ABSTRACT

The Effect of Neuropeptides on the Growth and Function of Bovine Articular Chondrocytes

(Abstract content here not to exceed 150 words)

Cartilage injury is a central issue in orthopedics to which there is no known physiological treatment. Neuropeptides play a role in the proliferative and reparative processes of many tissue types, but little is known about their effects on articular cartilage. The purpose of this study was to investigate the effects of the neuropeptides calcitonin gene-related peptide (CGRP), substance P (SP), neuropeptide Y (NPY), and vasoactive intestinal peptide (VIP) on the growth and metabolism of bovine chondrocytes cultivated in monolayer culture. Proliferation and DMMB assays were conducted over an eight day period. Insulin-like growth factor-1 was utilized as a positive control to validate the monolayer model. While CGRP showed little effect and NPY showed an inhibitive effect on proliferation, VIP demonstrated some ability to positively augment cartilage (n=8). Finally, SP showed the greatest reparative potential due to the significant increase in both proliferation and glycosaminoglycan production after SP 5 μg/mL treatment.

Amanda Herzog/Biochemistry
Author Name/Major

Amanda Herzog
Author Signature

4/23/07
Date

LEE KAPLAN/Dept of Orthopedics & Rehabilitation
Mentor Name/Department

LK
Mentor Signature

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AUTHOR'S NAME: Amanda Herzog

MAJOR: Biochemistry

DEPARTMENT: Biochemistry

MENTOR: Dr. Lee Kaplan

DEPARTMENT: Department of Orthopedics and Rehabilitation – UW Hospital

MENTOR(2): _____

DEPARTMENT(2):_____

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THE EFFECT OF NEUROPEPTIDES ON GROWTH AND FUNCTION OF BOVINE ARTICULAR CHONDROCYTES

Amanda Herzog

ABSTRACT:

Cartilage injury is a central issue in orthopedics to which there is no known physiological treatment. Neuropeptides play a role in the proliferative and reparative processes of many tissue types, but little is known about their effects on articular cartilage. The purpose of this study was to investigate the effects of the neuropeptides calcitonin gene-related peptide (CGRP), substance P (SP), neuropeptide Y (NPY), and vasoactive intestinal peptide (VIP) on the growth and metabolism of bovine chondrocytes cultivated in monolayer culture. Proliferation and DMMB assays were conducted over an eight day period. Insulin-like growth factor-1 was utilized as a positive control to validate the monolayer model. While CGRP showed little effect and NPY showed an inhibitive effect on proliferation, VIP demonstrated some ability to positively augment cartilage (n=8). Finally, SP showed the greatest reparative potential due to the significant increase in both proliferation and glycosaminoglycan production after SP 5 μg/mL treatment.

INTRODUCTION:

Cartilage consists of two main components: cells and matrix. Mature cartilage cells, known as chondrocytes, are round cells located in matrix cavities, or lacunae. Even though chondrocytes are the only cells in cartilage, they represent only 5-10% of total cartilage volume (Lin *et al*., 2006). Chondrocytes offer limited assistance to articular cartilage, but are instead essential due to their role in the synthesis and turnover of the extracellular matrix, which they accomplish through a constant turnover mechanism (Acosta *et al*., 2006). In turn, the matrix provides an environment for nutrition diffusion for chondrocytes, as well as provides the surface of the joint with biomechanical competence. The matrix also functions by maintaining a state of dynamic equilibrium between the cellular environment and the cartilage structure.

The structure of the matrix is composed of two main macromolecules: type II collagen and large chondroitin sulfate proteoglycan aggregates, or aggrecan. Aggrecan monomers consist of both a core protein and keratin sulfate glycosaminoglycan (GAG) chains, which fill the narrow spaces within the collagen. Due to its high negative charge, aggrecan can draw water into the tissue and swell against the collagen network, thereby resisting compression and allowing for proper joint movement. The composition and structural integrity of the matrix is responsible for the capacity of joints to withstand joint biomechanics (Lin *et al*., 2006).

Normal articular cartilage homeostasis is a balance between catabolic and anabolic agents. This balance is not only well synchronized but is very delicate and, therefore, can be disturbed in various ways. This includes the potential interruption of equilibrium by various proteinases and inflammatory mediators found within cartilage and the joint (Chubinskaya *et al*., 2000). Imbalance within the cartilage matrix between anabolic and catabolic pathways has been implicated in the development of osteoarthritis, and is thought be a determinant in the destruction and loss of articular cartilage that is characteristic of this disease (Loeser *et al*., 2003). Because this balance is so essential to cartilage maintenance and survival, bioactive factors with the

ability to stimulate chondrocyte anabolic activity, and in some cases inhibit catabolic activity, may be useful agents to combat the loss of cartilage matrix associated with osteoarthritis and traumatic articular cartilage defects.

The repair of cartilage is a central issue in orthopedic care, and is challenging because of the limited ability of chondrocytes for self repair. Cartilage degeneration not only leads to pain, but also loss of function and is a major cause of patient morbidity. Injured cartilage in adults usually leads to osteoarthritic changes of the joint when the injury involves a weight bearing area (Im *et al*., 2006). Because cartilage lacks regenerative ability, treatment for cartilage disease is primarily analgesic or surgical. Prosthetic joint replacement, while the most radical and reliable treatment for cartilage degeneration, carries with it significant risk and its results deteriorate over time. These issues have led to efforts to develop alternative means to restore damaged cartilage. Previous efforts to repair articular cartilage lesions have been restricted by the challenge of stimulating resident cells to form new cartilage, but a better understanding of cartilage biology and repair has given some hope of a biological treatment for cartilage degeneration.

Involved in articular synthesis are anabolic agents, including growth factors such as BMP-7, bFGF, TGF, and IGF-1 and anti-inflammatory cytokines such as tissue inhibitor metalloproteases (TIMP) and IL-4,10, and 13 (Webb, 1998). Due to their function, these agents have the potential to be utilized after articular cartilage injury in order to help compensate for cartilage damage. Many recent studies have examined the effects of growth factors on articular cartilage injuries and have found that these bioactive molecules enhance repair, to some extent, of these defects. Involved in many of these studies has been insulin-like growth factor-1 (IGF-1), an anabolic growth factor that has been found to be important to chondrocyte survival in cell culture. IGF-1 belongs to the family of insulin-like growth factors, which are expressed in tissues such as the heart, placenta, lungs, testes, brain, and bone. The IGF family includes peptide hormones such as relaxin and insulin, all of which have a single polypeptide homologous to proinsulin (Lin *et al*., 2006).

IGF-1 is a 7.6 kDa polypeptide growth factor that stimulates both chondrocyte proliferation and matrix synthesis. Specifically, IGF-1 increases chondrocyte production, both *in vivo* and *in vitro*, of proteoglycans and type II collagen, two principal constituents of cartilage (Madry *et al*., 2005, Martel *et al*., 1998). As well, IGF-1 plays a critical role in skeletal development. This has been demonstrated through studies using IGF gene knockout mice, which showed that the IGF deficiencies in these mice led to severe growth failure (Longobardi *et al*., 2005). IGF-1 has been shown to regulate many cellular functions by activating cell-surface receptors (Lin *et al*., 2006).

The potential to use IGF-1 for articular cartilage injuries partially stems from the fact that this growth factor shows positive expression in developing cartilage, mature cartilage, and synovial fluid in joints (Lin *et al*., 2006). Many studies have shown the beneficial effect of IGF-1 on chondrocytes, giving credence to the idea that IGF-1 can aid in the regeneration of chondrocytes after articular cartilage injury. In studies involving mesenchymal cell lines, embryonic limbs, and chondrocytes cell lines, IGF-1 was found to induce chondrocyte differentiation and proliferation (Oh and Chun, 2003, Worster *et al*., 2001, Phornphutkul *et al*., 2004). As well, this bioactive factor has been shown to inhibit nitric oxide induced dedifferentiation and apoptosis of articular chondrocytes, as well as promote new tissue formation in an *ex vivo* model of articular chondrocyte transplantation and enhance tissue engineering of cartilage (Lin *et al*., 2006, Madry *et al*., 2001, Madry *et al*., 2002).

Not only has IGF-1 been found to be beneficial to chondrocyte survival, it has been shown to facilitate cartilage improvement after damage. Chu *et al*. found that IGF-1 aided chondrocytes in their recovery from thermal stress by increasing proteoglycan synthesis by 32%, thereby abrogating the deleterious effects that occurred during articular cartilage degeneration (Chu *et al.,* 2004). In a canine model of osteoarthritis, articular delivery of 2 µg IGF-1 three times weekly for three weeks led to improvement in cartilage damage indices (Rogachefsky *et al*., 1993). As well, treatment of partial thickness (chondral) defects in adult rabbit and Yucatan mini pig models with 50 ng/mL IGF-1 applied in a fibrin clot improved the cellularity of the repair tissue (Hunziker and Rosenberg, 1996). Finally, treatment of extensive osteochondral (full-thickness) defects with 25 µg IGF-1, applied in a fibrin composite, enhanced repair in a horse model (Nixon *et al*., 1999) as did IGF-1 coupled with cell based treatment (Fortier *et al*., 2002).

However, while there has been some limited success of growth factor therapy in animal models of arthritis or cartilage damage, there is a lack of data regarding the use of this therapy in humans and therefore the feasibility of this as a clinical treatment option. This includes findings of a potential reduction in the capacity of older adult human chondrocytes to respond to growth factor stimulation. If this is true, this reduction would prove to be a major limiting factor in the use of growth factors to treat articular cartilage injuries. It is also somewhat controversial regarding whether human osteoarthritic chondrocytes may lack an anabolic response to IGF-1 (Schalwik *et al*., 1989 and McQuillan *et al*., 1986). As well, the use of IGF-1 as a therapeutic agent for articular cartilage lesions has been restrained by its short intra-articular residence time and the inherent rarity of articular chondrocytes to serve as target cells (Madry *et al*., 2005).

Based on background research, neuropeptides hold the same potential as growth factors to improve articular cartilage health. While little is known about the effect of neuropeptides on cartilage cells, these bioactive factors play a role in the proliferative and reparative processes of many tissue types, including fibroblasts, which arise from the same progenitor cells as chondrocytes (Burssens *et* al., 2005). Neuropeptides have also shown positive expression in developing cartilage. This includes evidence of the presence of SP and CGRP in the perichondrium, indicating the presence of nerve fibers preceding the development of cartilage canals. Nerve fibers may play a role in the development of synovial joints before and during the presence of cartilage canals. This information points toward a possible regulatory role of neuropeptides in cartilage development (Oliva *et al*., 2005, Edoff *et al*., 2000).

Along with this promising data, there are several advantages that neuropeptides confer over the use of growth factors in therapy. This includes the small size of neuropeptides, which allows for easier integration into systems for targeted drug delivery. As well, neuropeptides are less expensive than growth factors because they are easily synthesized without the need for harvesting, culturing, or transfecting cells. Most importantly, neuropeptides have been shown to have broad positive effects, including the promotion of angiogenesis and neurogenesis, modulation of the inflammatory response, and stimulation of cell proliferation (Appelgren *et al*., 1995). Therefore, neuropeptides orchestrate effects over a broader spectrum of soft tissue healing than targeted molecules.

Neuropeptides are powerful, controlling bioactive molecules that have been shown to have vasoregulatory, chemotactic, angiogenic, and proliferative roles (Edoff and Granseth, 2001, Salo P, 1999). These molecules are expressed in healthy cartilage *in vivo* where they are synthesized in dorsal root and autonomic ganglion neurons exterior to joints. They exhibit such a wide range of effects due to their numerous effector cells: mast cells, lymphocytes, fibroblasts,

chondrocytes, osteoclasts and osteoblasts. As well, once neuropeptides are bound, they tend to produce a cascade of reactions. These reactions eventually result in DNA transcription and protein expression, thereby further broadening their effect (Schaible *et al.*, 2005).

In the future, neuropeptides may be used to ameliorate the symptoms of articular cartilage injuries as well as other tissue ailments. Recently, these bioactive molecules have been used to positively affect osteoarthritis. For example, the autonomic neuropeptide VIP has been shown to effectively control collagen-induced arthritis in mice (Niissalo *et al.*, 2002). By utilizing neuropeptides in this manner, there is the possibility of decreasing the negative effects of osteoarthritis, an increasingly common disease. This solution may assist a large portion of the population; about 13% of people over 55 years of age have been diagnosed with osteoarthritis and this number is expected to double over the next 17 years (Melton, 2003). Just as important may be their effects on articular cartilage after acute injury, a problem to which there is currently no physiological treatment.

However appealing neuropeptides may be, to current knowledge there are only two studies that look at the effect of neuropeptides on chondrocyte behavior. In 2003, Edoff and Hildebrand showed that rat chondrocytes cultured in monolayer had a significant increase in cAMP in response to exposure to CGRP. In 1993, Halliday *et al*. reported a significant increase in prostaglandin E2 and collagenase in bovine articular chondrocytes exposed to a fragment of SP. These preliminary results indicate that neuropeptides play an unspecified role in chondrocyte biology. The aim of this paper is to investigate the effect of four candidate neuropeptides on the proliferation and glycosaminoglycan production of articular chondrocytes. The four neuropeptides to be tested are described in appendix figure A.1. This will be the most thorough investigation of the effect of neuropeptides on articular chondrocytes in the literature. It is hypothesized that the early delivery of neuropeptides will decrease the catabolic cascade associated with early articular injury and will therefore reduce the severity of articular degradation.

METHODS:

OVERVIEW:

Bovine chondrocytes were cultivated in monolayer culture in media alone or media containing one of four neuropeptides: neuropeptide Y (NPY), calcitonin gene-related peptide (CGRP), substance P (SP), or vasoactive intestinal peptide (VIP). Chondrocyte proliferation and glycosaminoglycan (GAG) production assays, which were considered indicative of a beneficial response, were conducted on days 2, 4, 6, and 8. Insulin-like growth factor-1 (IGF-1), which is known to stimulate chondrocyte proliferation and production, was utilized as a positive control to validate the monolayer model. Media exchanges were performed every two days, on the same days as the assays were performed.

The monolayer culture is a basic approach for chondrocyte propagation. It is used so frequently because of several important advantages over other culture systems, including its low cost and technical simplicity. As well, it allows for culture of large numbers of cells. While monolayer is used extensively, it fails to maintain the chondrogenic phenotype during long-term culture, thereby necessitating short trial times. If trials are extended too long in monolayer cultures, the chondrocytes become fibroblast like, losing their ability to secrete proteoglycan and changing collagen synthesis from type I to type II (Lin *et al.*, 2006).

Cartilage Dissection and Digestion:

Freshly slaughtered veal knees were obtained from Strauss Veal Co., Franklin, WI, and preferably consisted of mid tibia to mid femur with the capsule intact. In order to prevent unnecessary contamination in the sterile hood, all excess tissue was removed from the knee, attempting to leave only cartilage and bone. After this removal, the articular surface was rinsed profusely with sterile saline. Under the hood, available cartilage from the articular surface of the femoral trochlea, femoral condyles, and patella was harvested using a scalpel. The articular cartilage fragments were then rinsed twice with sterile saline, morcelized, and enzymatically digested overnight in Dulbeco's Modified Eagles Media (DMEM) containing 0.5 mg/mL hyaluronidase (Hyaluronidase Type IV-S, Sigma C-9407) and collagenase (Type XI Collagenase, Sigma C-9407) at 37°C.

Collecting and Plating Chondrocytes:

Falcon flasks containing enzymatically digested articular cartilage were agitated, and the supernatant was drawn out of the flaks and filtered through sterile 40 micron filters into 50 mL Falcon tubes. The tubes were balanced by transferring supernatent between the same knee. These tubes were then centrifuged at 1000 g for 20 minutes using a desktop centrifuge (2.5 k RPM). The supernatant was decanted and the pellets were resuspended in 5 mL of DMEM each. The number of cells was determined using a hemacytometer; cells were diluted 1:100 in Trypan blue and loaded in the hemacytometer, which created 10 μL of sample and 990 μL dye. Cells were resuspended in DMEM completed media to a concentration of $1x10^5$ cells/mL. Cells were plated using 0.5 mL of this suspension in each well of a 12 well tissue culture plate with 3 replicates for each treatment group to be used for glycosaminoglycan production analysis. Additionally, 100 μL of the cell suspension was placed in a 96 well, opaque-walled, tissue culture plate with 6 replicates for proliferation analysis. The plates were then placed in a culture incubator at 37°C and 5% CO_2.

Exchange of Media and Addition of Neuropeptides – Designated as Day 0:

After a 24 hour incubation period, the media was then exchanged with new DMEM media containing the appropriate treatment. The treatment groups consisted of a control (DMEM media only), a positive control (DMEM + 100 ng/mL IGF-1) and the neuropeptide treatment group (DMEM containing the neuropeptide to be tested). Based on preliminary dosage studies, the concentrations of each neuropeptide were determined and are listed in Table 1. Neuropeptide stock was diluted to the desired concentration in media. Dilutions occurred just prior to media exchange. This strategy was necessary due to the short half-life of these bioactive factors. Phosphate buffered saline (PBS) was added to the media to standardize the dilution.

Table 1. Neuropeptide Concentrations.

Neuropeptide	*1x Designation*	*Concentrations to be Tested*		
CGRP	10 ng/mL	1x	10x	100x
NPY	5 μg/mL	0.01x	0.1x	1x
SP	5 μg/mL	1x	10x	100x
VIP	10 ng/mL	10x	100x	1000x

Concentrations of neuropeptides to be used in treatment groups. Concentrations are based on dosage studies.

Media Exchange:

Media was exchanged every two days with new DMEM containing the appropriate treatment. The cultures were carried out to day 8.

Outcome Analysis Protocol:

Proliferation and glycosaminoglycan assays were run every two days. Proliferation was assessed with the Promega Cell Titer Glo® luminescence assay. This assay is a homogeneous method for determining the number of viable cells in culture based on the quantitation of the ATP present. The 96 well plates were removed from the incubator, 100 µL of Cell Titer Glo reagent was added to each well, and the plates were placed on a plate mixer for two minutes. The luminescence was then measured with a Fluoroscan.

Glycosaminoglycan production was assessed with the 1,9-dimethlymethylene blue (DMMB) assay, a commonly used indirect colorimetric method for measuring sulfated glycosaminoglycans (sGAG). The 12 well plates were removed from the incubator and digested by adding 1 mL of acetic acid containing papain to each well and incubating at 60°C for 4 hours. Then, 40 µL of each sample was transferred to a 96 well, opaque-walled plate. A standard curve was created using chondroitin sulfate at 5-50 µg/mL. To each well was added 250 µL of DMMB buffer and the samples were measured on a spectrophotometer within 5 minutes. Through utilization of the standard curve, the results were reported in µg/mL of sulfated glycosaminoglycans.

Evaluation of Data:

Assistance with the statistical analysis was provided by the statistics section of the department of general surgery. Analysis was performed using a split-plot ANOVA and pairwise t-tests between each group. The split-plot ANOVA accounted for multiple replicates per animal per treatment group. The pairwise t-test was used to determine if there was a significant difference between any two groups. A p value less than 0.05 was considered to be statistically significant. These statistical measures were performed on both GAG absorbance and proliferation data. The proliferation data was analyzed using percentages calculated by setting the mean of the control for a single knee as 100%. All of the values for the knee on a particular day were generated as a percent based on that mean, including back calculation of the control values. Statistics for DMMB procedure results were performed on the raw absorbance values, which corresponded to the GAG content. Determination of the quality of each monolayer was made through evaluation of the positive control, IGF, over time. Only those monolayer experiments demonstrating consistent increases in proliferation and DMMB absorbance, as expected with IGF, were considered to produce reliable results.

RESULTS:

The effects of neuropeptides on bovine chondrocytes was quantified through the use of proliferation and DMMB assays. Initially, pilot studies were performed in order to determine the proper range of neuropeptide concentrations to utilize in these experiments. Each of the neuropeptides was analyzed separately to determine their effects on both the proliferation and metabolism of bovine chondrocytes.

Effect of NPY on Bovine Chondrocytes:

Proliferation data, shown in figure 1, was assessed by comparing control data with the proliferation of chondrocytes after treatment with varying concentrations of NPY. IGF data, which served as a positive control, attested to the quality of the monolayer; IGF proliferation values were significantly higher than control values and steadily increased with time. An additional qualifier of the monolayer quality was a steady increase in control proliferation.

NPY 1x (5 µg/mL) was found to inhibit chondrocyte proliferation at all time points during the trial. This inhibition was statistically significant as shown by t-test p values (0.0064 for day 2, less than 0.0001 for day 4, 0.0003 for day 6, and 0.0313 for day 8). Treatment of cultured chondrocytes with NPY 0.1x (0.5 µg/mL) showed a similar inhibiting trend on days 2-6, but this reduction in proliferation was only significant on day 4, with a t-test p value of 0.0316. Finally, NPY 0.01x (0.05 µg/mL) provided the most favorable response, with stimulating effects on days 6 and 8, but this increase in proliferation was only significant on day 8, with a p value of 0.0208.

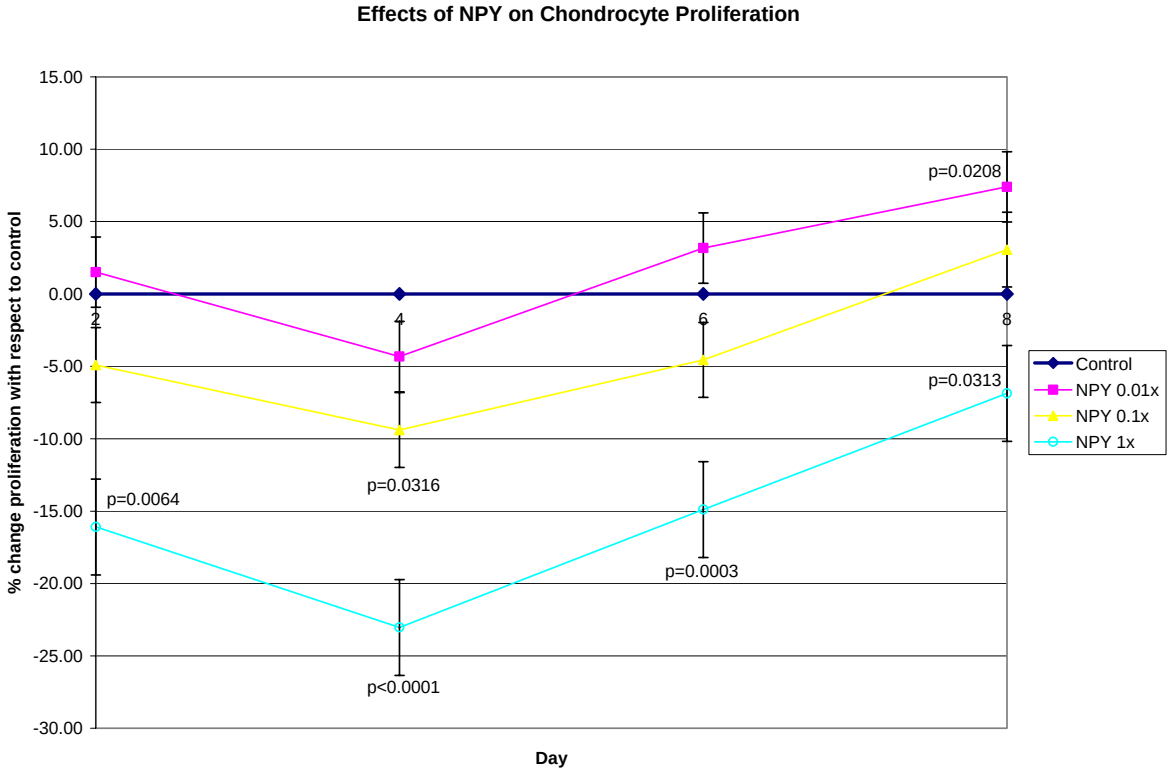

Figure 1. Effects of NPY on Chondrocyte Proliferation. NPY 0.01x (0.05 µg/mL) had a slight stimulatory effect on chondrocyte proliferation, while NPY 0.1x (0.5 µg/mL) and NPY 1x (5 µg/mL) had inhibitory effects. The stimulatory effect for NPY 0.01x was only significant on day 8 (p: 0.0208). The inhibitory effect of NPY 1x was significant at all time points (day 2 p: 0.0064, day 4 p<0.0001, day 6 p: 0.0003, and day 8 p: 0.0313). The decrease in proliferation resulting from NPY 0.1x was only significant on day 4 (p: 0.0316).

Results from the DMMB procedure in figure 2 showed no clear trends. These results were not considered to be reliable due to the lack of accurate IGF data; instead of increasing DMMB absorbance, and therefore GAG production over time, the effect of IGF created no

consistent pattern. Treatment of chondrocytes with IGF showed a large increase in metabolism that peaked on day 2 to later decrease and level off. Therefore, only proliferation results were used to characterize the effect of this neuropeptide on bovine chondrocytes. Overall, proliferation results verified that higher concentrations of NPY were detrimental to chondrocyte proliferation. A trend was observed that suggested a slight increase in proliferation, especially with increased time, with lower levels of this neuropeptide.

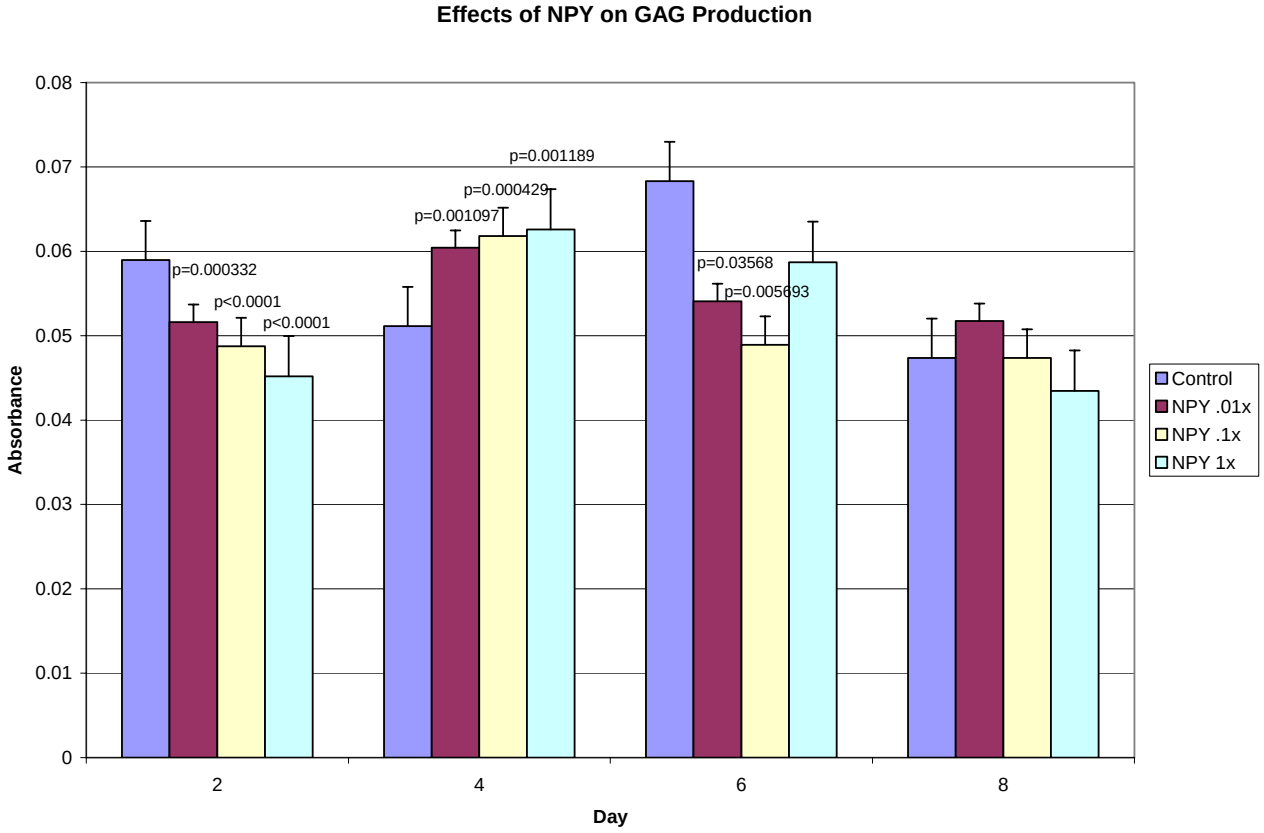

Figure 2. Effects of NPY on GAG Production. Results from the DMMB procedure showed no clear trends. These results were not considered to be reliable due to the lack of accurate IGF data; instead of increasing DMMB absorbance, and therefore GAG production over time, the effect of IGF created no consistent pattern.

Effect of CGRP on Bovine Chondrocytes:

Experiments testing the effect of treatment with CGRP on chondrocyte proliferation were validated through positive control data. IGF validated the quality of the monolayer model, as did the steady increase in control proliferation over time. Results, in figure 3, showed that treatment with all three concentrations of CGRP (1x: 10 ng/mL, 10x: 100 ng/mL, and 100x: 1000 ng/mL) had little significant effect on the proliferation of chondrocytes. Only slight trends were observed with this neuropeptide, including an increasing proliferation trend with CGRP 1x. However, this increase in proliferation was not found to be statistically significant, but approached significance on day 6 (t-test p values were 0.1592 for day 4, 0.0769 for day 6, and 0.1412 for day 8). As well, a trend was discovered showing that the highest CGRP

concentration, 100x, inhibited proliferation. This trend was only verified on day 4 as statistically significant (day 4 p: 0.0247, day 6 p: 0.0955, and day 8 p: 0.4122).

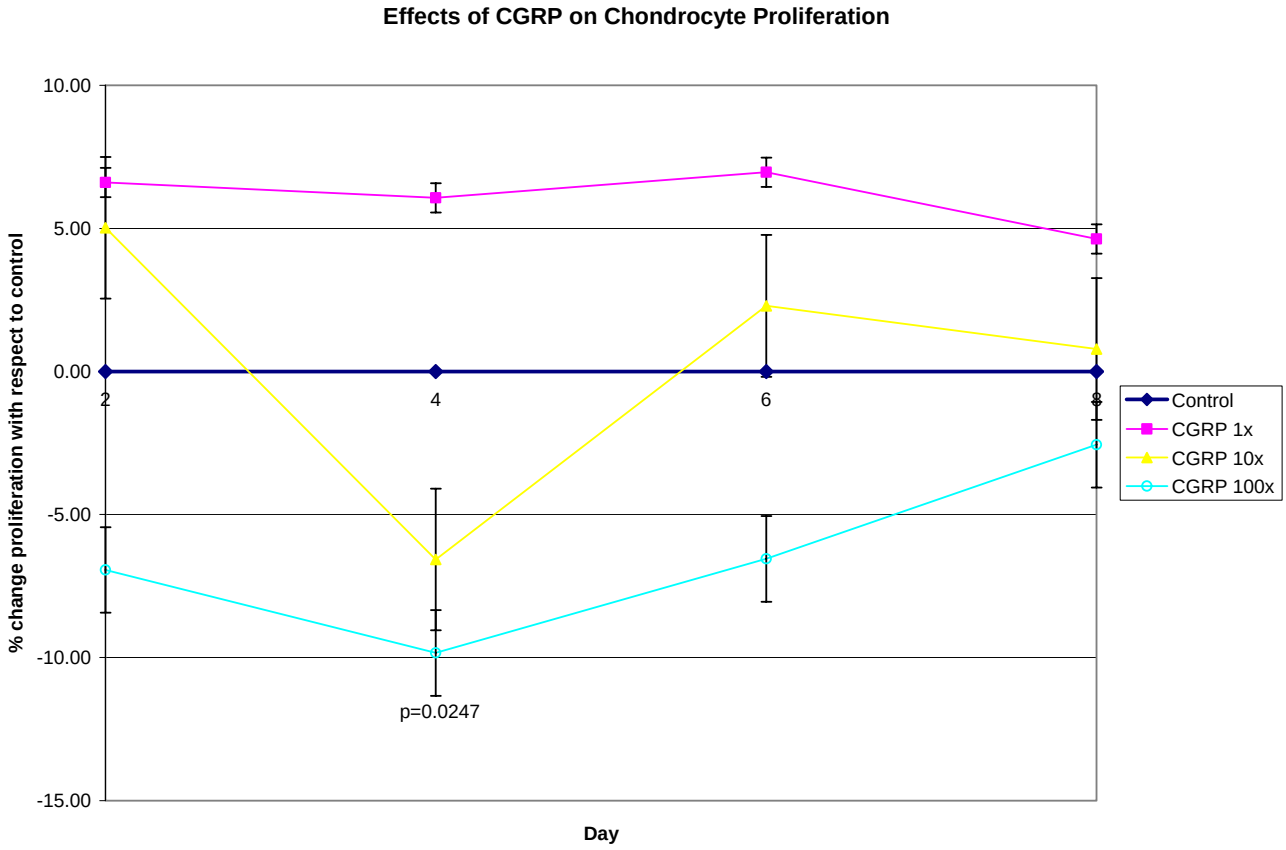

Figure 3. Effects of CGRP on Chondrocyte Proliferation. CGRP 1x (10 ng/mL) slightly stimulated chondrocyte proliferation with no statistical significance found. CGRP 10x (100 ng/mL) had varying effects on proliferation, none of which had any significance. The only significant value in the figure is for day 4 CGRP 100x (1000 ng/mL), with a significant decrease in chondrocyte proliferation as compared with control (p=0.0247). This concentration, CGRP 100x, showed the most clear trend, with a slight decrease in proliferation.

As the DMMB procedure for this neuropeptide was run simultaneously with NPY, these results, shown in figure 4, were similarly not considered to be reliable due to the lack of accurate IGF data. Therefore, only proliferation results were used to characterize the effect of CGRP on bovine articular chondrocytes. Overall, these proliferation results demonstrated that CGRP had little statistically significant effect on chondrocytes proliferation. The results only suggested slight increases with the lowest concentration, 1x, and decreases with the highest concentration, 100x.

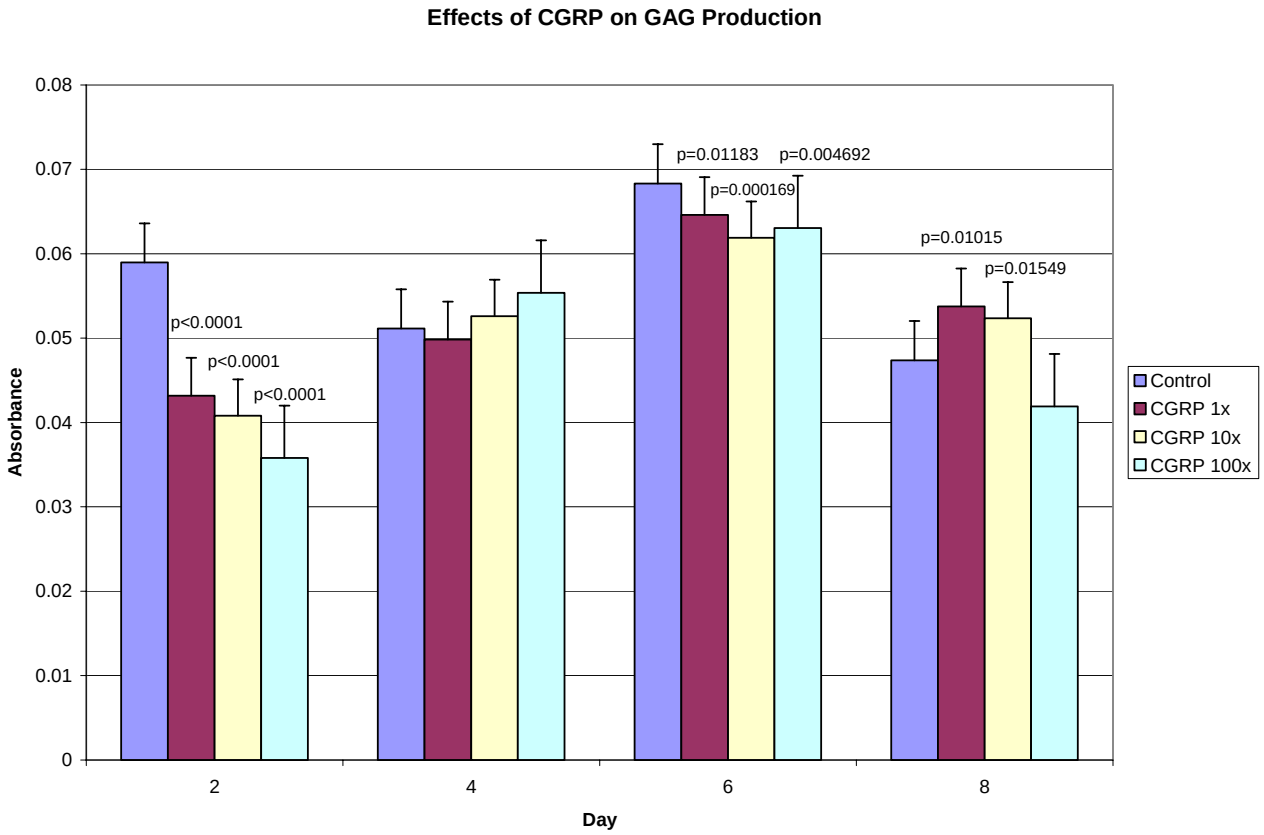

Figure 4. Effects of CGRP on GAG Production. Results from the DMMB procedure showed no clear trends. These results were not considered to be reliable due to the lack of accurate IGF data; instead of increasing DMMB absorbance, and therefore GAG production over time, the effect of IGF created no consistent pattern.

Effect of VIP on Bovine Chondrocytes:

Proliferation data, shown in figure 5, was determined through the Cell Titer Glo® assay. This data was validated through use of IGF as a positive control. IGF data was consistent with expected results and therefore the quality of the monolayer utilized for this assay was confirmed. Results from this assay demonstrated that treatment with VIP 1000x (10,000 ng/mL) decreased proliferation on all days, with statistically significant inhibition occurring after day 2 (day 2 p: 0.1872, day 4 p: 0.02216, day 6 p: 0.006698, day 8 p: 0.001393). Another lesser trend discovered through the proliferation assay was a stimulatory effect with the lowest concentration, VIP 10x (100 ng/mL). Chondrocyte proliferation increased on days 2-6, with only days 2 and 6 having statistically significant p values (day 2 p: 0.008592, day 4 p: 0.1476, and day 6 p: 0.02307). Day 8 proliferation with VIP10x treatment decreased slightly compared with control, but this decrease was not significant (day 8 p: 0.5279).

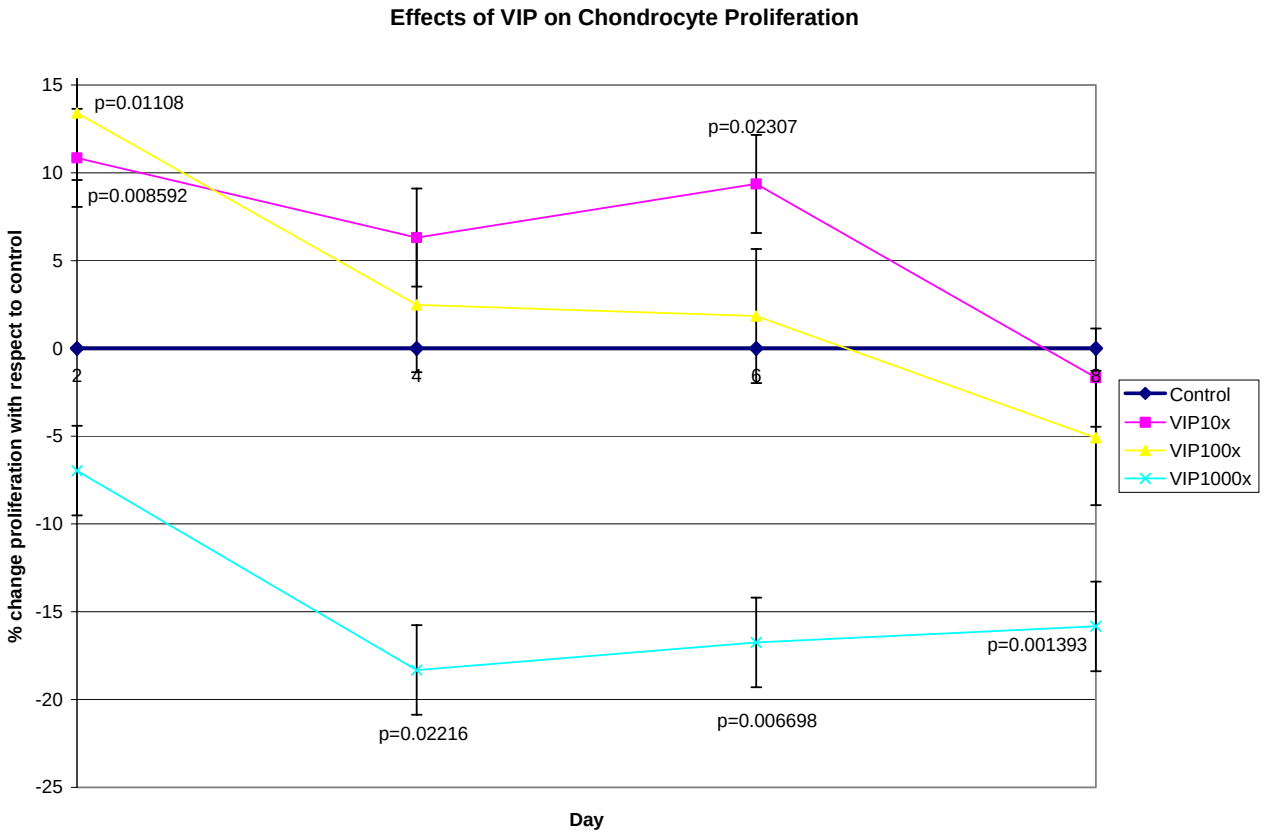

Figure 5. *Effects of VIP on Chondrocyte Proliferation.* Treatment with the highest concentration of VIP, 1000x: 10,000 ng/mL, inhibited chondrocyte proliferation on all days, a decrease that was statistically significant after day 2 (day 4 p: 0.02216, day 6 p: 0.006698, and day 8 p: 0.001393). VIP10x (100 ng/mL) and VIP100x (1000 ng/mL) had a slight stimulatory effect on chondrocyte proliferation. For the lowest concentration, 10x, this increase was significant on days 2 and 6 (p: 0.008592 and p: 0.02307 respectively). The effect on proliferation for VIP100x was only significantly higher on day 2 (p: 0.01108).

The GAG absorbance data was validated through accurate positive control data. The GAG data is visualized in figure 6. The lowest concentration of VIP utilized in this experiment, 10x, significantly increased GAG production on day 8 (p: 0.000158), suggesting a potential increase in GAG absorbance with increased time. As well, the 1000x concentration decreased GAGs on days 2-4 when compared with control, while day 6-8 data showed no change in GAG absorbance over control (day 2 $p<0.0001$ and day 4 p: 0.03007). The intermediate VIP concentration, 100x: 1000 ng/mL, showed decreased GAGs on all days, with statistically significant inhibition on days 2 and 8 (day 2 $p<0.0001$, day 4 p: 0.525, day 6 p: 0.2159, and day 8 p: 0.000398).

Combining data from both the DMMB and proliferation assays, it was possible to conclude that the lowest concentration increased both proliferation and GAG content, although at different time points. Proliferation stimulation was significant on days 2 and 6, while the only significant increase in GAG production occurred on day 8. The highest concentration of VIP, 1000x, decreased proliferation as well as GAG content, two effects which also occurred at

different time points. The decrease in proliferation was significant after day 2, while the decrease in GAG absorption was only significant on days 2 and 4.

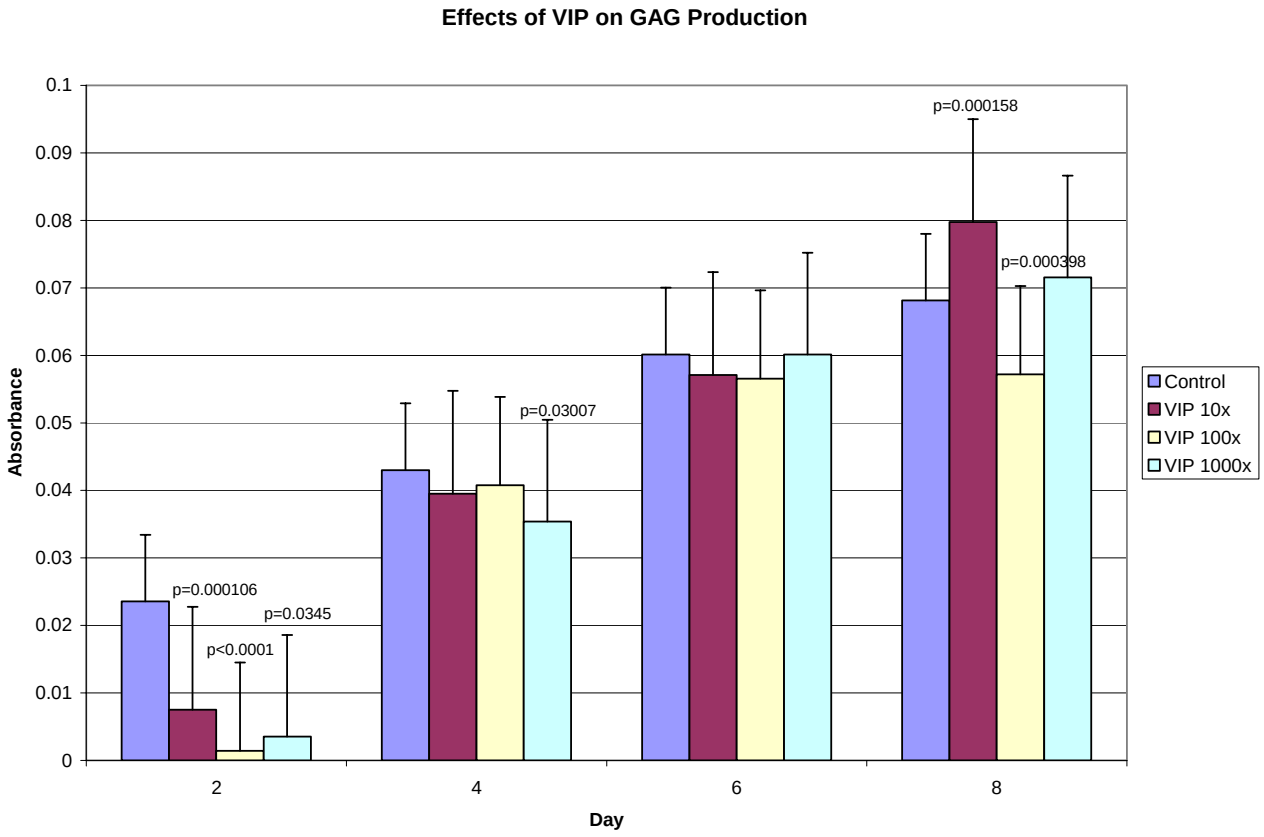

Figure 6. VIP10x (100 ng/mL) had lower GAG production on days 2-6, while the increase in GAG absorbance on day 8 was statistically significant (p=0.000158). VIP100x (1000 ng/mL) and 1000x (10,000 ng/mL) had no stimulatory effect on chondrocyte GAG production and instead had some inhibitory effect; on day 2 and 4 VIP1000x significantly decreased GAG absorbance (p=0.0345 and p=0.03007 respectively), while on day 8 VIP100x had a significant inhibitory effect (p=0.000398).

Effect of SP on Bovine Chondrocytes:

The effect of the neuropeptide SP on chondrocyte proliferation, shown in figure 7, was determined by comparing control proliferation results to those for chondrocytes treated with different SP concentrations. IGF, utilized as a positive control in this experiment, verified the quality of the monolayer culture and therefore validated the proliferation results. These results showed that no concentration of SP inhibited chondrocyte proliferation. Instead, the lowest SP concentration, 1x: 5 µg/mL, significantly increased proliferation on all days (day 2 p: 0.01233, day 4 p: 0.00061, day 6 p: 0.01866, day 8 p: 0.03422). Even the intermediate SP concentration, 10x: 50 µg/mL, increased chondrocyte proliferation. This stimulation was statistically significant on days 2-6 (day 2 p: 0.002005, day 4 p: 0.0244, day 6 p: 0.0012). The p value for day 8 approached significance (day 8 p: 0.09137). The highest concentration utilized, SP 100x: 500 µg/mL, had no significant effect on proliferation.

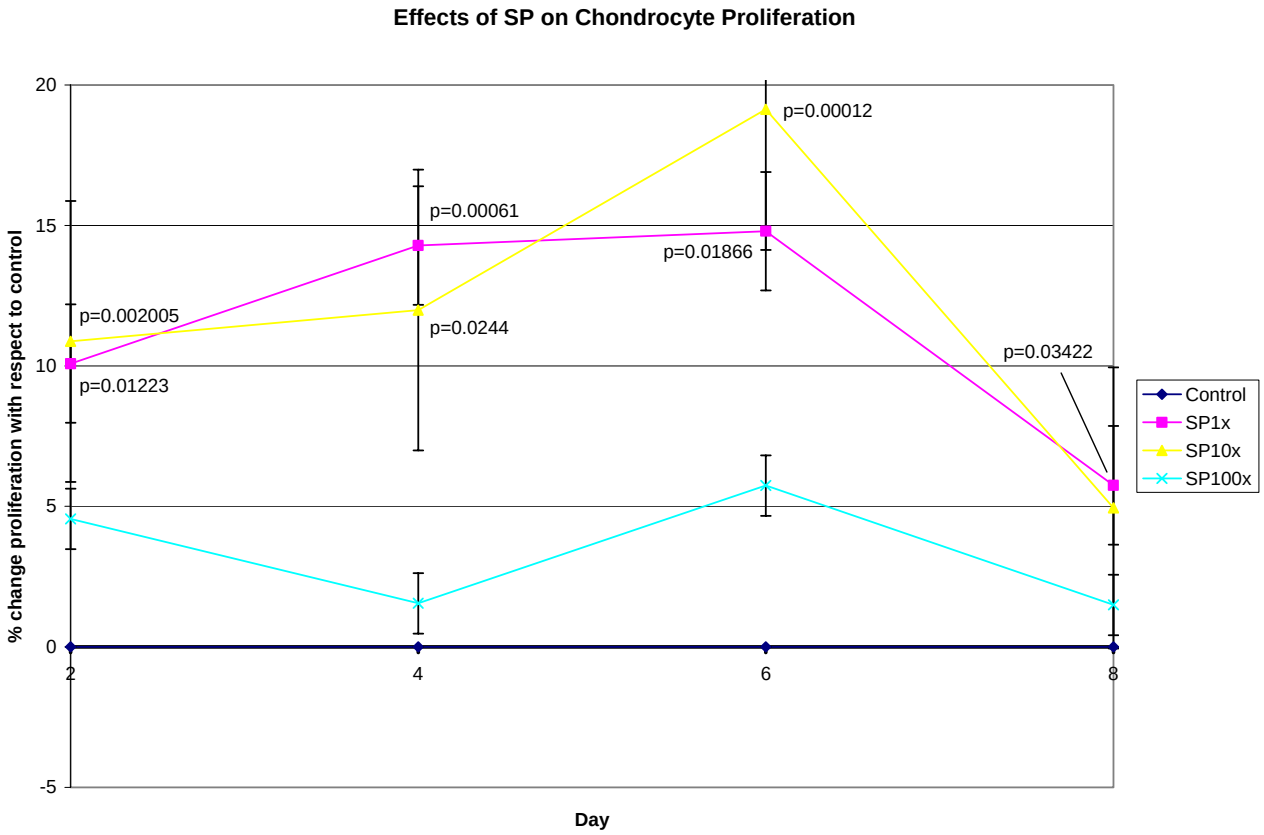

Figure 7. Effect of SP on Chondrocyte Proliferation. Overall SP had a stimulatory effect on chondrocyte proliferation, with statistically significant increases for both SP 1x (5 µg/mL) and SP10x (50 µg/mL). Treatment with SP1x significantly increased proliferation on all days (day 2 p: 0.01223, day 4 p: 0.00061, day 6 p: 0.01866, and day 8 p: 0.03422) while SP10x showed significant increases on days 2-6 (day 2 p: 0.002005, day 4 p: 0.0244, and day 6 p: 0.000120). The highest SP concentration utilized in this experiment, 100x: 500 µg/mL, had no significant effect on chondrocyte proliferation.

Data from the DMMB assay provided further information regarding the effect of this neuropeptide on chondrocytes, particularly on the metabolism of these chondrocytes. This data is visualized in figure 8. The IGF data verified that the results from this assay were reliable; treatment with IGF increased GAG absorbance over time. Results from this assay demonstrated that SP1x increased GAG production, although not steadily, after day 2. While days 4 and 8 significantly stimulated GAG synthesis, the p value for day 6 approached significance (day 4 $p<0.0001$, day 6 p: 0.07407, and day 8 $p<0.0001$). The other SP concentrations did not have any real, significant effect on GAG production; any increase was slight with only one data point on day 4 for SP10x showing significantly increased GAG production (p: 0.03645).

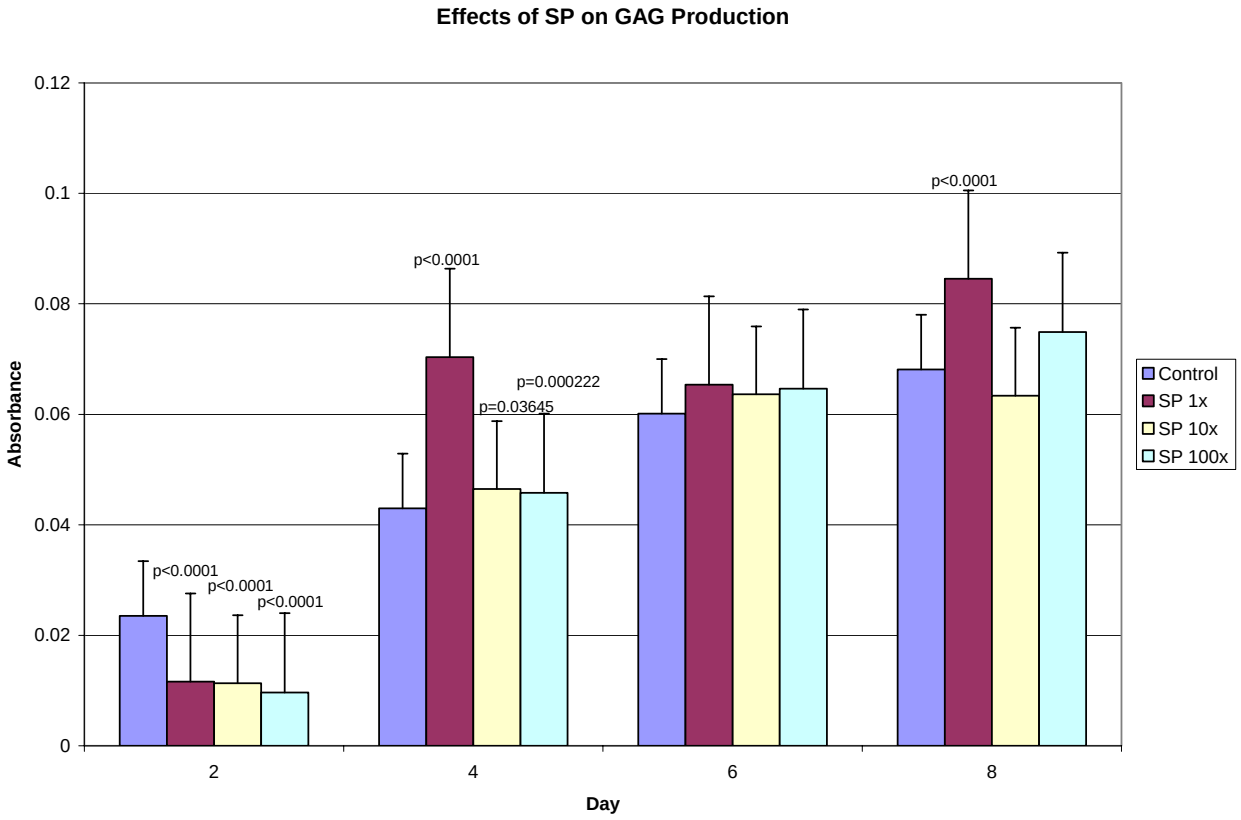

Figure 8. No concentration of SP inhibited GAG production significantly. Instead, SP 1x (5 µg/mL) increased GAG absorbance on days 4-8; this increase was significant on days 4 and 8 ($p<0.0001$ for both time points). SP 10x (50 µg/mL) and SP 100x (500 µg/mL) had significant stimulatory effects on day 4 only ($p=0.03645$ and $p=0.000222$ respectively).

Combined, this data provided information regarding the effect of SP on chondrocyte proliferation and GAG synthesis. The low SP concentration, SP 1x, stimulated both the proliferation and GAG absorbance values. Also a stimulant, the middle concentration, SP 10x, increased only proliferation. Finally, SP 100x, the highest SP concentration in this experiment, had no significant effect on proliferation or GAG production.

DISCUSSION:

The proposition that neuropeptides may provide some reparative functions on articular chondrocytes is both novel and remarkable as these bioactive molecules have been shown to play a role in the development of ailments of such cartilage. Evidence suggests that neuropeptides released from peripheral nerve endings play a pathogenic role in the development of arthritis and human joint inflammation (Appelgren *et al*., 1991, Hernanz *et al*., 1993). However, this paper is not the first to present substantiation that these molecules can be utilized successfully as an experimental treatment, such as the positive effects of VIP on collagen-induced arthritis in mice (Niissalo *et al*., 2002). It is, however, one of the first papers of its kind to look at the effects of neuropeptides on articular cartilage.

Effects of NPY on Bovine Chondrocytes:

In this experiment, a high concentration of NPY, 5 µg/mL, was found to significantly decrease proliferation throughout the length of the experiment. Additionally, while the lowest concentration, NPY 0.05 µg/mL, suggested a slight increase in proliferation, this increase was only significant on day 8. The results of this study correlate well with previous literature showing the harmful effects of this neuropeptide. While little research has been conducted to examine the effects of neuropeptide Y (NPY) on cartilage, one study found that NPY significantly stimulated the production of proinflammatory cytokines and chemokines in patients with rheumatoid arthritis. (Hernanz *et al*., 2003). The production of such detrimental regulatory proteins combined with the decrease in chondrocyte proliferation provides evidence that this neuropeptide holds little potential as a reparative molecule for chondrocytes.

However, this study also provided evidence that at minor concentrations this bioactive factor does not inhibit chondrocyte proliferation, and may also have a slight stimulatory effect. This trend was not fully established during the time period evaluated in this study; significant increases in proliferation were only reached on day 8. Without a longer trial, such as could be conducted using an alginate bead system that maintains phenotype for a longer period of time, this trend cannot be fully substantiated.

Effects of CGRP on Bovine Chondrocytes:

In this study CGRP was found to have little to no significant effect on chondrocyte proliferation. While the 10 ng/mL concentration showed a slight increase in proliferation that was not significant, the 1000 ng/mL concentration demonstrated a slight proliferation decrease that was only significant on day 4. The trivial effects of this neuropeptide shown in this experiment can be considered by taking into account previous research on CGRP, research that showed both its constructive and destructive effects.

Previous research on CGRP found this neuropeptide to have both potent vasodilatory and pain-transmitting effects. In a study by Brown and colleagues, patients with degenerative disc disease showed a noticeable increase in CGRP-containing sensory nerve fibers as compared with control patients. Also, as with NPY, CGRP is thought to play a pathogenic role in human joint inflammation (Hernanz *et al*., 1993). However, along with these injurious effects was reported that treatment with CGRP caused cultured chondrocytes and perichondrial cells to have a significantly increased cAMP level (Edoff and Hildebrand, 2003).

The lack of significant data in this experiment serves as an indicator that this neuropeptide holds little or no reparative ability for articular cartilage with respect to increasing chondrocyte proliferation. However, combined with other research, it may be possible to suggest that CGRP could be utilized as a treatment for articular cartilage injuries due to its ability to increase cAMP level. This positive effect, however, may be overshadowed by other harmful effects that CGRP may have on cartilage.

Effects of VIP on Bovine Chondrocytes:

This study showed that the lowest VIP concentration utilized, 100 ng/mL, increased both proliferation and GAG content. While proliferation was increased early in the experiment, with significant increases in proliferation on days 2 and 6, GAG production increased only on day 8. The VIP 1000 ng/mL concentration increased proliferation only. This increase was only significant on day 2. Finally, the 10,000 ng/mL concentration decreased both chondrocyte

proliferation and GAG content. The decrease in proliferation was significant on days 4-8 and the decrease in GAG production was significant earlier in the experiment, during days 2-4.

Previous research showed that VIP showed no discernable effect on DNA synthesis, as compared with the stimulation of DNA synthesis and proteoglycan content by human growth hormone and somatostatin (Johansson and Madsen, 1987). However, in this study the cultures were only incubated with bioactive factor for 18 hours. Therefore, the differences between these two experiments may be attributed to experimental design. Another study by Delgado and colleagues in 2001 suggested that VIP was a viable candidate for RA treatments. Similarly, Juarranz and colleagues in 2005 found that VIP treatment prevented experimental arthritis in animal models. This prevention of arthritis was suggested to occur by downregulation of both inflammatory and autoimmune components of the disease. These results correlate with those found in this experiment for the lower VIP concentration utilized. The increase in cell proliferation and GAG content suggests some reparative ability of this neuropeptide at low concentrations.

Effects of SP on Bovine Chondrocytes:

This experiment showed an increase in proliferation and GAG content with SP 5 μg/mL treatment. The increase in proliferation with this low concentration was significant at all time points and the GAG content increase was significant on days 4 and 8, with the day 6 GAG absorbance increase approaching significance. The intermediate SP concentration, 50 μg/mL, increased only chondrocyte proliferation and had no real effect on GAG content. Finally, the SP 500 μg/mL concentration had no significant effect on either cell proliferation or GAG production.

While it is widely known that SP is associated with pain, other research showed similar stimulatory effects of this neuropeptide including small stimulatory effects on disc cell proliferation (Ashton and Eisenstein, 1995). Therefore, the new findings in this study coordinate well with previous literature, thereby providing evidence that this neuropeptide holds reparative power for articular cartilage ailments. Additionally, this study provided comparison between the neuropeptides, thereby allowing for determination that SP has the greatest potential to positively influence articular chondrocytes. None of the concentrations utilized in this experiment inhibited chondrocyte growth and the increase, seen with both of the lower SP concentrations, was statistically significant throughout the course of the experiment.

Overall Effects of Neuropeptides on Bovine Chondrocyte:

The overall results of this study show that some neuropeptides have the ability to increase both chondrocyte proliferation and GAG production in cartilage. The enhancement of these indices was considered to be indicative of a beneficial response through treatment with these bioactive molecules. While CGRP showed little effect on bovine chondrocyte proliferation and NPY showed an inhibitive effect on proliferation, VIP demonstrated some ability to positively augment cartilage. Finally, SP showed the greatest reparative potential due to the large increase in both cell proliferation and GAG production after treatment with the low SP concentration.

Overall, this data provides evidence for the effect of neuropeptides on bovine chondrocytes. The effects of these bioactive factors on damaged human articular cartilage or *in vivo* are not known. However, there is no evidence to suggest that there are any large disparities between human and bovine cartilage. As well, this study was only conducted over eight days.

Therefore, the effects of these neuropeptides over longer periods of time are not known. However, this study was conducted in order to determine the potential for neuropeptides to act as reparative agents after cartilage damage. The short term effects of these neuropeptides, as shown through this study, may be enough to combat the catabolic cascade that is known to accompany cartilage injury.

The results of this study show the potential for some neuropeptides, especially SP, to treat articular cartilage injuries as well as arthritis. Along with improvement of the indices characterized in this study, neuropeptides may provide other beneficial effects. As stated previously once neuropeptides are bound, they tend to produce a cascade of reactions. These reactions eventually result in DNA transcription and protein expression, thereby further broadening their effect (Schaible *et al.*, 2005). Due to the therapeutic advantages that neuropeptides offer over growth factors, these molecules may be an improved treatment option. However, one set back to the use of neuropeptides is their short half-life, thereby requiring repeated insertion of the molecule in the body. While an issue of concern with regards to treatment, most known molecules with the potential for cartilage treatment success have similar issues. As well, a treatment method to localize the neuropeptide near the damaged chondrocytes is necessary due to their known detrimental effects within the body, such as pain and vasodilatory effects. However, the potential of neuropeptides to alleviate cartilage damage through reparative processes would provide patients suffering from articular cartilage injuries treatment options to improve quality of life and potentially lessen their chance of developing osteoarthritis.

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APPENDIX:

Figure A.1. Neuropeptide Table

Neuropeptide (NP) [peptides found in neural tissue]	Structure	Family	Receptor
Substance P (SP)	11-amino acid peptide Sequence: ARG-PRO-LYS-PRO-GLN-GLN-PHE-PHE-GLY-LEU-MET-NH_2	Tachykinin neuropeptide family (The tachykinin family is characterized by a common C-terminal sequence, Phe-X-Gly-Leu-Met-NH_2, where X is either an Aromatic or an Aliphatic amino acid.)	Endogenous receptor for Substance P is neurokinin 1 receptor (NK1-receptor, NK1R) (Belongs to the tachykinin receptor sub-family of GPCRs)
Vasoactive Intestinal Peptide (VIP)	28-amino acid peptide structurally related to secretin Sequence: HIS-SER-ASP-ALA-VAL-PHE-THR-ASP-ASN-TYR-THR-ARG-LEU-ARG-LYS-GLN-MET-ALA-VAL-LYS-LYS-TYR-LEU-ASN-SER-ILE-LEU-ASN-NH_2	Because VIP shows similarities to glucagon, secretin and gastric inhibitory peptide (GIP), it has been considered a member of the glucagon-secretin family.	The VIP receptor, VIPR mediated by G-proteins, activates adenylyl cyclase. The receptor is an integral membrane protein mainly expressed in lung, liver, kidney, heart and placenta and belongs to the family of G-protein coupled receptors.
Neuropeptide Y (NPY)	36-amino acid peptide Sequence: Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr -Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH_2	Member of a family of proteins that include pancreatic polypeptide, peptide YY and seminalplasmin	The receptor protein that NPY operates on is a G-protein coupled receptor in the rhodopsin like GPCR family. These receptors are metabotropic, causing metabolic changes in the target cell rather than directly opening ion channels. The protein contains seven membrane spanning domains (Michel *et al.*, 1998).
Calcitonin Gene Related Peptide (CGRP)	37-amino acid peptide with structural homology to salmon calcitonin. Sequence: H-Ala-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg- Ser-Gly-Gly-Val-Val-Lys-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2		Acts through G protein-coupled receptors whose presence and changes in function modulate the peptide's effects in various tissues. CGRP's signal transduction through the receptors is dependent on two accessory proteins: Receptor activity modifying protein1 (RAMP1) and receptor component protein (RCP). Several endogenous substances such as glucocorticoids, nitric oxide (NO), nerve growth factors (NGF), and steroid hormones modulate CGRP release and synthesis.

NP	Functions (General and With Respect to Cartilage)	Location
SP	Neurotransmitter and neuromodulator In CNS, associated in the regulation of mood disorders, anxiety, stress, reinforcement, neurogenesis, respiratory rhythm, neurotoxicity, nausea, emesis and pain. Acts as a potent vasodilator. This is caused by the release of nitric oxide from the endothelium. Its release can cause hypotension. Involved in the transmission of pain impulses from peripheral receptors to the central nervous system. Substance P has been shown to stimulate cellular growth in cell culture (Reid *et al.*, 1993), and it was shown that SP could promote wound healing of non-healing ulcers in humans (Brown *et al.*, 1997).	Central Nervous System
VIP	Its role in the intestine is to greatly stimulate secretion of water and electrolytes, as well as dilate intestinal smooth muscle, dilate peripheral blood vessels, stimulate pancreatic bicarbonate secretion, and inhibit gastrin-stimulated gastric acid secretion. Found in suprachiasmatic nuclei (SCN) region of the brain – the location of the master circadian pacemaker. Given this location, it is thought to play key role in the mammalian time keeping machinery. Also has significant effects on the cardiovascular system. It causes coronary vasodilation and also has a positive inotropic and chronotropic effect. In collagen-induced arthritis, treatment with VIP significantly reduced incidence and severity of arthritis, completely abrogating joint swelling and destruction of cartilage and bone. The therapeutic effect of VIP was associated with downregulation of both inflammatory and autoimmune components of the disease (Delgado *et al.*, 2001). It was concluded that VIP is a viable candidate for the development of treatments for rheumatoid arthritis.	Produced in the pancreas. Originally isolated from intestinal extracts, but later work demonstrated that VIP is very widely distributed in the peripheral and central nervous systems, and probably should not be considered a true GI hormone. Also found in brain, heart, and some autonomic nerves.
NPY	Augments the vasoconstrictor effects of noradrenergic neurons. Extremely potent stimulator of feeding behavior. Feeding behavior in rodents is blocked by injection of antibodies or antisense RNAs against NPY. Physiological roles such as involvement in circadian rhythms, sexual function, anxiety responses and vascular resistance. Associated with a number of physiologic processes in the brain, including the regulation of energy balance, memory and learning, and epilepsy (Colmers, 2003).	Most abundant neuropeptide in the brain. Found in the brain and autonomic nervous system.
CGRP	Most potent endogenous vasodilator currently known. Produces multiple biological effects and has both circulatory and neurotransmitter modes of action.	Primarily produced in nervous tissue, however, its receptors are expressed throughout the body. Co-localizes with Substance P in neurons

Referenced in this table: Colmers and El Bahh, 2003, Delgado *et al.*, 2001, Michel *et al*., 1998, and Reid *et al*., 1993.