nerve secretion

of these and other neuropeptides contributes to anabolic stimulation and maintenance of bone mass.

Central nervous system regulation of bone metabolism appears to originate in the hypothalamus. Recent studies are focused on leptin and the leptin receptor (Tartaglia 1997; Baldock, Sainsbury et al. 2002; Lerner and Persson 2008; Driessler and Baldock 2010; Qin, Bauman et al. 2010). The signaling form of the leptin receptor (obRb) is expressed in the hypothalamus (Tartaglia 1997; Baldock, Sainsbury et al. 2002). Knockout studies targeting the leptin receptor show that mice lacking the gene encoding the leptin receptor demonstrate high bone mass phenotype (Ducy, Amling et al. 2000; Baldock, Sainsbury et al. 2002). Furthermore, intra-cerebro-ventricular (icv) infusion of leptin normalized bone formation and bone mass in these mice (Ducy, Amling et al. 2000; Wolf 2008). Leptin-dependent inhibitory control of bone formation is mediated by the sympathetic nervous system (Cirmanova, Bayer et al. 2008; Driessler and Baldock 2010; Qin, Bauman et al. 2010). NPY, like leptin, is abundant in the hypothalamus and inhibits bone formation through activation of the sympathetic nervous system (Elefteriou 2008). ICV administration of NPY inhibits bone formation in normal mice through release of norepinephrine and activation of β2-adrenergic receptors on osteoblasts (Allison, Baldock et al. 2007). In leptin knockout mice,

ICV NPY administration reverses the high bone density phenotype of these mice, suggesting that NPY acts downstream of leptin in the hypothalamus (Cohen 2006). Neuromedin U (NMU) is another hypothalamic peptide that, like leptin and NPY, inhibits bone formation (Cirmanova, Bayer et al. 2008).

Networks of peptidergic nerve fibers are present throughout bone and are numerous in highly metabolic sites, e.g., periosteum, bone marrow, synovium, and osteochondral junctions of growth plates. CGRP- and SP-containing nerves have been found in almost every region of bone and surrounding soft tissue of rat limbs and are particularly concentrated in highly metabolic regions of bone (Bjurholm 1991; Hukkanen, Konttinen et al. 1993; Konttinen, Imai et al. 1996; Madsen, Wang et al. 1996; Goto, Ishizaki et al. 1999; Broome and Miyan 2000; Goto, Straka et al. 2002; Imai and Matsusue 2002; Irie, Hara-Irie et al. 2002; Aoki, Ohtori et al. 2003; Vandevska-Radunovic, Fristad et al. 2003; Kallakuri, Singh et al. 2004; Ljung, Alfredson et al. 2004; Mrak, Guidobono et al. 2010). The current studies were intended to assess the *in vitro* effects of these two neuropeptides – CGRP and SP – as potential effectors of bone formation and mineralization.

Calcitonin Gene Related Peptide

CGRP, a 37-amino-acid peptide, is synthesized as a result of alternative splicing, polyA addition, and differential proteolysis of the calcitonin gene mRNA and translation product, respectively. Calcitonin gene-related peptides belong to the calcitonin superfamily of peptides including also calcitonin, adrenomedullin, and the two recently discovered peptides intermedin and calcitonin receptor-stimulating peptide (Lerner 2006). CGRP was originally discovered in 1982, when Amara and colleagues showed that alternative RNA processing of the calcitonin gene generated mRNAs encoding a peptide they named CGRP (Coleman, Tran et al. 2003). Calcitonin mRNA is derived from exons 1-4 while CGRP mRNA is derived from exons 1-3, 5, and 6 of the calcitonin gene. Proteins produced from these mRNAs are cleaved within thyroid or neural cells to produce the mature calcitonin encoded by exon 4 or CGRP encoded by exon 5, respectively (Figure 1). In thyroid C cells, more than 98% of the mature mRNA from the calcitonin/CGRP gene encodes calcitonin (Coleman, Tran et al. 2003). In neurons, 99% of the transcripts are processed into an mRNA containing exons 1-3, 5, and 6, and translated into CGRP (Coleman, Tran et al. 2003). Regulation of cell type specific mRNA splicing is mediated by selective utilization of the 3' splice acceptor of exon 4 (active in thyroid cells, inactive in nerve cells) and consequent

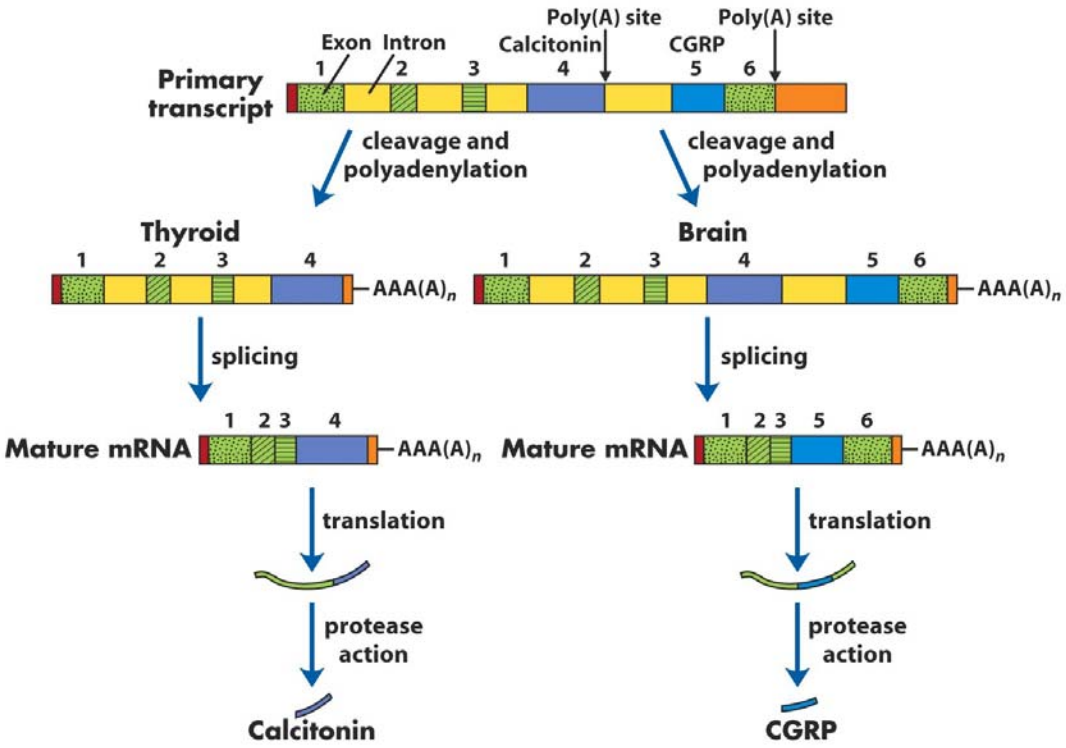

Figure 1. Calcitonin gene expression demonstrating alternative splicing, polyA addition, and differential proteolysis of the calcitonin gene mRNA and translation product, respectively. Calcitonin mRNA is derived from exons 1-4 while CGRP mRNA is derived from exons 1-3, 5, and 6. Proteins produced from these mRNAs are cleaved within thyroid or neural cells to produce the mature calcitonin encoded by exon 4 or CGRP encoded by exon 5, respectively.
From Lehninger Principles of Biochemistry 3rd ed.

alternate use of a poly-adenylation sequence in exon 4 that terminates the calcitonin mRNA (Lou and Gagel 1999; Coleman, Tran et al. 2003). Cell type specific regulation is mediated by an RNA binding protein Calcitonin Splice Regulator (CSR) that in nerve cells binds to sequences in exon 4 to inhibit utilization of the 3' exon 4 splice acceptor. In nerve cells this allows formation of a mature mRNA encoding CGRP from exon 5, and in thyroid cells the absence of CSR binding forces inclusion of exon 4 in the processed mRNA and excludes exon 5 due to poly-adenosine and sequence termination in exon-4 (Lou and Gagel 1999; Coleman, Tran et al. 2003).

The physiologic roles of CGRP are unclear, although it is postulated to regulate several processes in the central and peripheral nervous system and skin (Hoff, Catala-Lehnen et al. 2002; Han, Zhang et al. 2010). Although CGRP is a potent vasodilator, emerging roles for CGRP suggest that it has effects on immune cells and may be involved in the inflammatory response (Lundy and Linden 2004; Han, Zhang et al. 2010). Like calcitonin, CGRP inhibits osteoclast activity *in vitro* (Zaidi, Chambers et al. 1987; Akopian, Demulder et al. 2000; Cornish, Callon et al. 2001). In contrast to calcitonin, which targets only osteoclasts in bone, evidence indicates that CGRP also targets osteoblasts (Valentijn, Gutow et al. 1997; Villa, Dal Fiume et al. 2003; Villa, Mrak et al. 2006; Han, Zhang et al. 2010). Cultured osteoblasts

from multiple species demonstrate a characteristic rise in cyclic AMP concentration when exposed to CGRP directly (Michelangeli, Fletcher et al. 1989; Valentijn, Gutow et al. 1997; Villa, Mrak et al. 2006). CGRP exposure increases insulin-like growth factor –I (IGF-I) in osteoblasts (Michelangeli, Fletcher et al. 1989; Vignery and McCarthy 1996; Valentijn, Gutow et al. 1997). CGRP exposure increases interleukin-6 expression weakly (Sakagami, Girasole et al. 1993) and decreases tumor necrosis factor alpha expression (Millet and Vignery 1997). In addition, CGRP has been shown to control osteoblast production of cytokines *in vitro* (Valentijn, Gutow et al. 1997). Similar to calcitonin itself, CGRP inhibits osteoclast resorption directly in culture (Zaidi, Chambers et al. 1987) and decreases serum calcium *in vivo* (Roos, Fischer et al. 1986; Struthers, Brown et al. 1986).

CGRP is found in both central and peripheral neurons, notably including nerve fibers throughout bone (Ballica, Valentijn et al. 1999; Jimenez-Andrade, Mantyh et al. 2010; Wang, Guo et al. 2010). CGRP-positive nerve fibers are particularly concentrated along the epiphyseal trabeculae where they maintain cell-cell interactions with bone cells (Vignery and McCarthy 1996; Fan, Bouwense et al. 2010; Wang, Guo et al. 2010). With respect to these observations, it should be noted that the presence of CGRP-positive nerve fibers in and/or adjacent to epiphyseal trabecular bone is consistent with our hypothesis of

neuropeptide-dependent effects on bone (i.e., as opposed to effects of mechanical loading mediated by neuron-dependent muscular contraction). CGRP-producing nerve fibers increased three-fold in number during fracture healing (Hukkanen, Konttinen et al. 1993) and increased during osteogenesis in post-arthritic bone (Imai and Matsusue 2002). Interestingly, osteoblasts demonstrate functional CGRP receptors (Figures 2 and 3) (Bjurholm, Kreicbergs et al. 1992; Togari, Arai et al. 1997; Naot and Cornish 2008).

CGRP binds to a seven transmembrane-spanning G protein-coupled receptor and CGRP has been observed to induce a dose-dependent increase of cyclic AMP formation in chick, rat, and mouse calvarial osteoblasts and osteoblastic cell lines (Michelangeli, Fletcher et al. 1989; Bjurholm, Kreicbergs et al. 1992). In fetal rat osteoblasts, CGRP increased cell number and stimulated thymidine and phenylalanine incorporation (Cornish, Callon et al. 1999). CGRP has also been demonstrated to increase thymidine incorporation in human osteoblast-like cells (Villa, Melzi et al. 2000). Bernard and Shih reported that CGRP had an osteogenic stimulating effect by increasing the number and size of bone colonies in nucleated rat bone marrow cells (Bernard and Shih 1990). Furthermore, Vignery et al. showed that CGRP increased both the accumulation of mRNA encoding IGF-I

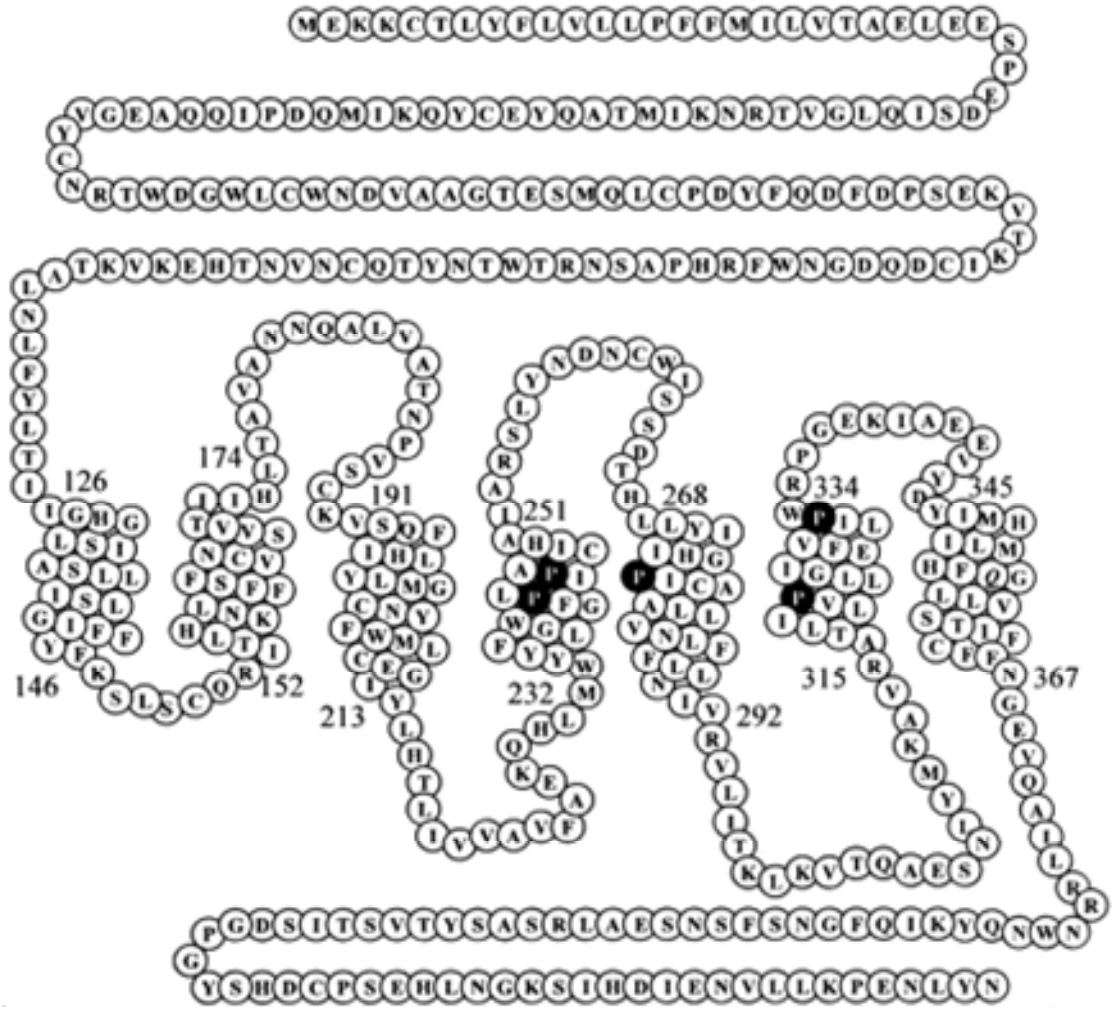

Figure 2. Amino acid sequence of the human CGRP receptor. Some residues are conserved within the calcitonin receptor family.
From Conner et al., 2007

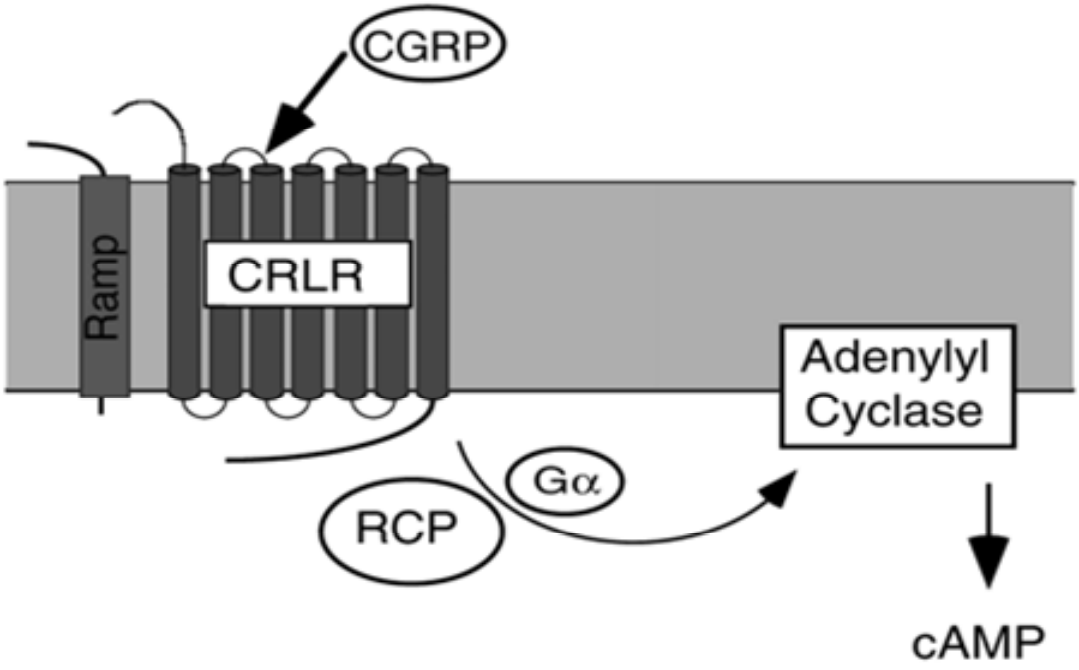

Figure 3. Model for functional CGRP receptor, including ligand-binding protein (CRLR), coupling protein (RCP), and chapter one protein (RAMP).
From Conner et al., 2005

and the production of IGF-I polypeptide by osteoblasts (Vignery and McCarthy 1996).

In vivo studies have demonstrated that targeted expression of CGRP in osteoblasts of transgenic mice increased trabecular bone density and bone volume at 6 weeks, 3 months, and 10 months (Ballica, Valentijn et al. 1999). In this study, mice were engineered to express CGRP in osteoblasts by placing the human CGRP gene under the control of the rat osteocalcin promoter. Trabecular bone density and bone volume were increased in mice expressing CGRP, compared to their control littermates (Ballica, Valentijn et al. 1999) were interpreted as evidence that CGRP may affect coupled bone formation (i.e., bone formation in response to bone remodeling coupling factors) rather than bone modeling (Ballica, Valentijn et al. 1999).

As shown in Figure 1, CGRP is produced by alternative splicing of mRNA transcribed from the calcitonin gene. Transgenic mouse studies in which the coding sequence for the gene is deleted resulted in loss of both calcitonin and CGRP expression. Mice lacking the entire gene have significantly greater trabecular bone volume and a 1.5- to 2-fold increase in bone formation (Hoff, Catala-Lehnen et al. 2002); however, it is not known if the effect was due to eliminating calcitonin, CGRP, or both. Interestingly, specific deletion of the αCGRP coding sequence of the calcitonin gene resulted in osteopenia caused by

decreased bone formation in mice (Schinke, Liese et al. 2004). Furthermore, treatment with CGRP and calcitonin inhibited bone resorption in ovariectomized rats (Vignery and McCarthy 1996).

Substance P

Substance P (SP) is an 11-amino-acid peptide richly distributed in sensory nerve fibers, often co-localizing with CGRP. SP is a member of the tachykinin family of neuropeptides, also known as neurokinins. In mammals, two separate genes designated preprotachykinin I (PPT-I) and preprotachykinin II (PPT-II) encode the tachykinins . The PPT-I gene can express four distinct forms of mRNA, all of which encode synthesis of SP (Nawa, Kotani et al. 1984). The four products of the tachykinin peptide hormone family include SP, neurokinin A, neuropeptide K, and neuropeptide gamma (Carter, Deibel et al. 1990). Since its discovery in 1931, SP has been studied as a therapeutic target for conditions as diverse as pain, inflammation, emesis, and depression (Prabhu, Malde et al. 2005). This undecapeptide is widely distributed in the central and peripheral nervous systems and is thought to participate in regulating neuronal survival and degradation, cardiovascular and respiratory function, modulation of autonomic reflexes, and neurotransmission of pain (O'Connor, O'Connell et al. 2004). SP is secreted by nerves as well as inflammatory cells

including macrophages, eosinophils, lymphocytes, and dendritic cells and acts by binding to the neurokinin-1 receptor (Figure 4) (O'Connor, O'Connell et al. 2004).

With up to four times as much SP transported to peripheral branches of primary sensory neurons compared with the dorsal root (Harmar, Armstrong et al. 1984), considerable quantities of neuropeptides are therefore stored peripherally for subsequent release (Figure 5) (Lundy and Linden 2004). SP has been shown to regulate the vasodilator activity of CGRP (Brain, Williams et al. 1985; Brain, MacIntyre et al. 1986; Brain and Grant 2004) suggesting that there may be a functional significance to the co-localization of the two peptides in neurons.

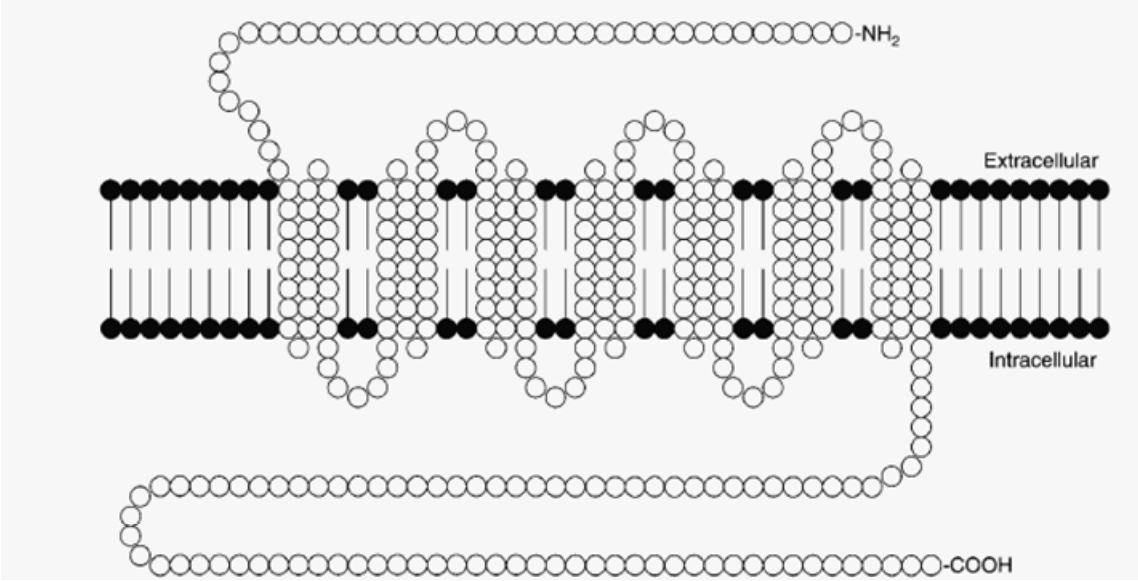

Figure 4. The neurokinin-1 receptor (NR-1R) is a glycoprotein with seven putative α-helical transmembrance segments, an extracellular amino-terminus, and an intracellular carboxyl tail. The human NK-1R consists of 407 amino acid residues and has a relative molecular mass of 46 kDa. The second and third membrane-spanning domains are involved in agonist/antagonist binding, the third cytoplasmic loop is responsible for G-protein interaction, while the cytoplasmic carboxyl terminal contains many serine and threonine residues which when phosphorylated, cause desensitization of the receptor in response to repeated application of agonist.
From O'Connor et al., 2004

The mechanisms of action and physiological functions of CGRP and SP in tissues receiving innervation from neurons that release these peptides are not well understood. Observed functions of CGRP and SP include activation of immune and inflammatory responses, blood flow regulation, osteoclast inhibition, activation of second messenger systems, IGF-I stimulation, Ca^{2+} regulation, and NO regulation in some cells (Michelangeli, Fletcher et al. 1989; Kawase, Howard et al. 1995; Vignery and McCarthy 1996; Ballica, Valentijn et al. 1999; Villa, Melzi et al. 2000; Fernandez, Chen et al. 2003; Kawase, Okuda et al. 2003; Lundy and Linden 2004). As in many physiological modulators, CGRP and SP likely affect cells in the context of many other hormones and growth factors, so effects of these peptides observed in any experimental model will depend on cell types and physiological conditions (Michelangeli, Fletcher et al. 1989; Kawase, Howard et al. 1995; Vignery and McCarthy 1996; Ballica, Valentijn et al. 1999; Villa, Melzi et al. 2000; Fernandez, Chen et al. 2003; Kawase, Okuda et al. 2003; Lundy and Linden 2004).

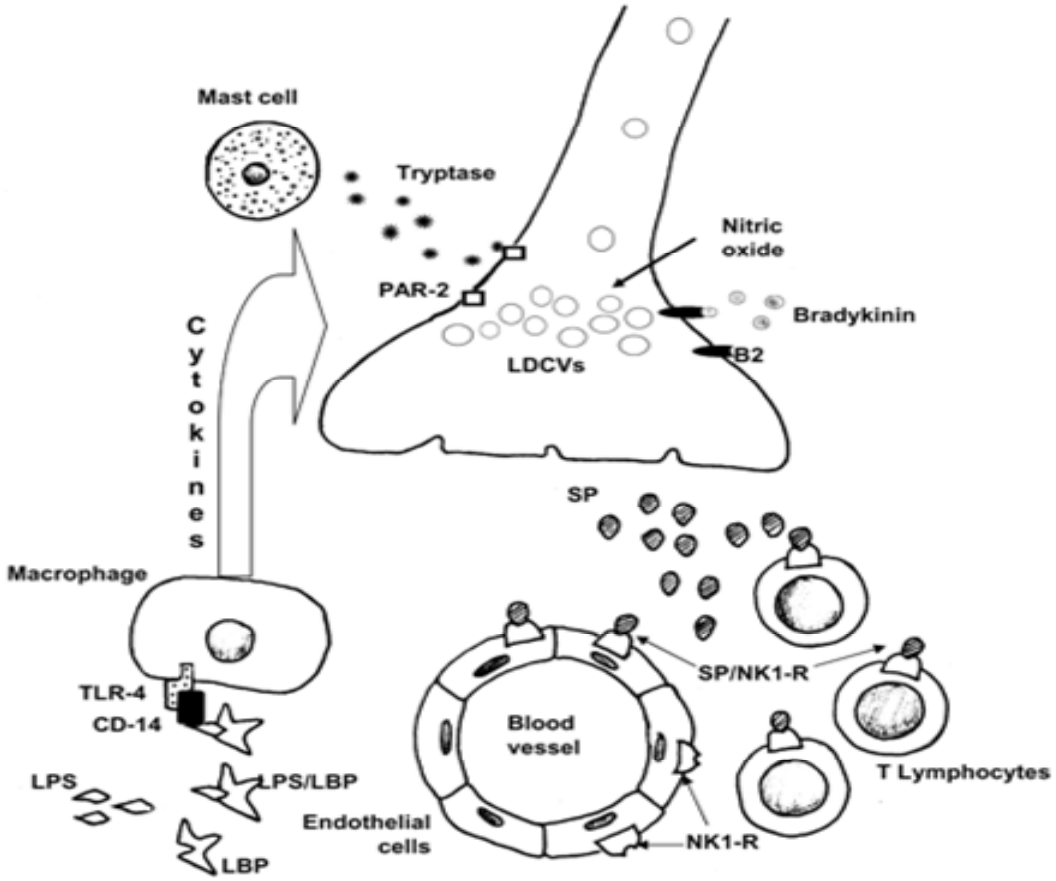

Figure 5. Factors influencing the release of substance P (SP) from sensory nerves. SP is released from dense core vesicles (LDVCs) following (1) cleavage of protease-activated receptor-2 (PAR-2) by tryptase, (2) bradykinin binding to B2 receptors, (3) sensitization by cytokines induced by LPS, or (4) nitric oxide. Activation of the vanilloid receptor and sensitization of sensory nerves by prostaglandins are not shown. Exocytosed SP binds to the neurokinin-1 receptor (NK1-R) expressed on endothelial cells causing vaosodilation and edema formation, prominent features of neurogenic inflammation. SP also interacts with immune cells inducing T-lymphocyte proliferation. NK-1 receptor expression on macrophages and monocytes is not shown. As a result of these interactions, SP is believed to act as an important mediator of crosstalk between the nervous and immune systems. SP, Substance P; NK1-R, neurokinin 1 receptor; LDCVs, large dense core vesicles (containing neuropeptdies); PAR-2, protease-activated receptor-2; B2, bradykinin receptor; TLR-4, Toll-like receptor-4; LPS, lipopolysaccharide; and LBP, lipopolysaccharide binding protein. From Lundy and Linden, 2004

Osteoblast Differentiation

The central thesis of this proposal is that bone growth and metabolism are regulated, in part, by the nervous system, specifically by neuropeptides and, more specifically, by CGRP and/or SP. The studies summarized in this report were intended to assess the *in vitro* effects of CGRP and SP on osteoblast growth, differentiation, and mineralization; and, because our studies did reveal neuropeptide-dependent differences in differentiation and mineralization, we conducted supplemental studies to determine whether these effects reflected changes in the patterns of gene expression. Previous studies in several *in vitro* model systems have shown that temporal expression of osteoblast phenotype-related genes may be used to define the growth period, the postproliferative matrix-maturation stage, and the mineralization stage (Figure 6). For example, c-fos and c-myc are among the genes expressed during the proliferation period (Kitching, Qi et al. 2002). The matrix maturation period is characterized by peak levels of alkaline phosphatase as well as the transcription factors osterix and Runx2/Cbfa1 (Lian, Stein et al. 1998; Kitching, Qi et al. 2002; Arnett 2008). Runx2/Cbfa1 and osterix are essential for bone development as demonstrated by the inhibition of bone tissue formation in gene deletion mouse models (Komori 2000; Nakashima, Zhou et al. 2002). Runx2/Cbfa1 enhances transcription of bone

marker genes including type I collagen and osteocalcin (Gutierrez, Javed et al. 2002; Lee, Lim et al. 2006). Runx/Cbfa1 expression was not affected in osteoblast lineage cells of osterix-/- mice, while osterix expression was absent in Runx2/Cbfa1-/- mice, suggesting that osterix functions downstream of Runx/Cbfa1 in transcriptional regulation of osteoblast differentiation (Nakashima, Zhou et al. 2002). Both genes, as well as transcription factor Dlx5, are induced by BMP-2, which induces osteoblast differentiation. Dlx5 was reported to independently induce expression of Runx/Cbfa1 and osterix in C2C12 mesenchymal cells (Lee, Javed et al. 1999).

During the mineralization period, mRNA transcripts of osteocalcin and bone sialoprotein are increased (Lian, Stein et al. 1998; Kitching, Qi et al. 2002). These non-collagenous bone matrix proteins are considered important for bone mineralization. Both mineralization and expression of osteoblast stage-specific genes in MC3T3-E1 osteoblasts are affected by hormones, growth factors, and extracellular matrix proteins (Lian, Stein et al. 2003; Luppen, Leclerc et al. 2003; Jadlowiec, Koch et al. 2004; Pornprasertsuk, Duarte et al. 2004). To determine effects of CGRP and SP on osteoblast differentiation, our studies examined the effects of these neuropeptides on mRNA levels of selected marker genes.

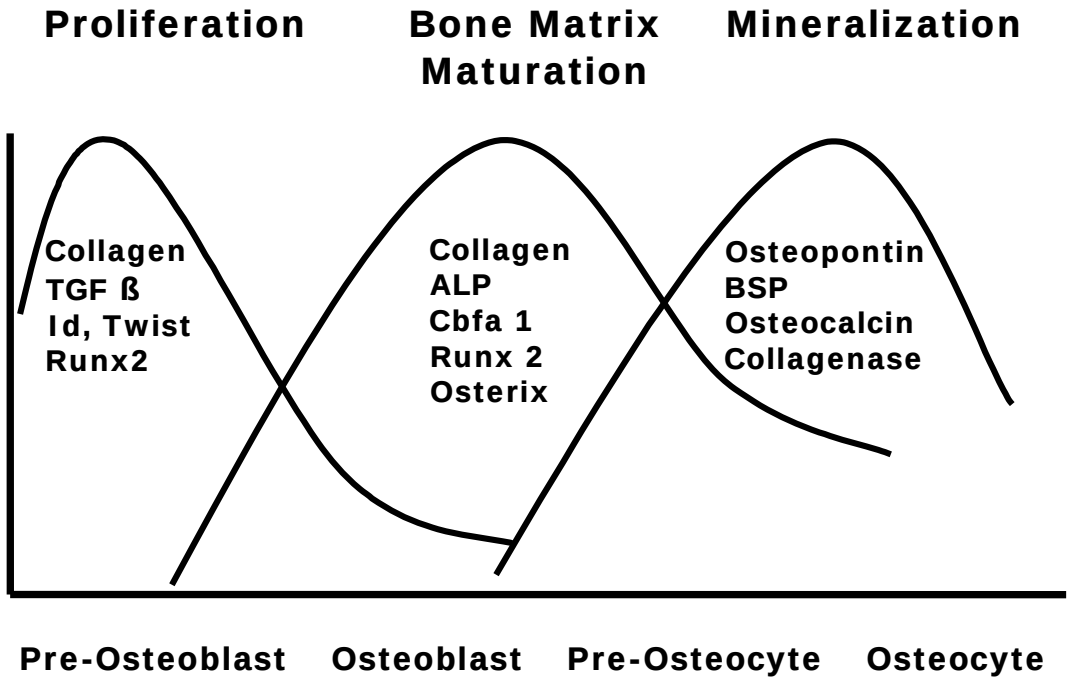

Figure 6. Temporal expression of cell growth and osteoblast phenotype-related genes during 35 days of culture of fetal rat calvarium-derived osteoblasts as they synthesize a mineralizing matrix. Peak expression levels of each gene, which were determined in total cellular RNA prepared at 3-day intervals, define the growth period, the post-proliferative matrix maturation stage, and mineralization stage.
(Lian, Stein et al. 1998)

We also assessed the effects of CGRP and SP on COX-2 mRNA, which codes for a specific inducible isoform of the enzyme, cyclooxygenase. Cyclooxygenase, also known as prostaglandin synthase, is the rate limiting enzyme of the synthetic pathway which converts arachidonic acid to prostaglandins and related molecules (Chikazu, Li et al. 2002). Recent studies suggest a possible role for COX-2 in bone repair (Zhang, Kuroda et al. 2002; Boursinos, Karachalios et al. 2009). A retrospective study by Giannoudis et al., (Giannoudis, Pape et al. 2002) described the association between nonunion and the use of non-steroidal anti-inflammatory drugs (NSAIDS) after injury. Sixty-three percent of the patients who developed nonunions admitted to taking NSAIDS versus 13% in the control group (Dahners and Mullis 2004). Using a rat spine fusion model, Dimar et al., reported that posterior fusion rate dropped from 45% in the placebo group to 10% in the indomethacin-treated group (Dimar, Ante et al. 1996). Simon et al. showed that fracture healing failed in mice treated with selective COX-2 inhibitors (celecoxib and rofecoxib) and fracture healing also failed in mice homozygous for a null mutation for the COX-2 gene (Simon, Manigrasso et al. 2002). Osteoblasts increase expression of COX-2 and increase production of PGE_2 in response to a wide variety of ligands (Wadleigh and Herschman 1999) as well as mechanical stress (Norvell, Ponik et al.

2004). COX-2 is produced in bone-forming cells such as osteoblasts and osteocytes (Forwood and Turner 1995) and its expression is highly regulated by hormones, growth factors, and cytokines both systemically and locally (Jones, Tsigkou et al. 2007). Recent studies have shown that SP and CGRP may influence COX-2 expression and/or PG release in cells (Ghilardi, Svensson et al. 2004; Bachar, Rose et al. 2005). For example, intrathecal administration of SP in rats increased PGE_2 release into the spinal fluid and SP NK-1 receptor antagonists blocked this effect (Vanegas and Schaible 2001). Activation of CGRP or SP receptors results in prostaglandin release in spinal nerves and astrocytes (Malmberg and Yaksh 1992; Hua, Chen et al. 1998); Peripheral inflammation was found to increase COX-2 expression in neurons adjacent to SP CGRP positive neurons in dorsal horn areas that transmit pain signals from inflammation (Yamada, Kamiya et al. 1999). Prostaglandins in turn act on nerve terminals of primary afferents to further release CGRP and SP in a positive feedback loop (Figure 7) (Trang, Sutak et al. 2002) and treatment of cultured dorsal root ganglion cells with inflammatory cytokine IL-1 ß increased SP release and COX-2 expression (Inoue, Muneyuki et al. 1997; Inoue, Yamada et al. 1999). Interleukin 1- ß induced SP release from primary afferent neurons through the cyclooxygenase-2 system (Inoue, Muneyuki et al. 1997; Inoue, Yamada et al. 1999). In this

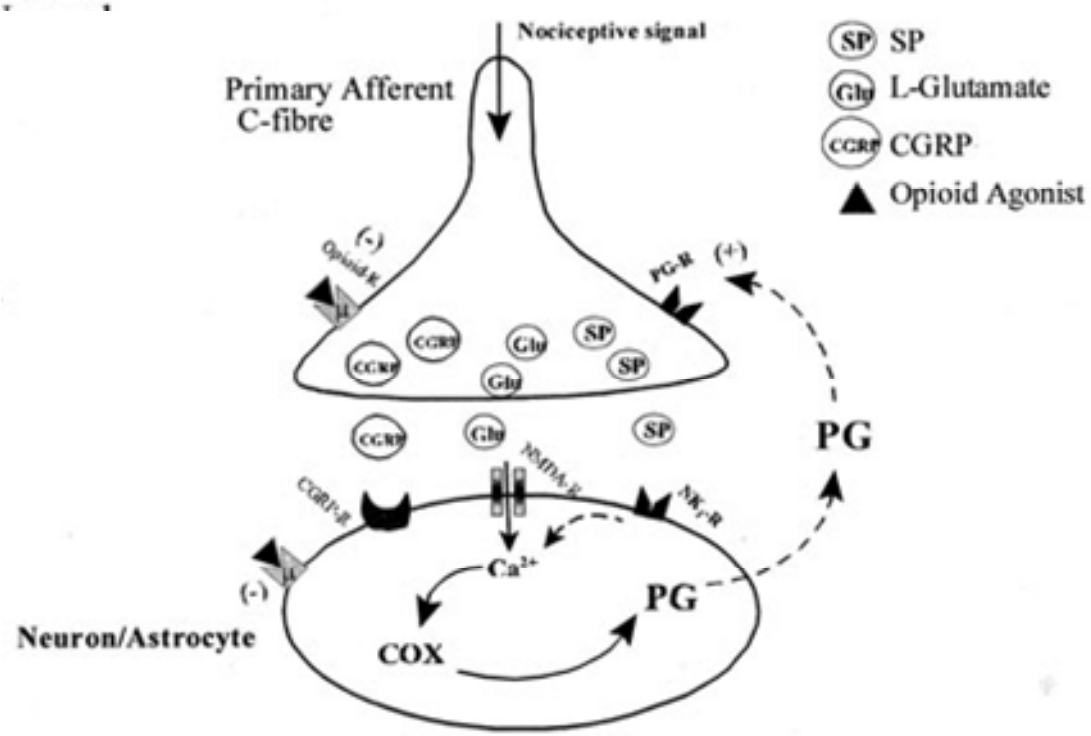

Figure 7. Model illustrating the reciprocal relationship between sensory transmitters and prostaglandins in the dorsal horn. Activity of primary afferents in the dorsal horn releases glutamate, SP, and CGRP, which activate specific receptors located on neurons and astrocytes and mobilize prostaglandins (PG), which act on presynaptic receptors to release more sensory transmitters. These processes constitute a positive feedback loop.
From Trang et al., 2002

study, we measured the effects of CGRP and/or SP on COX-2 mRNA expression in osteoblastic cells. The results of these studies are anticipated to advance our general understanding of the neuropeptidergic influence on osteoblasts and bone growth, and our specific understanding of the potential roles of CGRP and SP in this process.

Hypothesis

Proper regulation of bone growth and metabolism is necessary for the skeleton to provide its vital mechanical, protective, and metabolic functions. Histomorphometric studies of bone from the human iliac crest have revealed that bone formation is determined primarily by changes in osteoblast number and, to a lesser degree, by changes in osteoblast biosynthetic activity. Osteoblasts are the bone-forming cells, which produce bone matrix and respond to the physiological and mechanical demands on the skeleton. In addition, osteoblasts relay signals from osteolytic hormones and cytokines to osteoclasts by producing RANKL, which activates osteoclast formation and bone resorption. Osteoblast number and function are regulated by a number of endocrine, paracrine, and autocrine factors (Goad and Tashjian 1993; Konttinen, Imai et al. 1996; Canalis 2000; Goltzman, Karaplis et al. 2000; Sooy, Sabbagh et al. 2005). Recent studies

suggest that osteoblasts may also be regulated by the nervous system (Hill and Elde 1991; Hukkanen, Konttinen et al. 1993; Grills and Schuijers 1998; Asaumi, Nakanishi et al. 2000; Elefteriou 2008; Wang, Zhao et al. 2009; Franquinho, Liz et al. 2010). The central thesis of this proposal is that bone growth and metabolism are regulated, in part, by the nervous system and, specifically, by neuropeptides. Accordingly, knowledge of these regulatory mechanisms would provide new insights to the etiologies of neuropeptide-linked bone volume regulation and the possible development of neuropeptides as treatment modalities for disorders of bone metabolism. The goal of the studies summarized in this proposal was to determine the role of the nervous system in the regulation of osteoblast function and bone volume regulation. The specific aims of this proposal were intended to assess the following hypotheses.

Hypothesis 1. CGRP and/or SP have direct, osteogenic effects on the number of osteoblast-line cells in bone and/or the extent of osteoblastic differentiation.

Hypothesis 2. CGRP and/or SP have direct effects on osteoblast-line cells to increase mineralization.

Hypothesis 3. The osteogenic effects of CGRP and/or SP are dependent on changes in the expression of stage-specific osteoblast genes.

Specific Aims

Specific Aim#1. Determine whether the exogenous application of the neuropeptides CGRP and SP increases osteoblast growth and differentiation, as assessed by changes in cell layer protein and skeletal alkaline phosphatase activity, respectively. (Skeletal alkaline phosphatase is an ecto-enzyme, and is regarded as a marker of mature osteoblasts (Manolagas, Burton et al. 1981; Bianco, Riminucci et al. 1993; Lian, Stein et al. 1998). Neonatal mouse calvaria cells and the MC3T3-E1 cell line were used for Aim #1.

Specific Aim #2. Determine whether the exogenous application of the neuropeptides CGRP and SP increases osteoblast mineralization, when mineralization as assessed by comparing calcium deposition after 7–14 days of growth with and without neuropeptides. Mineralization is determined by the quantitation of alizarin red staining (Hessle, Johnson et al. 2002; Dallas, Veno et al. 2009). The mouse osteoblast cell line MC3T3-E1 was used for Aim #2.

Specific Aim #3. Determine whether the osteogenic effects of exogenous neurotrophic factors (i.e., CGRP and SP) are associated with or determined by changes in the expression of stage-specific osteoblast genes. The most significant genes that are transcribed and translated in osteoblasts are those dealing with the synthesis of an extracellular matrix, i.e., collagenous and noncollagen matrix proteins

(Canalis 2000), and transcription factors that regulate osteoblast differentiation. Relative levels of mRNA for a variety of osteoblast-specific proteins and regulatory enzymes were quantified by real-time polymerase chain reaction (RT-PCR). The MC3T3-E1 cell line was used for these studies.

CHAPTER TWO

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle Medium (DMEM) was purchased from Mediatech (Herndon, VA). α-Minimum Essential Medium was from Gibco-Invitrogen (Grand Island, NY). Bovine calf serum (CS) and Fetal bovine serum (FBS) were obtained from Atlanta Biologicals (Norcross, GA). Tissue culture ware was obtained from Becton Dickenson (Franklin Lakes, NJ) and Corning Life Sciences (Fisher Scientific, Pittsburg, PA). All other chemicals were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Chino, CA). RNA purification kits were purchased from Gentra Systems (ISIS, Wicklow, Ireland), First-Strand cDNA Synthesis kits for RT-PCR were from Invitrogen (Carlsbad, CA), and Quantitech SYBER Green PCR kits were from Qiagen (Valencia, CA).

Cell Culture

Calvarial osteoblasts were isolated by collagenase digestion from 2–3 day old Swiss-Webster mice obtained from Harlan, using standard techniques (Michelangeli, Fletcher et al. 1989; Bjurholm, Kreicbergs et

al. 1992; Arnett 2003). Osteoblasts were collected and grown to 80% confluence in DMEM containing 10% CS at 37°C with 5% CO_2. For measurements of mineralization, MC3T3-E1 cells obtained from Dr. Baruch Frenkel at the University of Southern California (Smith, McCabe et al. 2006) were cultured in α-MEM containing 10% FBS at 37°C with 5% CO_2. The NIH 3T3 mouse embryo fibroblast cell line was from ATCC.

Neuron Osteoblast Co-Cultures

Neurons were isolated from fetal rat neocortex and included 65% cortical neurons and 35% glial cells. Blocks of tissue were placed on glass cover slips and cultured in 35 mm culture dishes. Osteoblasts were divided into three experimental conditions: (1) osteoblasts only, in which two cover slips were placed into a small culture dish; (2) osteoblasts plus neurons, in which a single cover slip containing osteoblasts was placed into a culture dish together with a single cover slip of neurons placed at a distance to prevent physical contact with osteoblasts, and (3) osteoblasts plus fibroblasts, in which a cover slip containing osteoblasts was placed into a petri dish containing a cover slip of fibroblasts. Culture dishes contained 2 ml of serum-free F12/Delbecco's Modified Essential Medium (Sigma, St. Louis, MO). Serum-free media was chosen to eliminate the possibility of trophic

factors in fetal calf serum that could affect the growth of the osteoblasts. A total of five repeat experiments were performed, in which each experiment contained five or more petri dishes for each experimental condition. All cultures were maintained at 37°C with 5% CO_2 and visually inspected each day for seven days at which time the osteoblasts were stained for alkaline phosphatase activity as described (Lundy et al. 1991).

Following staining, the cover slips containing osteoblasts were attached to glass slides with mounting medium and analyzed on a Nikon Optiphot microscope with Normarski Optics. In practice, a 10x10 net eyepiece graticule was used to calculate cell area and density on each cover slip. Each adjacent non-overlapping area, constituting one vertical sweep through the center of the cover slip, was photographed and analyzed. Alkaline phosphatase positive areas were photographed with a digital microscope camera (Aphogee Inc., Temecula, CA), and analyzed using a computer image software program (Scion Image). All statistical analyses were made using ANOVA (Microsoft Excel, Redmond, WA) with Bonferroni post-hoc comparisons. A probability of less than 0.05 was considered significant.

Measurement of ALP Activity

Swiss Webster calvarial osteoblasts were plated in 24-well plates in DMEM with 10% CS at 30,000–40,000 cells/ml (Lundy et al. 1991; Farley et al. 2005). Twelve hours later, the medium was replaced with serum-free DMEM and neuropeptides or equivalent amounts of dilution buffer were added to replicate wells (n=3). After 48 hours of continuous exposure, replicate cell cultures were rinsed with PBS, then scraped and homogenized by agitation with a disposable transfer pipette and extracted in 0.01% Triton X-100 (vol/vol). Alkaline phosphatase activity was measured in these extracts by the rate of hydrolysis of ρ-nitrophenylphosphate (10 mM), in 200 mM carbonate/tris buffer at pH 10.3, in the presence of 1 mM $MgCl_2$. Using the method of Lundy (Lundy et al. 1991) and Farley (Farley et al. 2002), ρ-nitrophenolate production was measured as the time-dependent increase in optical density at 410 nm, using an automated microtiter-plate spectrophotometer (SLT LabInstruments Easy Reader Ear 400AT). Alkaline phosphatase specific activity was calculated as U/mg protein, where 1 U ALP activity = 1 μmol product (ρ-nitro-phenolate) formed per minute at 22°C. The dye binding method of Bradford (Bradford 1976; Schleicher and Wieland 1978; Compton and Jones 1985; Noble and Bailey 2009) was used to determine total cell layer protein concentration in the extracts, using BSA standards and

blanks also prepared in 0.01% triton X-100. Alkaline phosphatase levels were normalized to protein levels in order to calculate alkaline phosphatase specific activity.

Alkaline Phosphatase Assay Protocol

1. Remove media and rinse cells 3 times with PBS.
2. Add 0.25 ml 0.01% Triton X-100 to each well (24 well plate) to extract cells.
3. Scrape the cell layer, using cell scraper or plastic pipette tip, and mix the solution by repeat pipetting.
4. Transfer aliquots of cell layer extract of each sample (0.05 ml) into wells of 96-well plate and add 0.250 ml of the ALP substrate solution to every well to start the reaction.
5. Take an initial OD reading (410 nm with a reference wavelength of 490 nm) as soon as the substrate is added and a final reading after incubating at room temperature when the solutions are visibly yellow.
6. Calculate ALP activity: 1 milliunit equals 1 nm of product produced per minute (milliunit/ml of extract = Change in OD x 410 / OD standard x 1/time in minutes)

Mineralization

MC3T3-E1 cells were plated in 6-well plates at 30,000–40,000 cells/ml, in a volume of 2.0 ml/well. When the cells were 80% confluent, the culture medium was supplemented with 50 μg/ml ascorbic acid and 10 mM ß-glycerolphosphate to support mineralization with and without addition of neuropeptides; 3 replicate wells for each dose of neuropeptide and 3 replicate control wells containing an equivalent amount of the dilution buffer were compared. Media was replaced and fresh peptides were added every other day. After 7–14 days, cells were rinsed 5 times with PBS and fixed for one hour at 4°C with 10% formalin in PBS. Alizarin red solution (40mM, pH 4.2) was applied to the fixed cells for 10 minutes at room temperature. Alizarin red staining was quantified by extracting the calcium-bound dye with cetylpyridinium chloride (a 10% w/v solution in water, 1.0 mL per 6-place well) and reading the absorbance at 570 nm (Hessle, Johnson et al. 2002).

Alizarin Red/Cetylpyridinium Chloride (CPC) Protocol

1. Remove media and wash cells 5 times with PBS.
2. Fix with 10% Formalin in PBS (Fischer Scientific) for 60 minutes at 4°C. (1 ml /well for 6-well plates)
3. Wash culture gently 5 times with PBS and Nanopure water.

4. Stain with 40 mM Alizarin Red (p.H. 4.2) at room temperature for 15 minutes. (1 ml/well for 6-well plates)
5. Wash gently 5 times with Nanopure water, rinse with PBS for 15 minutes to reduce nonspecific staining.
6. Photograph using light box.
7. Destain with 10% (w/v) CPC in 10 mM sodium phosphate buffer (p.H. 7.0) for 60 minutes at room temperature with gentle rotation. (1 ml/well in 6-well plates)
8. Dilute aliquots 10-fold in 10% CPC solution in 96-well plate in triplicate.
9. Measure absorbance at 562 nm for quantification.

mRNA Measurements by RT-PCR

Replicate 100 mm-diameter dishes of Swiss-Webster calvarial osteoblasts and/or MC3T3-E1 cells were exposed to doses of effectors (including zero effector controls). Following treatment (i.e., 24–96 hours of continuous exposure) with neuropeptides, total cellular RNA was extracted from individual dishes of control and experimental cells using a Versagene RNA purification kit. RNA concentration was determined at A260 using Nanodrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). cDNA templates for RT-PCR were synthesized from 1 μg of total RNA by reverse transcription reaction

using a SuperScript III First Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA) and a PTC-150 MiniCycler (MJ Research Inc., Waltham, MA). Relative levels of mRNA were quantified using real-time polymerase chain reaction with a Quantitech SYBER Green PCR kit (Qiagen, Valencia, CA) and DNA Engine Opticon System (MJ Research/ BioRad, Hercules, CA). For each 20 µl reaction, 2 µl of Superscript III RT reaction product was used as template with 10 µM primer pairs described in Table 1. Fluorescence of SYBER Green dsDNA binding dye was measured by the Opticon detector and cycle threshold data were analyzed using Opticon Monitor software.

Briefly, the PCR parameters were denatured at 95°C for 10 minutes; followed by 35 cycles of 95°C for 1 minute, 56.6°C for 30 seconds, 72°C for 1 minute. Melting curve-analysis was performed by the Opticon System to analyze product homogeneity; and gel electrophoresis was performed for further confirmation. Osteoblast stage-specific primers for mouse type Iα1 collagen, alkaline phosphatase, bone sialoprotein, osteocalcin, Runx2/Cbfa1, COX-2, and osterix (Table 1) were used to determine the effect of neuropeptides on mRNA levels of osteoblast stage-specific proteins. Cyclophilin was used as a housekeeping gene for normalizing each sample. The characteristics of each of the PCR primer pairs are summarized in the following table (Table 1).

Table 1. RT-PCR Primer List. Primer names indicate 5' base pair position in sequence of Genbank Accession number, and forward (F) or reverse (R).

Protein	Primer Name	Accession Number	% GC	Tm ºC	# bp	sequence
Osterix	mOSX 1152F	NM_130458	55.0	57.8	20	5'-cac ttg cct gct ctg ttc ca-3'
Osterix	mOSX 1350R	NM_130458	50.0	55.6	20	5'-cgg ctg att ggc ttc ttc tt-3'
Cbfa1	mCbfa1 41F	NM_009820	55.0	57.9	20	5'-gcg gtg caa act ttc tcc ag-3'
Cbfa1	mCbfa1 144F	NM_009820	42.9	55.0	21	5'-ggc tgc aag cag tat tta caa-3'
Cbfa1	mCbfa1 273R	NM_009820	55.0	58.3	20	5'-ggt gct cgg atc cca aaa ga-3'
Cbfa1	mCbfa1 523R	NM_009820	63.2	59.9	19	5'-cca tgg tgc ggt tgt cgt g-3'
Osteocalcin	mOC 362F	NM_031368*	55.0	55.3	20	5'-gct acc ttg gag ctt cag tc-3'
Osteocalcin	mOC 485R	NM_031368	55.0	58.8	20	5'-atg cgt ctg tag gcg gtc tt-3'
Collagen (I)	mcol1a1f1432	BC003198	55.0	58.3	20	5'-gac ctt cct gcg cct aat gt-3'
Collagen (I)	mcol1a11602R	BC003198	50.0	55.5	20	5'-ctg tag gtg aag cga ctg tt-3'
Bone Sialoprotein	mBSPf733	NM_008318	55.0	58.2	20	5'-agg tgc aga agg aac cac ag-3'
Bone Sialoprotein	mBSP989R	NM_008318	50.0	54.8	20	5'-cgt cct cat aag ctc ggt aa-3'
Alkaline Phosphatase	mALP 1984F	X13409	60.0	60.5	20	5'-cca gac cct gca acc tcc at-3'
Alkaline Phosphatase	mALP 2253R	X13409	55.0	58.8	20	5'-ggt tgg cga gtc tct gca at-3'
Alkaline Phosphatase	mALP 2272R	X13409	55.0	55.9	20	5'-gag agc cac ttc agt gaa gg-3'
Cyclophilin	rmcyclophilin 315 F	BC059141	55.0	57.9	20	5' gca tac agg tcc tgg cat ct 3'
Cyclophilin	rmcyclophilin 505 R	BC059141	50.0	56.6	20	5' tct tgc tgg tct tgc cat tc 3'
Cyclophilin	rmcyclophilin 596 R	BC059141	55.0	55.1	20	5' gct ctc ctg agc tac aga ag 3'

Subsequent to finishing these experiments, NM_031368 was identified as mouse osteocalcin-related gene, and not actual osteocalcin (Petrucci et al. 2006).

Total RNA was isolated at 0, 1, and 3 days from control cells and cells treated with CGRP plus SP. Relative mRNA expression levels of each sample were determined by quantitative RT-PCR with the comparative C_t method. The C_t (cycle threshold) value represents the PCR amplification cycle at which the fluorescent signal exceeds a background threshold level (Figure 24). After determining C_t for each gene, relative levels of each osteoblast marker gene were normalized to mRNA levels of the housekeeping gene cyclophilin. Each sample was analyzed for each osteoblast gene in duplicate in at least three experiments, and cyclophilin was assessed in the same assay each time. A ΔC_t value was obtained for each osteoblast gene mRNA by subtracting the C_t value of cyclophilin mRNA from the C_t value of the mRNA for each sample. To compare relative mRNA levels between treated and untreated cells at each time point, $\Delta\Delta C_t$ was calculated as Control ΔC_t – Treated ΔC_t. The ratio of Treated/Control mRNA levels was calculated as $2^{(\Delta\Delta Ct)}$. C_t value represents threshold cycle for PCR amplification cycle between cycle and reference.

Versagene Total RNA Purification Protocol

1. Remove media and rinse cells 3 times with PBS.
2. Thoroughly homogenize sample in appropriate volume calculated using cell number ($3 \times 10^4 - 1 \times 10^6$ cells use 200 μl of Lysis solution).
3. Pipette 400μl of lysate onto Purification Column and centrifuge at 13,000–16,000 x g for 1 minute.
4. Apply lysate onto column in 400 μl aliquots and centrifuge at maximum speed after each addition of 400 μl lysate.
5. Transfer basket containing Purification Column to a new tube.
6. Add 400 μl Wash 1 to Purification Column and centrifuge at 13,000–16,000 x g for 1 minute.
7. Transfer basket containing Purification Column to new tube.
8. Apply 50 μl DNase solution to the column and incubate at room temperature for 15 minutes.
9. Add 200 μl DNase Wash Solution to the column and centrifuge at 13,000–16,000 x g for 1 minute.
10. Add 200 μl DNase Wash Solution to the column and centrifuge at 13,000–16,000 x g for 2 minutes.
11. Transfer column to new tube.
12. Add 200 μl Wash 2 Solution to the Purification Column and centrifuge at 13,000–16,000 x g for 1 minute.

13. Add an additional 200 μl Wash 2 Solution to the purification Column and centrifuge at 13,000–16,000 x g for 2 minutes.
14. Transfer basket containing Purification Column to a new tube.
15. Add 50–100 μl Elution Solution to the Purification Column and centrifuge at 13,000–16,000 x g for 1 minute.
16. Discard basket, place tube containing purified RNA on ice and store at -80°C.
17. Make A_{260} and A_{280} readings with Nanodrop.

First-Strand cDNA Synthesis Using SuperScript™ III for RT-PCR

1. Add the following components to a nuclease-free tube:

   1 μl of oligo $(dT)_{20}$ (2 nM)

   1 μg total RNA (in up to 11 μl)

   1 μl 10mM dNTP Mix, pH7.0 (10 mM each dATP, dGTP, dCTP)

   RNAse, DNAse free H_2O to final volume of 11 μl
2. Heat mixture to 65°C for 5 minutes and incubate on ice for at least 1 minute.
3. Collect the contents of the tube by brief centrifugation and add:

   4 μl 5X First-Strand Buffer

   1 μl 0.1 M DDT

   1 μl RNase OUT™ Recombinant RNase Inhibitor

   1 μl SuperScript™ III RT (200 units/ μl)

4. Mix by pipetting gently up and down.
5. Incubate at 50°C for 30–60 minutes.
6. Inactivate the reaction by heating at 70°C for 15 minutes.
7. To remove RNA complementary to the cDNA, add 1 μl (2 units) *E. coli.* RNase H and incubate at 37°C for 20 minutes.

Statistical Analysis

Data are presented as averages of replicates (mean +/- standard error). Statistical analyses were performed with Systat software (Point Richmond, CA). Group comparisons were made by one- and two-way analysis of variance with Tukey's post hoc comparison. Correlations were assessed by linear regression. In order to minimize the effects of interassay variation, statistical methods were limited to intraassay group comparisons (i.e., except where specified as otherwise). All reported observations were confirmed in a minimum of three experiments. Minimal statistical significance was defined as $p < 0.05$.

CHAPTER THREE

RESULTS

Preliminary data demonstrated that rat calvarial osteoblasts cultured in media shared with primary cortical neurons demonstrated more intense staining for alkaline phosphatase (Figure 8). In this study, neurons were isolated from fetal rat neocortex and included 65% cortical neurons and 35% glial cells. Osteoblasts were divided into three experimental conditions: (1) osteoblasts only, in which two cover slips containing osteoblasts were placed into a small petri dish; (2) osteoblasts plus neurons, in which a single cover slip containing osteoblasts was placed into a petri dish together with a single cover slip of neurons placed at a distance to prevent physical contact with osteoblasts, and (3) osteoblasts plus fibroblasts, in which a cover slip containing osteoblasts was placed into a petri dish containing a cover slip of fibroblasts. Osteoblasts cultured in the presence of neurons demonstrated significant increases in cell number and cell area compared to osteoblasts cultured without neurons (Figures 8–10). From this, we inferred that the neurons can produce growth and/or differentiation factors which can be active on osteoblasts.

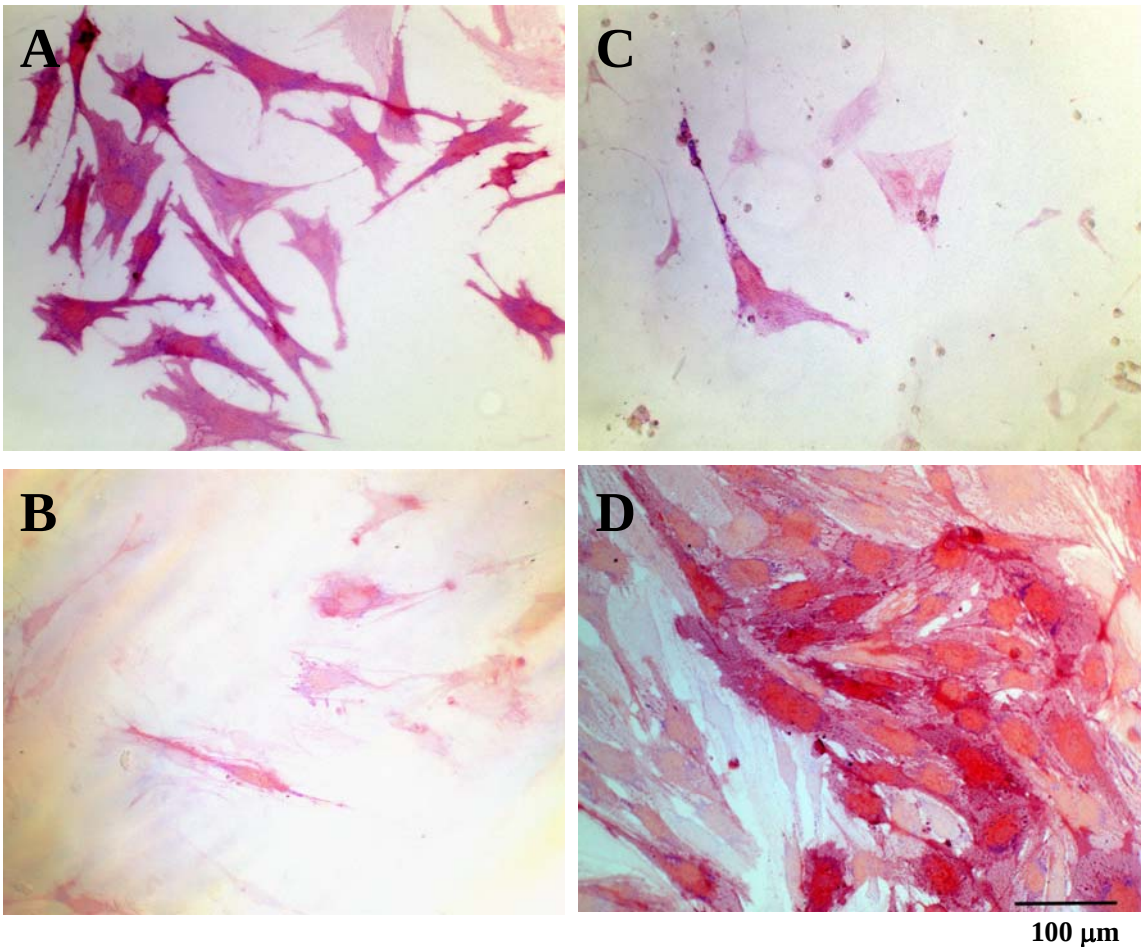

Figure 8. Photomicrograph of alkaline phosphatase staining (A) control rat osteoblasts, (B and C) osteoblast grown in the presence of fibroblasts, and (D) osteoblasts grown in the presence of primary rat cortical neurons.

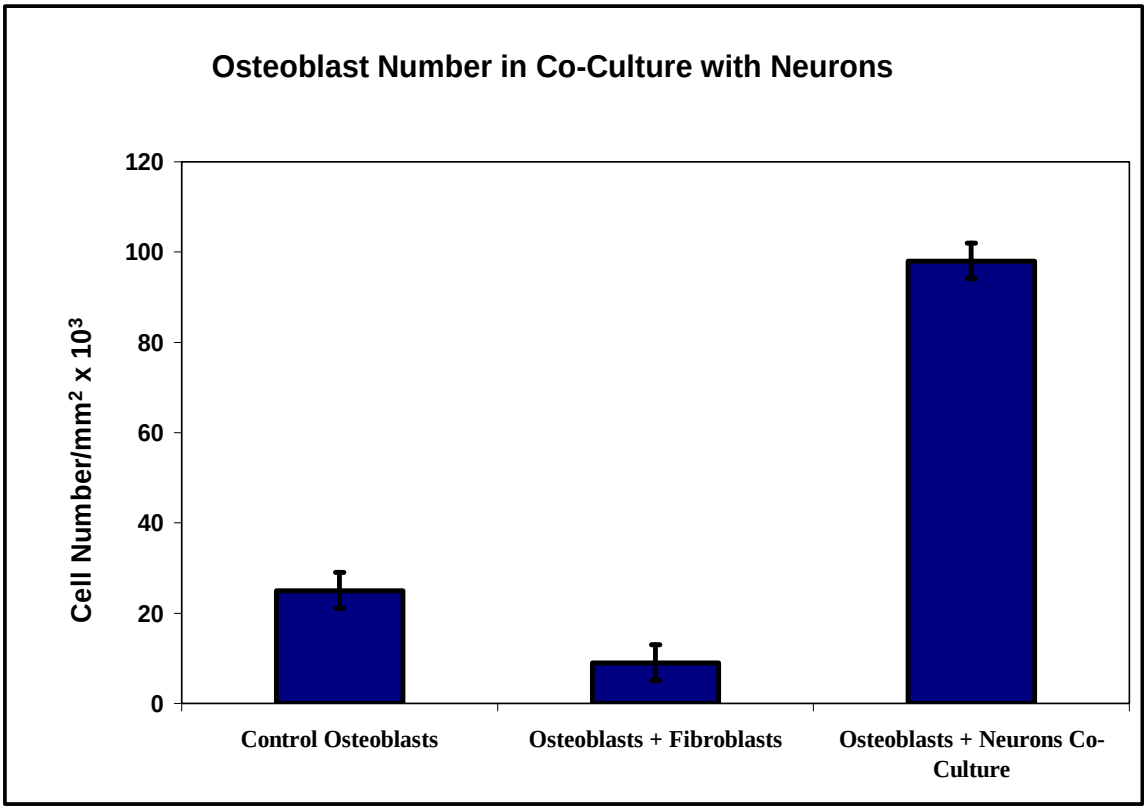

Figure 9. Number of osteoblasts after 7 days in culture as described in Methods. Significantly greater numbers of osteoblasts were obsevered when co-cultured with primary cortical neurons than in the other two conditions ($p<0.0001$). n=5

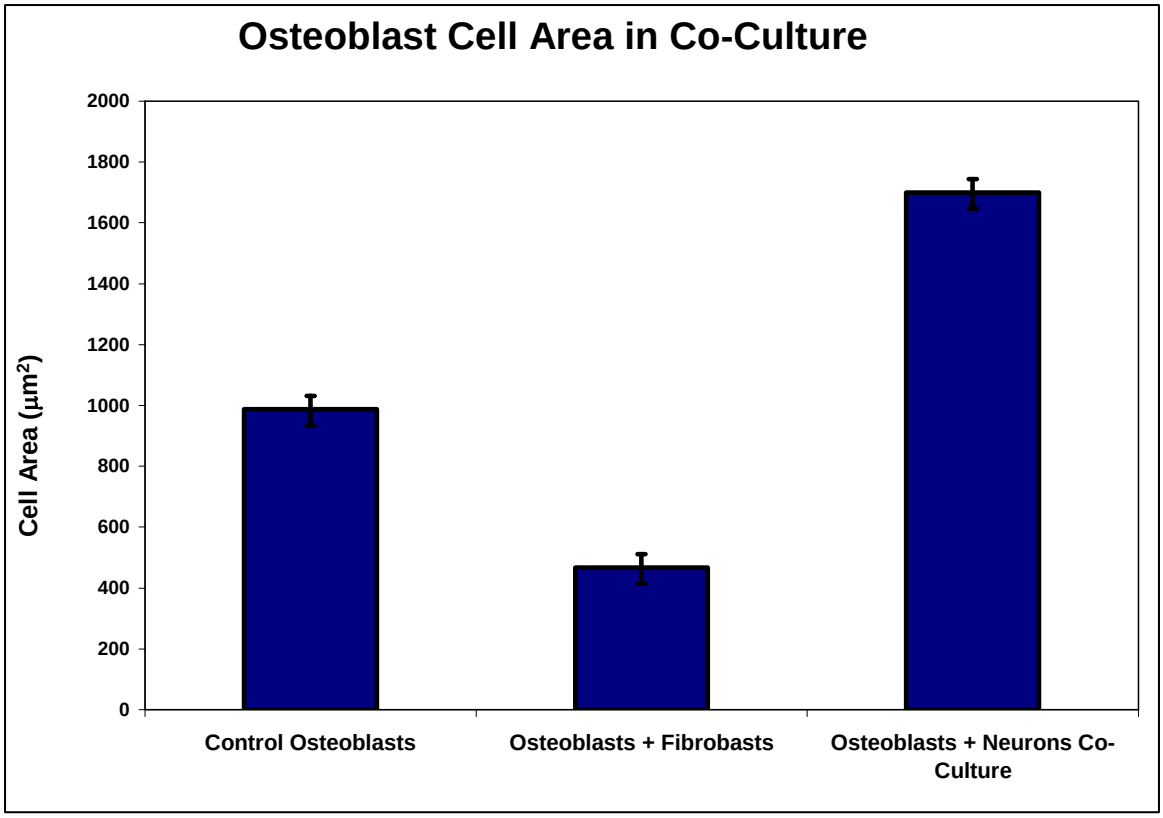

Figure 10. Measurement of the area of osteoblast cell bodies in culture for 7 days. Osteoblasts co-cultured with primary cortical neurons had significantly larger cell bodies compared to two other groups ($p<0.001$). n=5

Effects of Neuropeptides on Cell Layer Protein and AlkalinePhosphatase Levels

Initially, *in vitro* experiments with osteoblast cell cultures were done with mouse calvarial osteoblasts. A series of experiments established that treatment of calvarial osteoblasts with CGRP (0.01μg/mL, 0.1μg/mL, 1.0μg/mL, 10μg/mL) induced a significant dose dependent increase in alkaline phosphatase specific activity when compared with controls (Figures 11-13). Pearson correlation indicates r=0.950 (Figure 13). Total cell protein was not significantly affected, suggesting that the neuropeptides did not affect osteoblast proliferation. When cells were treated with SP (tested at 0.01μg/mL, 0.1μg/mL, 1.0μg/mL, and 10μg/mL), analysis by ANOVA did not reveal similar group-specific differences in alkaline phosphatase specific activity. However, these experiments consistently showed a significant correlation between SP dose and ALP specific activity when the data were analyzed by Pearson correlation (Figures 14-17). Interaction studies showed that osteoblasts treated with both CGRP and SP had significantly higher alkaline phosphatase specific activity compared to controls and these levels were higher than in cultures treated with CGRP or SP alone (Figures 16-21).

Effects of the neurotrophin nerve growth factor (NGF) were also examined using various dilutions (0.01μg/mL, 0.1μg/mL, 1.0μg/mL,

and 10μg/mL). Nerve growth factor did not affect protein or ALP levels after 48 hours of treatment.

The same series of experiments was performed using MC3T3-E1 osteoblastic cells; however, there was no effect on protein or ALP levels following 48 hours of treatment with any of the neuropeptides alone or in combination.

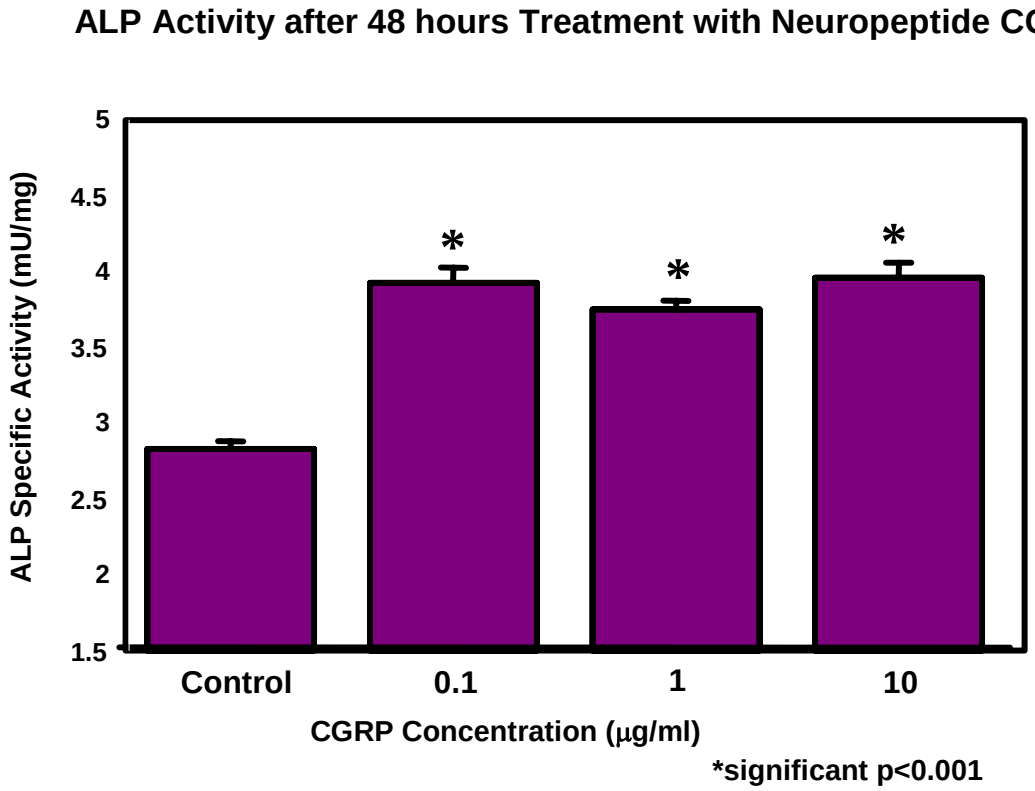

Figure 11. Swiss-Webster mouse calvarial osteoblasts treated with CGRP for 48 hours. Alkaline phosphatase (ALP) specific activity was measured as described in the Methods. Significant increase in ALP specific activity was observed at 0.1, 1.0, and 10 μg/ml CGRP ($p<0.001$). n=3

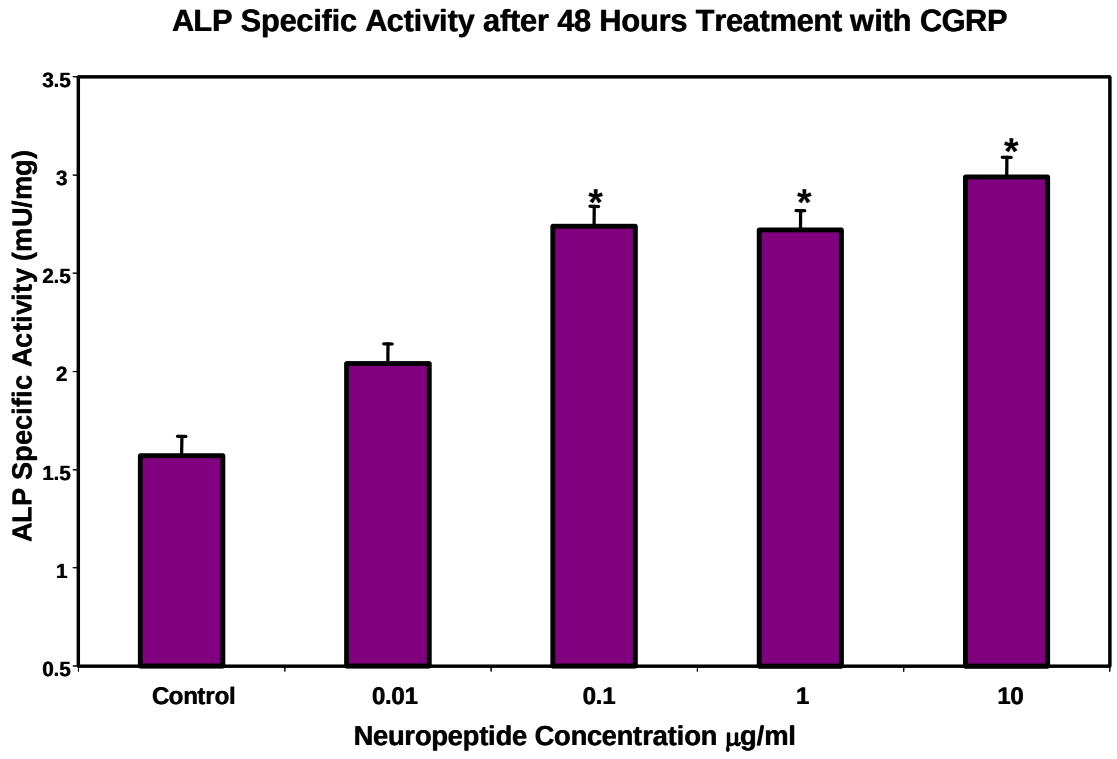

Figure 12. Swiss-Webster mouse calvarial osteoblasts treated with CGRP for 48 hours. Alkaline phosphatase (ALP) specific activity was measured as described in the Methods. Significant increase in ALP specific activity was observed at 0.1, 1.0, and 10 μg/ml CGRP ($p<0.001$).

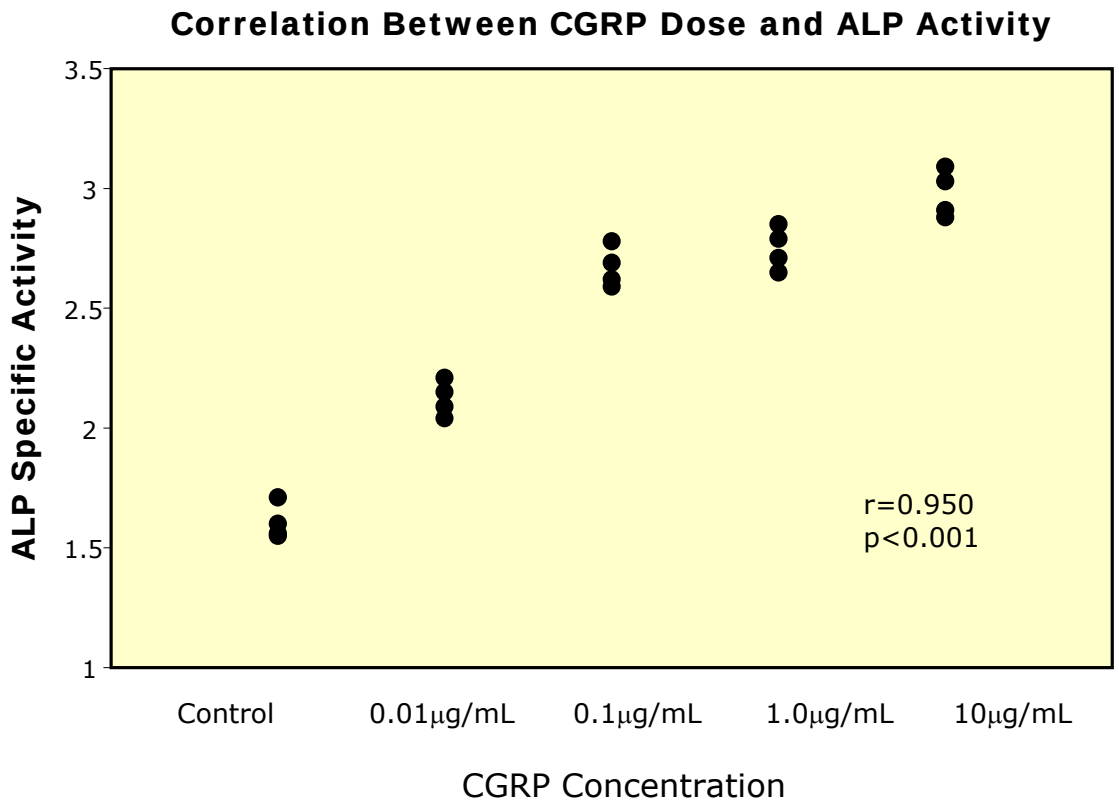

Figure 13. Dose-dependent increase in alkaline phosphatase specific activity (mU/mg) in Swiss-Webster mouse calvarial osteoblasts after 48 hours of treatment with CGRP concentration 0.01, 0.1, 1.0, 10μg/mL as described in Methods. Alkaline phosphatase specific activity was measured as described in Methods. Data shown as samples of replicates (n=4). Pearson correlation indicates r=0.950, p<0.001.

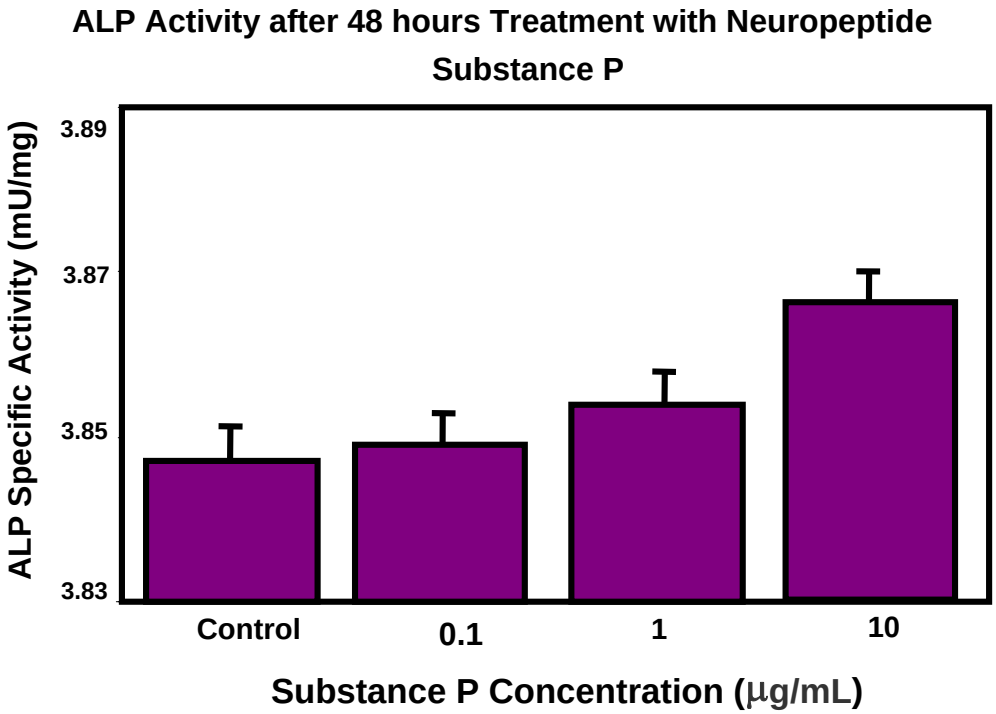

Figure 14. Swiss-Webster mouse calvarial osteoblasts treated with SP for 48 hours. Alkaline phosphatase (ALP) specific activity was measured as described in the Methods. ALP specific activity was correlated with dose of SP at 0.1, 1.0, and 10 µg/ml SP ($r=0.979$; $p<0.001$).

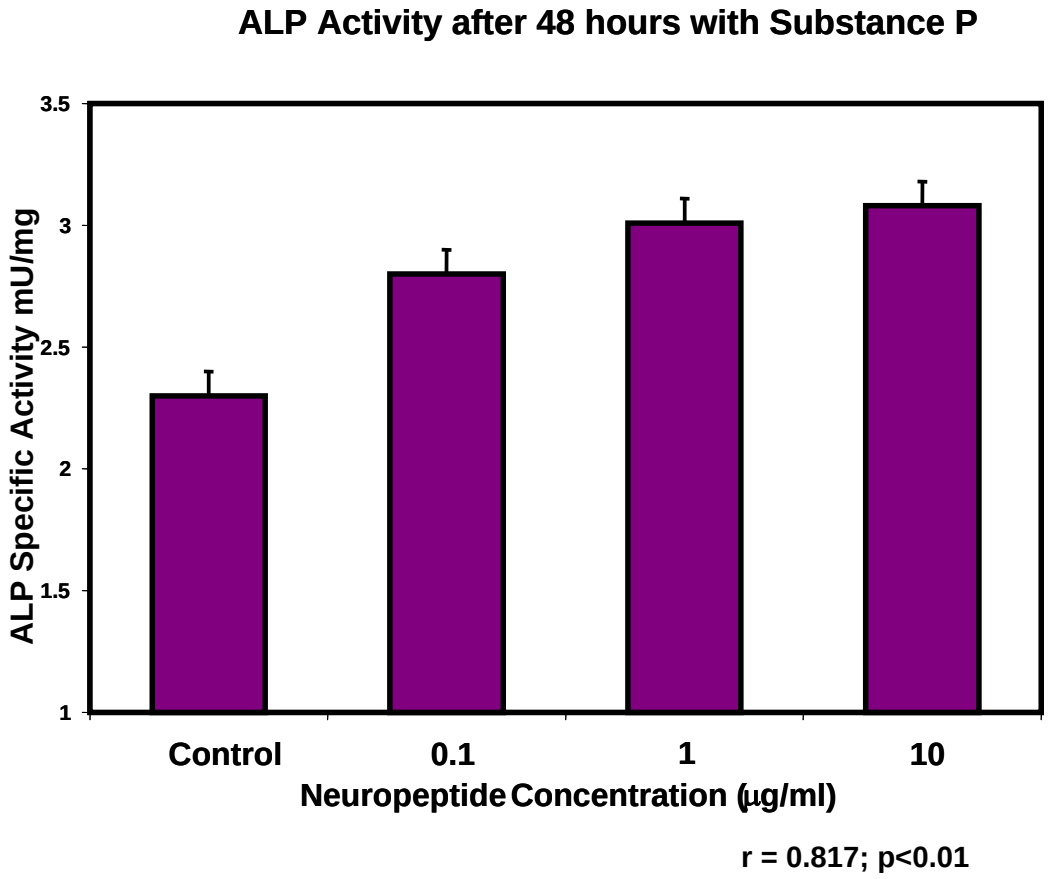

Figure 15. Swiss-Webster mouse calvarial osteoblasts treated with SP for 48 hours. Alkaline phosphatase (ALP) specific activity was measured as described in the Methods. ALP specific activity was correlated with dose of SP at 0.1, 1.0, and 10 µg/ml SP ($r=0.817$; $p<0.01$).

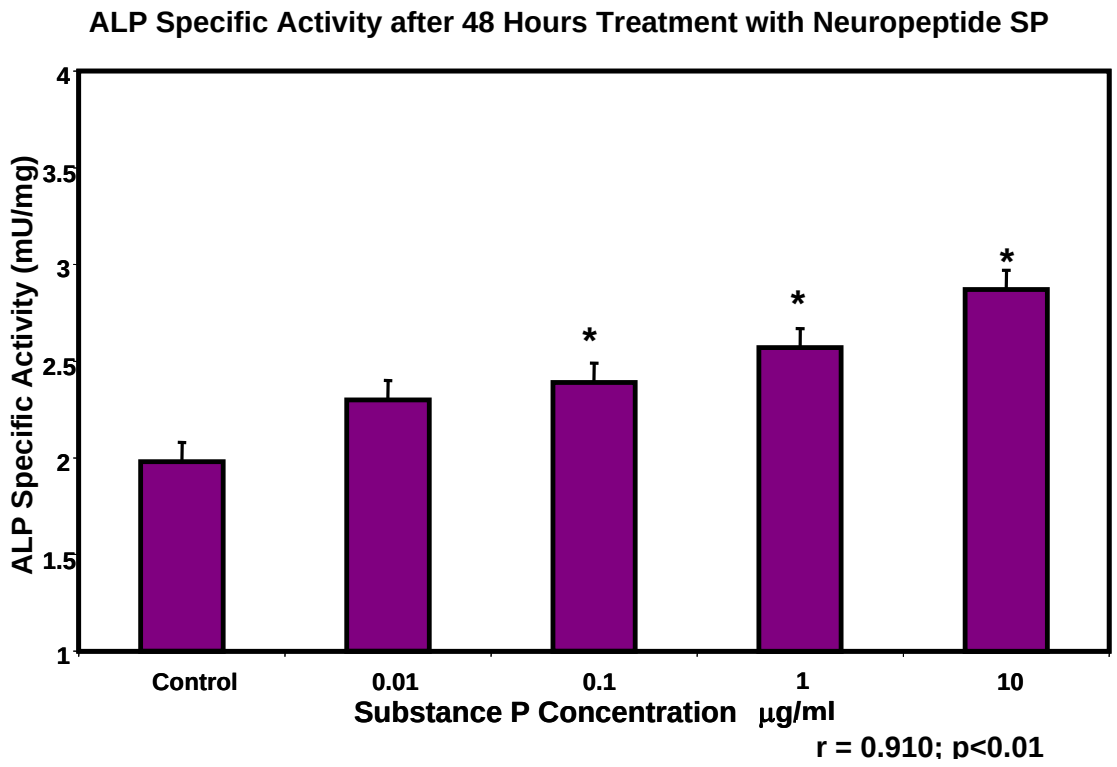

Figure 16. Swiss-Webster mouse calvarial osteoblasts treated with SP for 48 hours. Alkaline phosphatase (ALP) specific activity was measured as described in the Methods. ALP specific activity was correlated with dose of SP at 0.1, 1.0, and 10 µg/ml SP ($r=0.910$; $p<0.01$). * r= 0.910

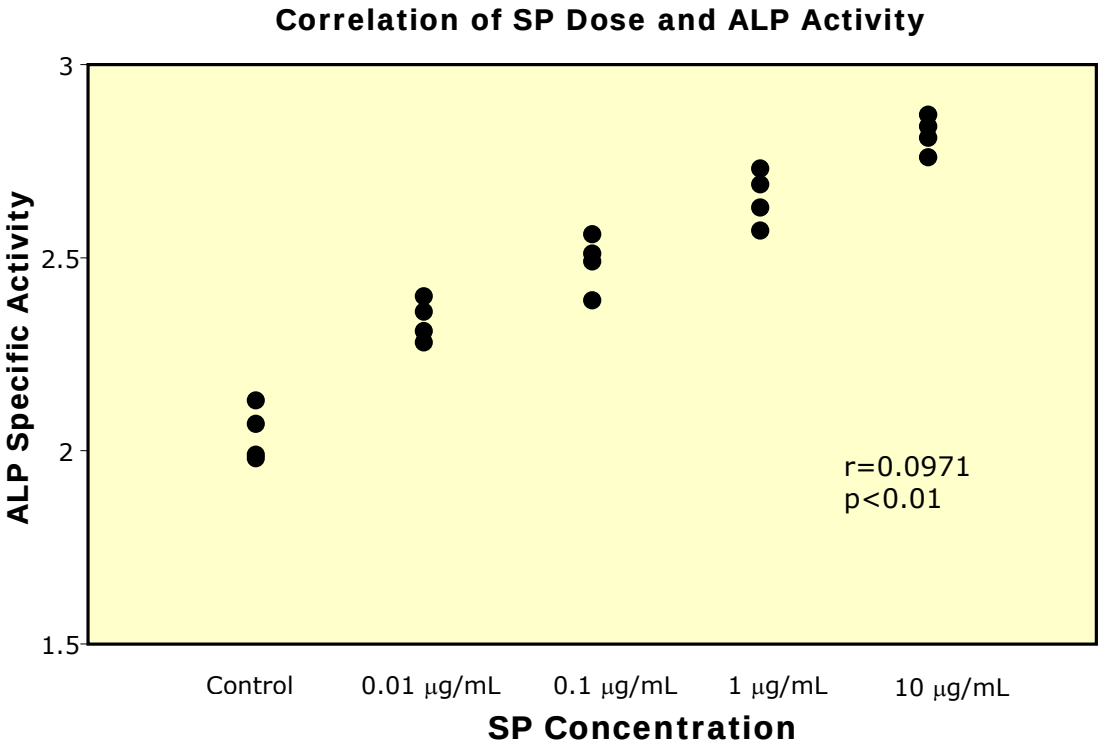

Figure 17. Dose-dependent increase in alkaline phosphatase specific activity (mU/mg) in Swiss-Webster mouse calvarial osteoblasts after 48 hours of treatment with SP concentration 0.01, 0.1, 1.0, 10μg/mL as described in Methods. Alkaline phosphatase specific activity was measured as described in Methods. Data shown as samples of replicates (n=4). Pearson correlation indicates r=0.971, p<0.01.

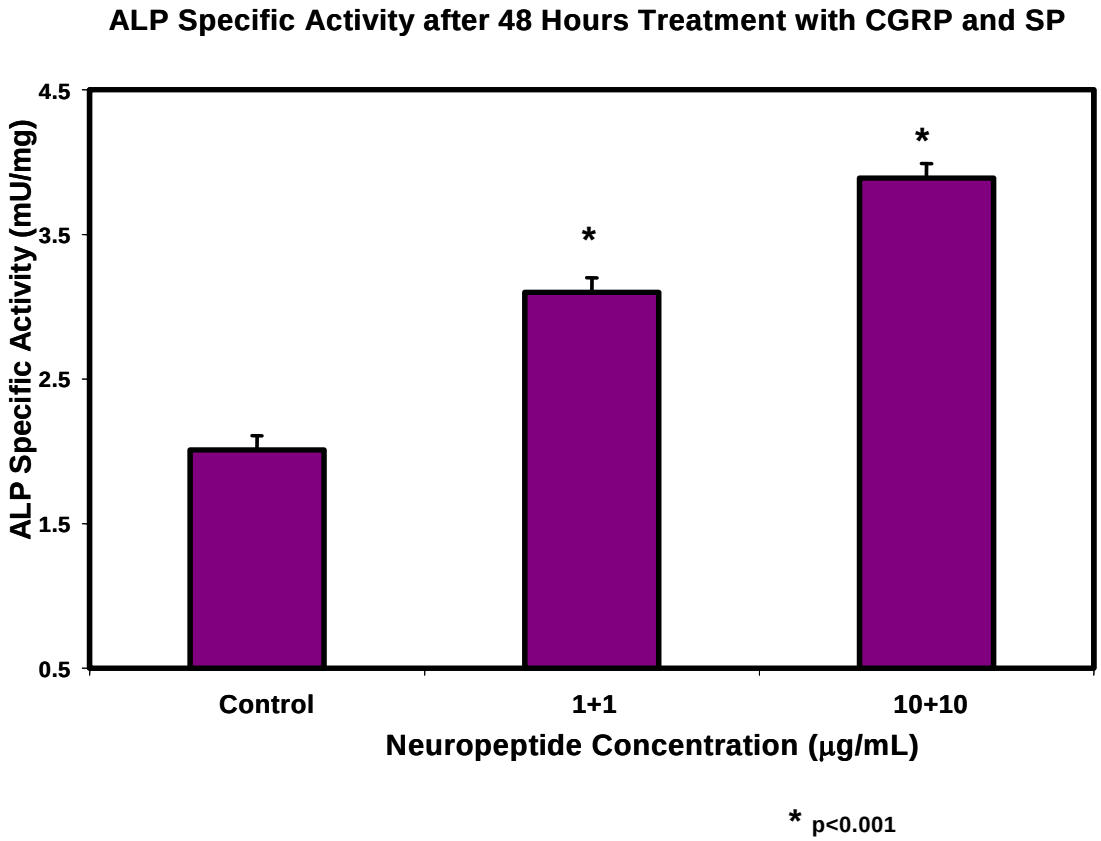

Figure 18. Swiss-Webster mouse calvarial osteoblasts treated with CGRP and SP for 48 hours. Alkaline phosphatase (ALP) specific activity was measured as described in the Methods. Significant increase in ALP specific activity was observed at combinations of 1 μg/ml CGRP and SP and 10 μg/ml CGRP and SP ($p<0.001$).

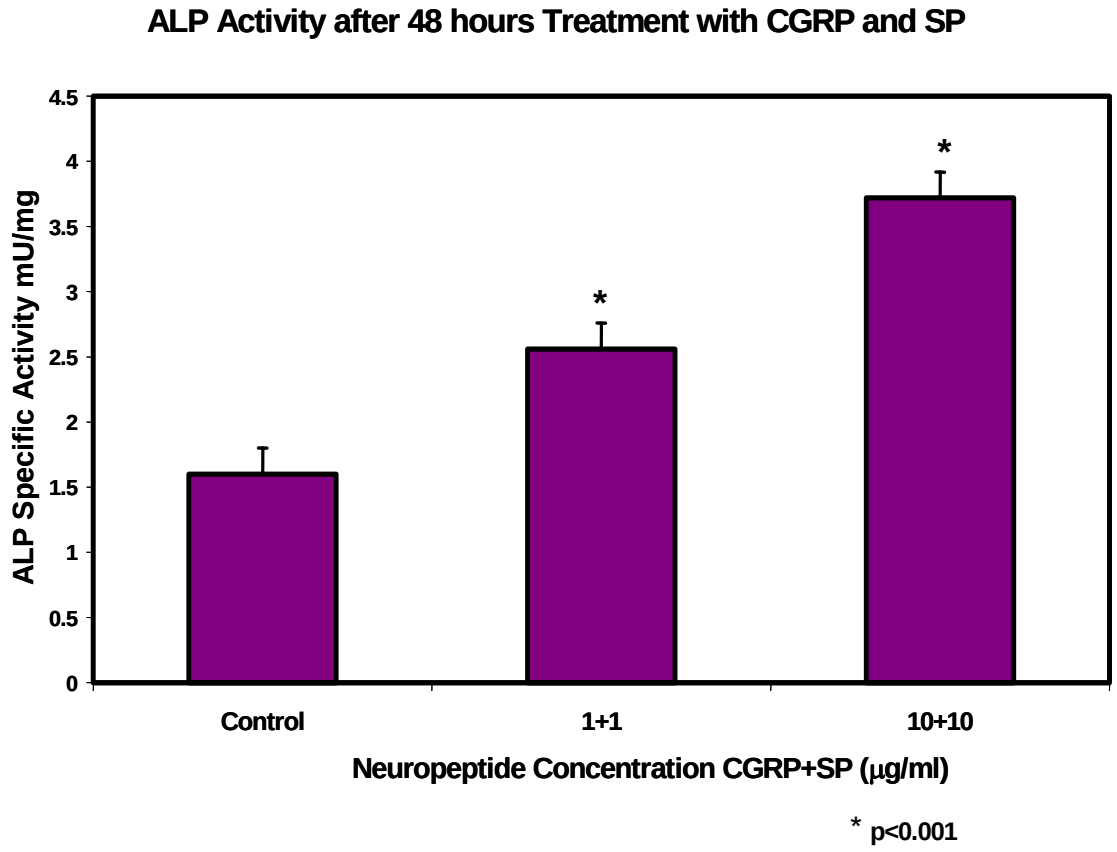

Figure 19. Swiss-Webster mouse calvarial osteoblasts treated with CGRP and SP for 48 hours. Alkaline phosphatase (ALP) specific activity was measured as described in the Methods. Significant increase in ALP specific activity was observed at combinations of 1 μg/ml CGRP and SP and 10 μg/ml CGRP and SP ($p<0.001$).

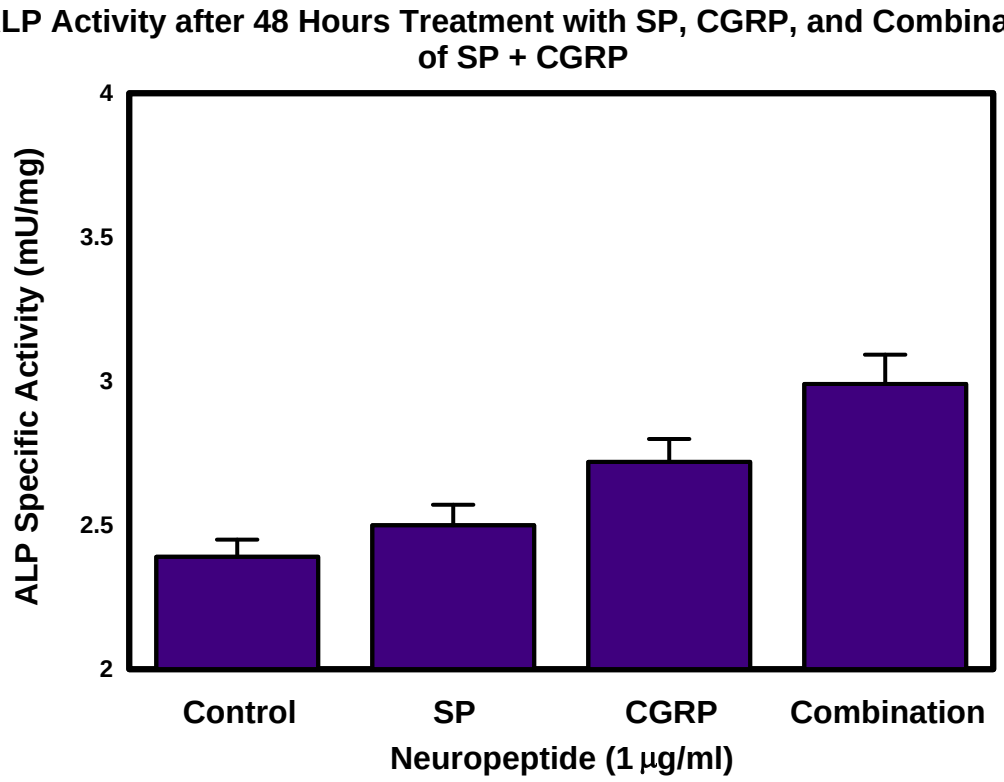

Figure 20. Swiss Webster mouse calvarial osteoblasts treated with SP, CGRP, and SP + CGRP in combination. Alkaline phosphatase (ALP) specific activity was measured as described in the Methods. Significant increase in ALP specific activity was observed at 1 μg/ml CGRP and 1 μg/ml CGRP + 1 μg/ml SP ($p<0.001$).

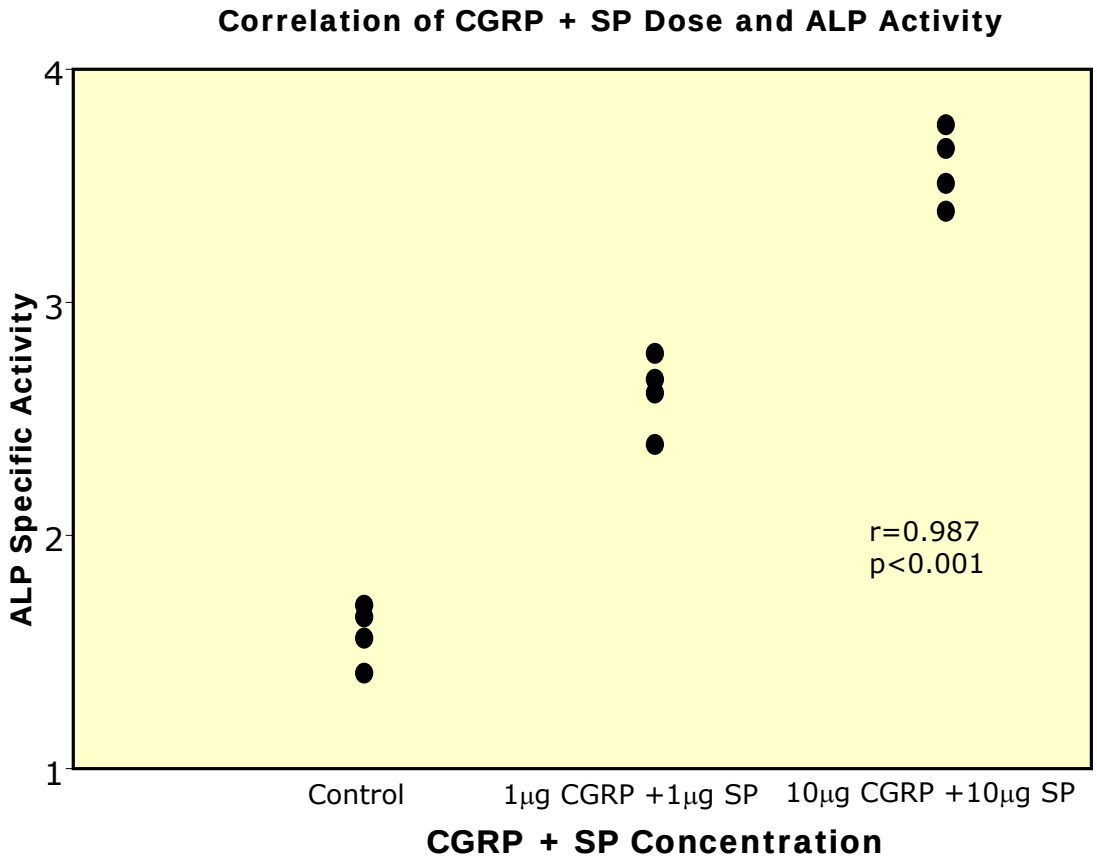

Figure 21. Dose-dependent increase in alkaline phosphatase specific activity (mU/mg) in Swiss-Webster mouse calvarial osteoblasts after 48 hours of treatment with CGRP + SP concentration 1μg/mL CGRP + 1μg/mL SP, 10μg/mL CGRP +10μg/mL SP as described in Methods. Alkaline phosphatase specific activity was measured as described in Methods. Data shown as samples of replicates (n=4). Pearson correlation indicates r=0.987, p<0.001.

Effects of Neuropeptides on Osteoblastic Mineralization

A series of studies revealed that both CGRP and SP reproducibly increased the rate and the extent of mineralization in MC3T3-E1 osteoblastic cells as measured by alizarin red staining. Alizarin red staining correlates with Von Kossa staining, infrared spectroscopy, and calcium content as an indicator of bone mineral accumulation in cultured MC3T3-E1 cells and other *in vitro* models (Luppen, Leclerc et al. 2003; Jadlowiec, Koch et al. 2004; Naito, Kaji et al. 2005). We found that CGRP produced an effect on mineralization, as evidenced by greater area and intensity of staining after 7–14 days of treatment (Figures 22-24). SP produced a similar effect on mineralization, as evidenced by greater area and intensity of staining (Figure 25). After 5–6 days of growth, the treated cells appeared to form groups of cells on top of each other, which is typical of these cells just prior to mineralization, and there was robust mineralization by 8–10 days. Untreated cells underwent the same morphological changes after 10–12 days and mineralized by 14–16 days. Extraction of the alizarin red stained cultures with CPC allowed quantitative comparisons and those extraction studies showed significant increases in alizarin red absorbance following treatment with CGRP, SP, and combination of the two peptides (Table 2).

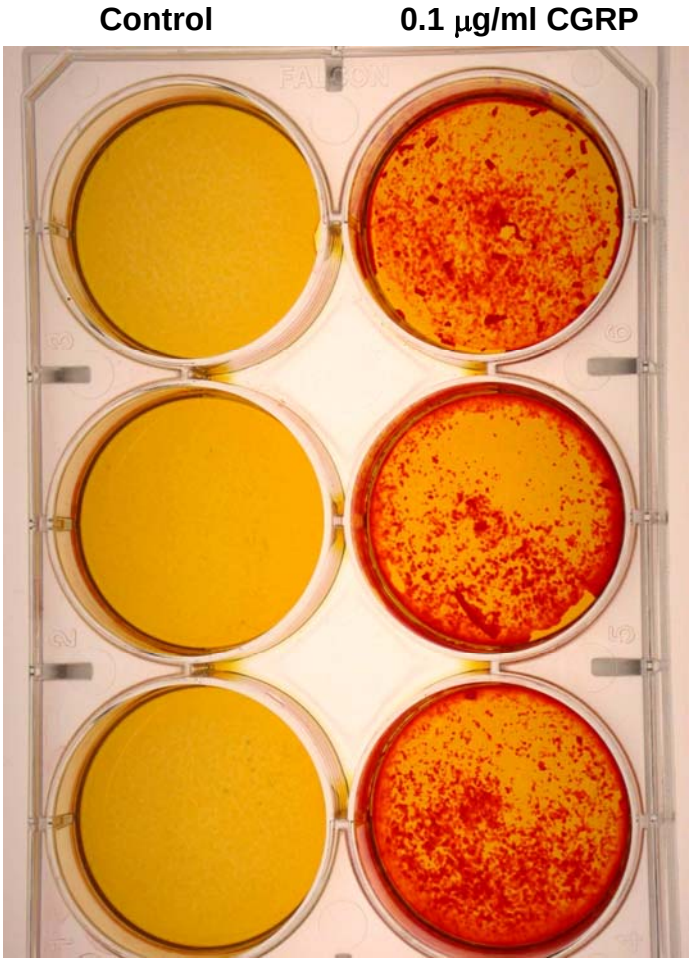

Figure 22. Mineralization of MC3T3-E1 osteoblastic cells after 7 days of growth with and without CGRP. On day 7, cells were stained with alizarin red for calcium deposition. Alizarin red staining demonstrates increased calcium deposition with CGRP treatment compared to controls.

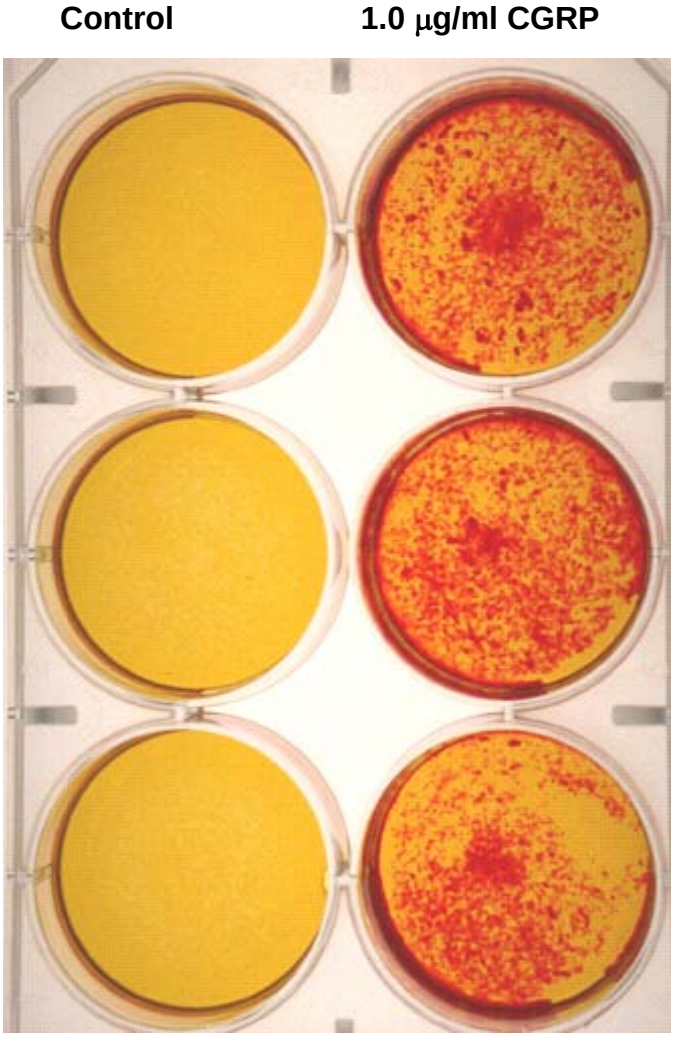

Figure 23. Mineralization of MC3T3-E1 osteoblastic cells after 7 days of growth with and without CGRP. On day 7, cells were stained with alizarin red for calcium deposition. Alizarin red staining demonstrates increased calcium deposition with CGRP treatment compared to controls.

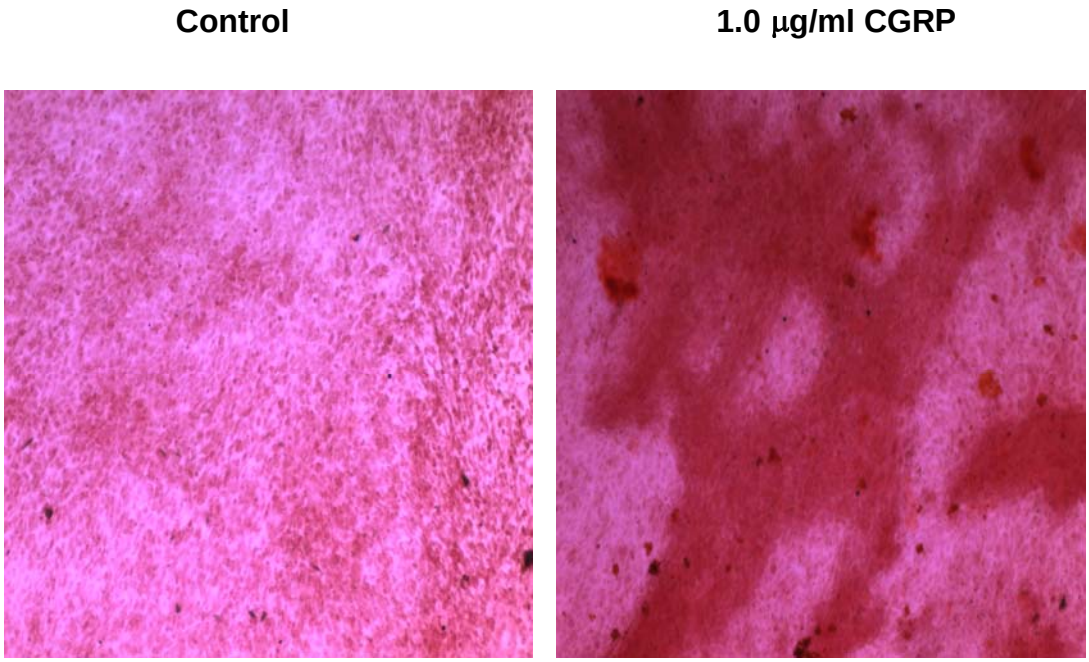

Figure 24. Histochemical demonstration of Alizarin Red stain in MC3T3-E1 cells after 7 days growth with and without CGRP. Photomicrographs show mineral deposition in cells treated with CGRP compared to control; magnification, 4x.

Control **1.0 μg/ml SP**

Figure 25. Mineralization of MC3T3-E1 osteoblastic cells after 14 days of growth with and without SP. On day 14, cells were stained with alizarin red for calcium deposition. Alizarin red staining demonstrates increased calcium deposition with SP treatment compared to controls.

Control **1.0 μg/ml SP & 1.0 μg/ml CGRP**

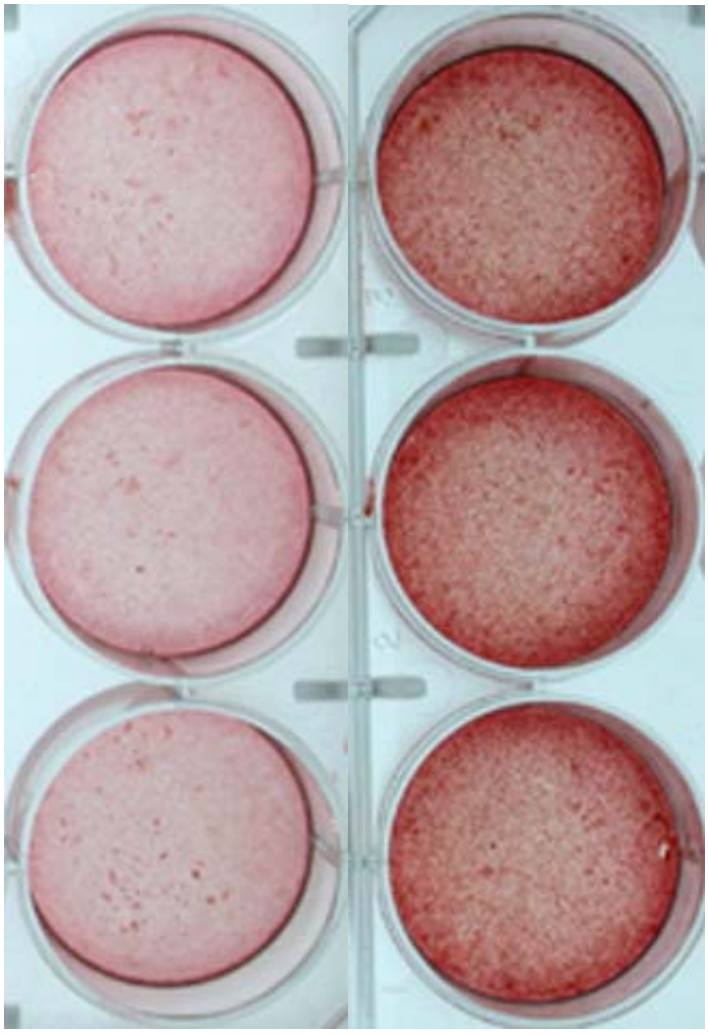

Figure 26. Mineralization of MC3T3-E1 osteoblastic cells after 7 days of growth with and without SP & CGRP in combination. On day 7, cells were stained with alizarin red for calcium deposition. Alizarin red staining demonstrates increased calcium deposition with SP & CGRP combined compared to controls.

Table 2. Alizarin red staining of MC3T3-E1 osteoblastic cells was quantified by extracting the calcium-bound dye with cetylpyridinium chloride (CPC) and reading the absorbance at 562 nm as described in Methods. Extraction studies showed significant increases in alizarin red absorbance following treatment with 1.0 μg/mL CGRP, 1.0 μg/mL SP, and combination of 1.0 μg/mL of the two peptides ($p<0.001$).

CPC Measurements A_{562}

Control	CGRP
179.8	378.0
209.6	356.9
198.7	320.2

Control	SP
183.2	225.1
170.0	253.7
171.5	272.3

Control	CGRP + SP
177.3	375.4
206.9	403.3
187.4	396.9

mRNA Measurements

Total RNA was isolated at 0, 1, and 3 days from control cells and cells treated with CGRP plus SP. Relative mRNA expression levels of each sample were determined by quantitative RT-PCR with the comparative C_t (cycle threshold) method. The C_t value represents the PCR amplification cycle at which the fluorescent signal exceeds a background threshold level. After determining C_t for each gene, relative levels of each osteoblast marker gene were normalized to mRNA levels of the housekeeping gene cyclophilin. Each sample was analyzed for each osteoblast gene in duplicate in at least three experiments, and cyclophilin was assessed in the same assay each time. A ΔC_t value was obtained for each osteoblast gene mRNA by subtracting the C_t value of cyclophilin mRNA from the C_t value of the mRNA for each sample. To compare relative mRNA levels between treated and untreated cells at each time point, $\Delta\Delta C_t$ was calculated as Control ΔC_t – Treated ΔC_t. The ratio of Treated/Control mRNA levels was calculated as $2^{(\Delta\Delta Ct)}$.

Relative mRNA expression levels of alkaline phosphatase, bone sialoprotein, osterix, osteoclacin, cbfa1, and collagen did not demonstrate measurable differences at the time points tested (1 and 4 days of treatment with neuropeptides) using RT-PCR. Relative mRNA expression levels of COX-2 were significantly increased in MC3T3-E1

osteoblastic cells treated with neuropeptides compared to control (Table 3-5). This effect was significant after 4 days of continuous treatment with CGRP and/or SP ($p<0.001$).

Table 3. Original: RT-PCR data after 4 days culture with and without neuropeptides showing cycle threshold $C_{(T)}$ value, which represents the PCR amplification cycle at which the fluorescent signal exceeds a background threshold level. A ΔC_t value was obtained for each osteoblast gene mRNA by subtracting the C_t value of cyclophilin mRNA from the C_t value of the mRNA for each sample. To compare relative mRNA levels between treated and untreated cells at each time point, $\Delta\Delta C_t$ was calculated as Control ΔC_t – Treated ΔC_t. The ratio of Treated/Control mRNA levels was calculated as $2^{(\Delta\Delta Ct)}$. C_t value represents threshold cycle for PCR amplification cycle between cycle and reference. $\Delta\Delta C_t$ represents the difference between $\Delta C_{t(\text{treated sample})}$ and $\Delta C_{t(\text{untreated sample})}$ for the same gene.

	C(T)	ΔCt	ΔΔCt	$2^{(\Delta\Delta Ct)}$
CONTROL COX-2	23.25	9.33		
CGRP COX-2	22.03	9.94	-0.61	0.66
SP COX-2	23.87	8.53	0.80	1.74
BOTH COX-2	21.64	7.78	1.55	2.93
RT NEG	NONE			
CONTROL CYCLO	13.92			
CGRP CYCLO	12.09			
SP CYCLO	15.34			
BOTH CYCLO	13.86			
RT NEG	NONE			

Table 4. Replicate 1: RT-PCR data after 4 days culture with and without neuropeptides showing cycle threshold $C_{(T)}$ value, which represents the PCR amplification cycle at which the fluorescent signal exceeds a background threshold level. A ΔC_t value was obtained for each osteoblast gene mRNA by subtracting the C_t value of cyclophilin mRNA from the C_t value of the mRNA for each sample. To compare relative mRNA levels between treated and untreated cells at each time point, $\Delta\Delta C_t$ was calculated as Control ΔC_t – Treated ΔC_t. The ratio of Treated/Control mRNA levels was calculated as $2^{(\Delta\Delta Ct)}$. C_t value represents threshold cycle for PCR amplification cycle between cycle and reference. $\Delta\Delta C_t$ represents the difference between $\Delta C_{t(\text{treated sample})}$ and $\Delta C_{t(\text{untreated sample})}$ for the same gene.

	C(T)	ΔCt	ΔΔCt	$2^{(\Delta\Delta Ct)}$
CONTROL COX-2	21.51	9.45		
CGRP COX-2	22.27	10.29	-0.84	0.56
SP COX-2	22.05	8.75	0.70	1.62
BOTH COX-2	21.88	7.94	1.51	2.84
RT NEG	NONE			
CONTROL CYCLO	12.07			
CGRP CYCLO	11.98			
SP CYCLO	13.30			
BOTH CYCLO	13.94			
RT NEG	NONE			

Table 5. Replicate 2: RT-PCR data after 4 days culture with and without neuropeptides showing cycle threshold $C_{(T)}$ value, which represents the PCR amplification cycle at which the fluorescent signal exceeds a background threshold level. A ΔC_t value was obtained for each osteoblast gene mRNA by subtracting the C_t value of cyclophilin mRNA from the C_t value of the mRNA for each sample. To compare relative mRNA levels between treated and untreated cells at each time point, $\Delta\Delta C_t$ was calculated as Control ΔC_t – Treated ΔC_t. The ratio of Treated/Control mRNA levels was calculated as $2^{(\Delta\Delta Ct)}$. C_t value represents threshold cycle for PCR amplification cycle between cycle and reference. $\Delta\Delta C_t$ represents the difference between $\Delta C_{t(\text{treated sample})}$ and $\Delta C_{t(\text{untreated sample})}$ for the same gene.

	C(T)	ΔCt	ΔΔCt	$2^{(\Delta\Delta Ct)}$
CONTROL COX-2	23.10	9.14		
CGRP COX-2	23.29	9.48	-0.34	0.79
SP COX-2	22.19	8.07	1.07	2.10
BOTH COX-2	21.27	7.28	1.86	3.63
RT NEG	NONE			
CONTROL CYCLO	13.96			
CGRP CYCLO	13.81			
SP CYCLO	14.12			
BOTH CYCLO	13.99			
RT NEG	NONE			

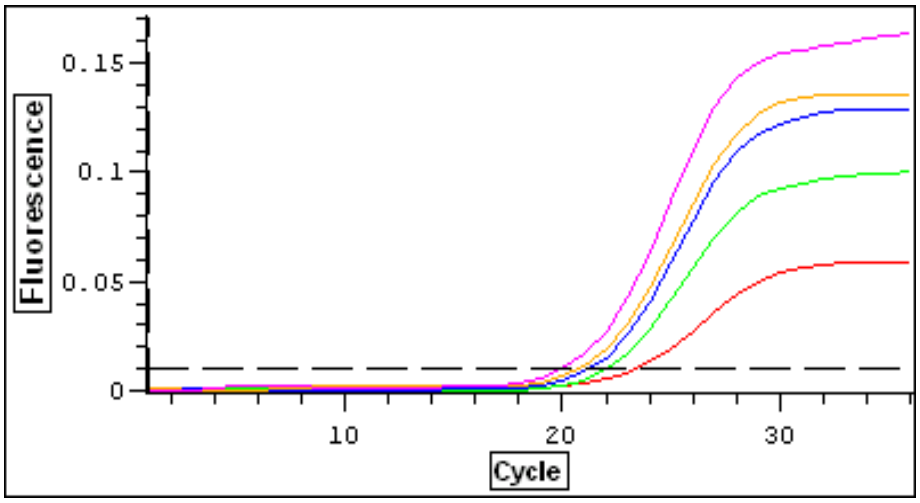

Figure 27. Real Time PCR data graph demonstrating the cycle number and fluorescence level for each sample. Cycle threshold C(T) value represents the amplification cycle number at which the fluorescent signal exceeds the background threshold level (dotted line). A ΔC_t value was obtained for each osteoblast gene mRNA by subtracting the C_t value of cyclophilin mRNA from the C_t value of the mRNA for each sample. To compare relative mRNA levels between treated and untreated cells at each time point, $\Delta\Delta C_t$ was calculated as Control ΔC_t – Treated$\Delta\Delta C_t$. The ratio of Treated/Control mRNA levels was calculated as $2^{(\Delta\Delta Ct)}$. C_t value represents threshold cycle for PCR amplification cycle between cycle and reference. $\Delta\Delta C_t$ represents the difference between $\Delta C_{t(\text{treated sample})}$ and $\Delta C_{t(\text{untreated sample})}$ for the same gene.

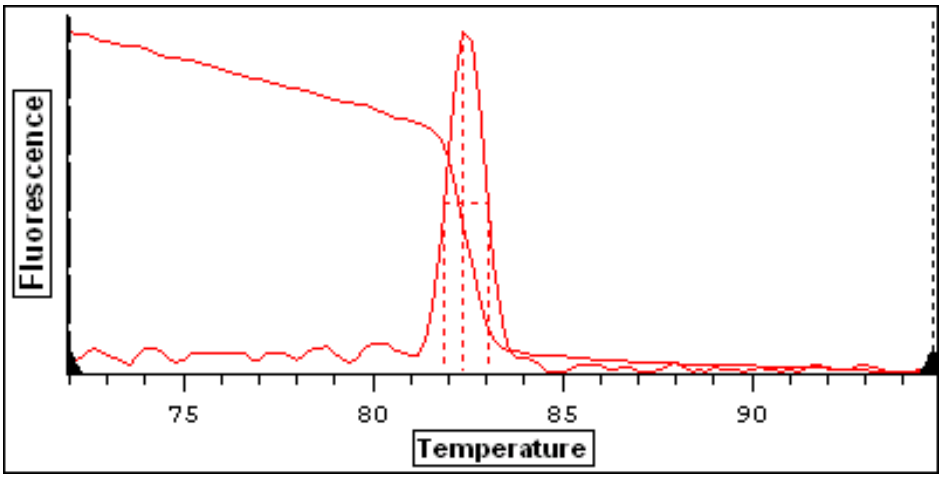

Figure 28. Real time PCR melting curve of a single sample representing product homogeneity as shown by a single peak at the given melting temperature.

Table 6. Replicate 3: RT-PCR data after 4 days culture with and without neuropeptides showing cycle threshold $C_{(T)}$ value, which represents the PCR amplification cycle at which the fluorescent signal exceeds a background threshold level. A ΔC_t value was obtained for each osteoblast gene mRNA by subtracting the C_t value of cyclophilin mRNA from the C_t value of the mRNA for each sample. To compare relative mRNA levels between treated and untreated cells at each time point, $\Delta\Delta C_t$ was calculated as Control ΔC_t – Treated ΔC_t. The ratio of Treated/Control mRNA levels was calculated as $2^{(\Delta\Delta Ct)}$. C_t value represents threshold cycle for PCR amplification cycle between cycle and reference. $\Delta\Delta C_t$ represents the difference between $\Delta C_{t(\text{treated sample})}$ and $\Delta C_{t(\text{untreated sample})}$ for the same gene.

	C(T)	ΔC(T)	ΔΔC(T)	$2^{(\Delta\Delta C(T))}$
mALP CONTROL	15.10	3.03		
mALP CGRP	16.72	5.00	1.97	3.91
mALP SP	15.20	3.25	0.21	1.16
mALP BOTH	15.45	3.99	0.96	1.95
RT NEG	None			
mBSP CONTROL	13.51	1.44		
mBSP CGRP	13.15	1.44	-0.00	0.99
mBSP SP	13.98	2.02	0.57	1.48
mBSP BOTH	14.09	2.63	1.18	2.27
RT NEG	None			
mCBFA1 CONTROL	16.8	4.73		
mCBFA1 CGRP	16.06	4.34	-0.38	0.76
mCBFA1 SP	16.87	4.92	0.18	1.13
mCBFA1 BOTH	16.14	4.68	-0.04	0.97
RT NEG	None			

Table 6. *Continued*

	C(T)	ΔC(T)	ΔΔC(T)	$2^{(\Delta\Delta C(T))}$
mCOL CONTROL	8.36	-3.70		
mCOL CGRP	8.71	-3.00	0.70	1.62
mCOL SP	7.95	-3.99	0.77	2.08
mCOL BOTH	7.60	-3.84	-0.14	0.90
RT NEG	None			
mOC CONTROL	15.28	3.21		
mOC CGRP	15.57	3.86	0.64	1.56
mOC SP	15.87	3.92	0.70	1.63
mOC BOTH	15.60	4.14	0.93	1.90
RT NEG	None			
mOSX CONTROL	18.06	5.99		
mOSX CGRP	17.94	6.22	0.22	1.17
mOSX SP	18.06	6.10	0.10	1.07
mOSX BOTH	18.04	6.58	0.10	1.07
RT NEG	None			
mCYCLO CONTROL	12.06			
mCYCLO CGRP	11.71			
mCYCLO SP	11.95			
mCYCLO BOTH	11.45			
RT NEG	None			

As shown above, COX-2 mRNA expression levels demonstrated measurable differences in osteoblastic cells treated with neuropeptides CGRP and SP. Although the mechanisms are not known, the effect to increase COX-2 message levels may suggest a role for prostaglandins. Initial studies using COX-2 inhibitor, indomethacin, demonstrate a visible decrease in osteoblast mineralization when indomethacin is present in levels 1-10 μM. For these studies, MC3T3-E1 cells were plated in 6-well plates at 30,000-40,000 cells/ml, in a volume of 2.0 ml/well. When the cells were 80% confluent, the culture medium was supplemented with 50 μg/ml ascorbic acid and 10 mM ß-glycerol-phosphate to support mineralization in the presence of indomethacin with and without addition of neuropeptides; 3 replicate wells containing indomethacin and 3 replicate wells containing indomethacin with neuropeptides. Media was replaced and fresh peptides were added every other day. After 7–14 days, cells were rinsed 5 times with PBS and fixed for one hour at 4°C with 10% formalin in PBS. Alizarin red solution (40mM, pH 4.2) was applied to the fixed cells for 10 minutes at room temperature.

Initially, cells were not surviving in the presence of indomethacin at 10 μM; therefore, concentrations were diluted to 1 μM for the experiment. Peptides were used in concentrations from prior

experiments (1.0μg/mL SP + 1.0μg/mL CGRP). Equal amount of dilution buffer was added to indomethacin-only wells.

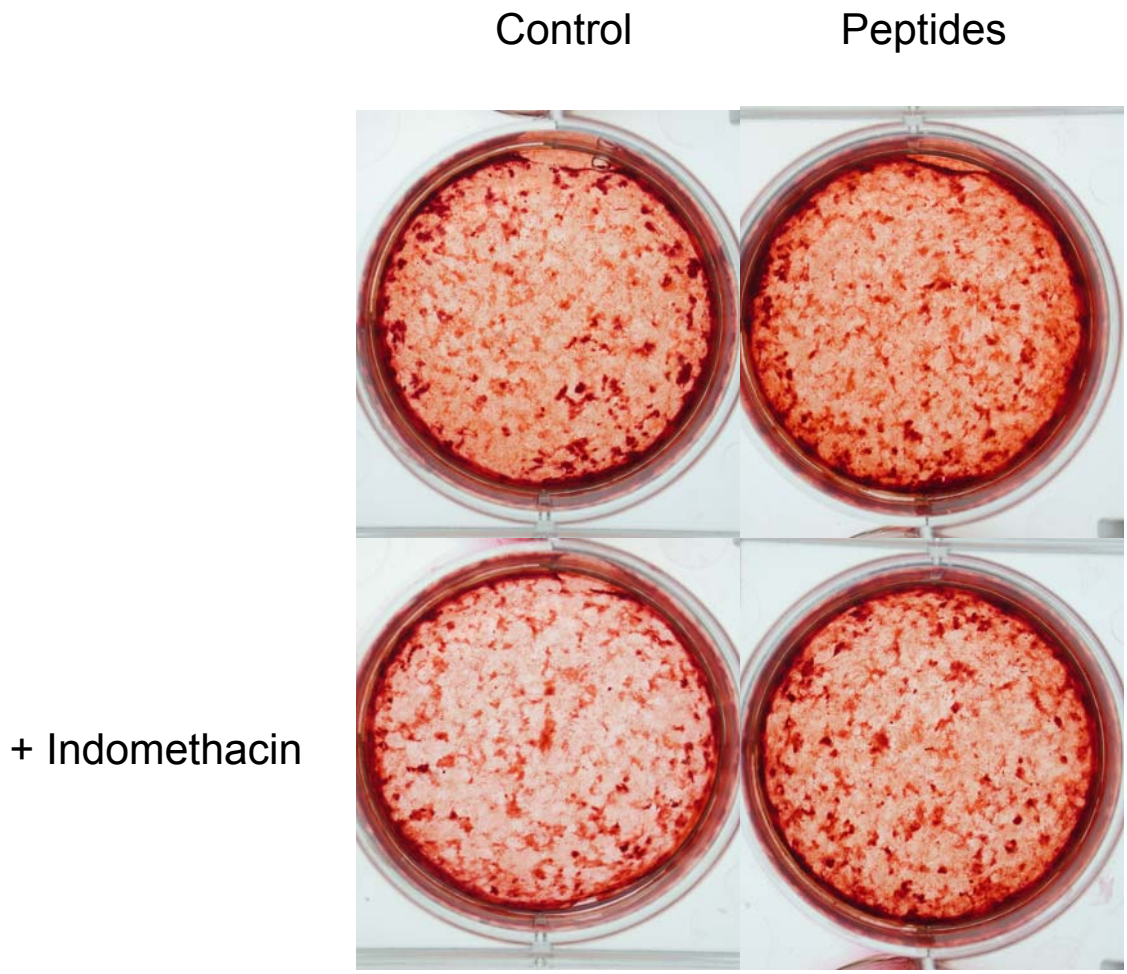

Figure 29. Mineralization of MC3T3-E1 osteoblastic cells after 7 days of growth with (1) Control, (2) Neuropeptides, (3) COX-2 inhibitor, indomethacin, and (4) Indomethacin plus neuropeptides. Osteoblasts treated with COX-2 inhibitor, indomethacin, appear with decreased mineralization. Addition of neuropeptides, even in presence of indomethacin, appears to increase mineralization, as shown by alizarin red staining.

CHAPTER FOUR

DISCUSSION

The present study examined the effects of two neuropeptides, CGRP and SP, on osteoblast growth and mineralization. Although the presence of these neuropeptides throughout bone has already been established (Hill and Elde 1991; Hukkanen, Konttinen et al. 1993), the potential role(s) of CGRP and SP as direct effectors of osteoblasts has not been assessed. Previous studies have shown that CGRP can increase cAMP production in osteoblasts (Michelangeli, Fletcher et al. 1989), and that osteoblasts possess receptors for both CGRP and SP (Michelangeli, Fletcher et al. 1989; Bjurholm 1991; Bjurholm, Kreicbergs et al. 1992). The results of the present studies have confirmed and extended those findings by demonstrating direct, *in vitro* effects of these two neuropeptides on the differentiation and mineralization of murine osteoblasts.

The effects of CGRP on alkaline phosphatase activity are consistent with previous studies (Michelangeli, Fletcher et al. 1989; Bjurholm 1991; Bjurholm, Kreicbergs et al. 1992). The effects of SP on alkaline phosphatase activity have not been previously reported.

Our observations of the interactive effects of CGRP and SP on differentiation, as reflected by the increase in alkaline phosphatase specific activity are also novel. Previous studies have not investigated the effects of these two peptides in combination; it is interesting to speculate that the effects of the combination of CGRP and SP may be physiologically relevant as these peptides frequently co-localize.

Although previous studies have demonstrated effects of CGRP on bone marrow mononucleated cells, i.e., CGRP stimulated bone colony formation both *in vitro* and *in vivo* (Bernard and Shih 1990), the present study is the first to demonstrate the effects of SP on osteoblastic mineralization. The current data demonstrate the neuropeptides, CGRP and SP, alone or in combination, can increase the rate and the extent of mineralization, at least, in our cell culture model. These findings suggest that neuropeptides released from neurons in bone may be involved in the regulation of bone formation, by means of direct effects on osteoblast growth and mineralization.

The mechanisms for these neuropeptides to affect osteoblasts are not clear; however the results of our RT-PCR studies have revealed significant effects of CGRP and SP on COX-2 mRNA expression. This, in addition to the known role of neuropeptides in neurogenic inflammation, suggests a possible role of neuropeptides in bone metabolism during inflammatory processes. With respect to the

osteogenic responses of osteoblast-line cells to CGRP and SP, the effect to increase COX-2 message levels may also suggest a mechanistic role for prostaglandin(s). Further studies (e.g., using COX-2 inhibitors) will be required to assess this new hypothesis.

Physiologically, the results of this study are consistent with the possible functional significance of these neuropeptides in bone. Physiologic levels of neuropeptides are difficult to measure as amounts vary widely at different times in various tissues. Measurable values in body fluids range from 3 pg/ml-1 ng/ml of peptide (Itoh, Nagaya et al. 2004); we have observed effects at concentrations in the range of 1 ng/ml-10 ug/ml.

Proposed physiologic mechanisms for these neuropeptides include regulation of blood flow, osteoclast inhibition, activation of second messenger systems, increased IGF-1 production, Ca2+ regulation, and immune and/or inflammatory response (Michelangeli, Fletcher et al. 1989; Kawase, Howard et al. 1995; Vignery and McCarthy 1996; Villa, Melzi et al. 2000; Fernandez, Chen et al. 2003; Kawase, Okuda et al. 2003; Lundy and Linden 2004). Since both peptides are known to increase vasodilation, many different physiological effects would be possible through this pathway. Nerves and blood vessels typically travel together throughout the body and

usually enter bone as a neurovascular bundle allowing nerves to regulate blood flow in and around bone.

Clinically, patients with spinal cord injuries often develop abnormal bone formation known as heterotopic ossification. The cause of this painful condition is unknown. Since these patients obviously have altered nerves and neuropeptides, the relationship between bone cells and neuroptides may be involved. Alternatively, decreased norepinephrine release or action due to impaired sympathetic innervation at tissue trauma sites may contribute to heterotopic ossification. β2-adrenergic receptor knockout mice have increased bone formation, and beta adrenergic antagonists increase bone formation (Elefteriou 2008).

Interestingly, COX-2 inhibitors have been shown to be effective in preventing heterotopic ossification (Murat, Hocaoglu et al. 2005; Grohs, Schmidt et al. 2007; Tsailas, Babis et al. 2009; Xue, Zheng et al. 2011). This medical "enigma" may be explained using the knowledge that neuropeptides are involved in both regulation of bone cells and regulation of COX-2 production. Therefore, this bone disorder occurring after neural injuries could be explored further regarding relationship between bone cells, neuropeptides, and COX-2.

Although further studies are, clearly, required to assess the possible role of neuropeptide secretion as a local, site-specific

determinant of bone formation (and the possible mechanistic role of COX-2 as a mediator of that process), several findings from previous studies are consistent with the premise. Increased sensory and sympathetic innervation of fracture calluses have been reported in previous animal studies (Hukkanen, Konttinen et al. 1993; Grills and Schuijers 1998). Clinical treatments, such as nerve blocks and nerve resections, may affect bone metabolism partially through neuropeptide pathways. Other treatments, including non-steroidal anti-inflammatory medications, inhibit COX-2; since COX-2 is potentially involved with fracture healing, these treatments may affect the healing process partially through the neuropeptide pathway as well.

Additional studies may include an *in vivo* fracture-healing model to determine the effect of neuropeptides which we have found to be effective in cell cultures on fracture callus formation, healing rate, and gene expression. *In vitro* studies might include examining the effect of CGRP and SP on apoptosis. Receptor studies might include measuring the effect of NK-1 and/or CGRP receptor antagonists on differentiation and mineralization. Although we examined a number of osteoblast-related mRNAs, there are many other genes, proteins, and transcription factors that could be measured, including osteopontin, osteonectin, fibronectin, c-myc, c-fos, TGF, FGF, IGF, etc. Since COX-2 mRNA was affected by treatment with neuropeptides, it would be

interesting to examine interleukins, prostaglandins, arachadonic acid, and other inflammatory substances. The COX-2 culture experiments could be expanded by testing effects of various COX-2 inhibitors with and without neuropeptides. The clinical relevance could be examined through retrospective or prospective studies; clinical studies could examine the effects of COX-2 inhibitors on patients who have suffered fractures.

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