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AN ABSTRACT OF THE THESIS OF

Austin K. Viall for the degree of Master of Science in Veterinary Science presented on April 25, 2014

Title: The Expression, Function, and Potential Therapeutic Targeting of Serotonin Receptors 1B and 2A in a Canine Osteosarcoma Cell Line

Abstract approved:

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Shay Bracha

Abstract:

The serotonergic system plays a significant regulatory role in osteoblast differentiation and proliferation. Serotonin (five-hydroxytryptamine or 5HT) may promote or inhibit osteoblast proliferation depending upon the serotonin receptor isoforms expressed by the cell. Classically, 5HT receptor 1B (5HTR1B) reduces osteoblast proliferation by inhibiting phosphorylation of the cAMP response element binding protein (CREB) transcription factor. Conversely, 5HT receptor 2A (5HTR2A) activation positively influences osteoblast proliferation through extracellular signal-regulated kinase (ERK) signaling. Despite the serotonergic system being well characterized in normal osteoblasts, minimal information concerning the influence of serotonin on malignant osteoblasts is reported. The objectives of our study were to elucidate the expression, function, and potential of therapeutic targeting of these two receptors in a canine osteosarcoma cell line (COS) and primary normal canine osteoblasts (CnOb).

Equal levels of *htr1b* (gene) and 5HTR1B (protein) expression were found in normal and malignant osteoblasts. Treatment with serotonin enhanced viability of osteosarcoma cells but not normal osteoblasts. Challenge with the 5HTR1B agonist anpirtoline did not change cell viability. Rather, incubation with the specific receptor antagonist SB224289 caused reduction in osteoblast viability and osteosarcoma cells were more sensitive to this inhibitory effect. Investigation of this inhibitory activity showed 5HTR1B antagonism induces capase-3/7 activation in malignant cells. Evaluation of phosphorylated levels of CREB and ERK revealed abnormal 5HTR1B signaling in COS.

Htr2a (gene) and 5HTR2A (protein) expression was observed in COS and CnOb, with osteosarcoma cells demonstrating five-fold greater receptor expression than normal osteoblasts. Antagonism of 5HTR2A with the inhibitor ritanserin attenuated viability in neoplastic and normal cells. COS appeared more

sensitive to the inhibitory activity of ritanserin than did CnOb. 5HTR2A blockade induced increased activity of caspase-3/7 in COS, thus promoting apoptosis in osteosarcoma cells. Discordant patterns of CREB and ERK phosphorylation were found between malignant and normal osteoblasts following 5HTR2A pharmacologic manipulation, suggesting aberrant receptor signaling in neoplastic cells.

Cumulatively, our findings demonstrate that functional aspects of the serotonergic signaling system are present in a canine osteosarcoma cell line. 5HTR1B and 5HTR2A could plausibly have roles in the genesis and progression of osteosarcoma. Importantly, both serotonin receptors appear to be novel therapeutic targets. Future investigations into the relationship between canine osteosarcoma and other serotonergic signaling components may highlight previously unknown aspects of osteosarcoma biology.

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The Expression, Function, and Potential Therapeutic Targeting of Serotonin Receptors 1B and 2A in a Canine Osteosarcoma Cell Line

by
Austin K. Viall

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented April 25, 2014
Commencement June 2014

Master of Science thesis of Austin K. Viall presented on April 25, 2014

APPROVED

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Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Austin K. Viall, Author

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my wonderfully amazing wife Jennifer for all the years of friendship and support. Without you, none of this would have been possible!

Cheri, Nadette, Kevin, and Pat – thank you all for the technical support, supply pillaging, and entertaining conversations in our shared laboratory.

Shay, I greatly appreciate all your guidance and mentorship with our project. You gave me just the right balance of direction and freedom to explore – and excel – in this endeavor.

Sue and Elena, you two simply rock! I am so very grateful for all the time, energy, and effort you have placed into my pathology mentorship. I will greatly miss our little clin path trifecta when I leave... Thank you for everything!

TABLE OF CONTENTS

Page

1. Introduction…… 1

2. Literature Review…… 3

2.1 The serotonergic signaling system………………………………………………………………………… 3

2.2 The role of the serotonergic system in bone physiology…………………………………………… 6

2.3 Canine osteosarcoma………………………………………………………………………………………………. 13

2.4 The serotonergic signaling system in osteosarcoma……………………………………………… 22

3. Experimental Design and Methodology………………………………………………………………………. 23

3.1 Experimental design……………………………………………………………………………………………… 23

3.2 Methodology………. 24

4. Results……. 30

4.1 Evaluation of 5HTR1B……………………………………………………………………………………………… 30

4.2 Evaluation of 5HTR2A……………………………………………………………………………………………. 36

5. Discussion……… 42

5.1 5HTR1B in malignant and normal canine osteoblasts…………………………………………… 42

5.2 5HTR2A in malignant and normal canine osteoblasts…………………………………………… 45

6. Conclusion……… 48

7. Bibliography…… 50

8. Appendix…… 62

LIST OF FIGURES

Figure | Page

Figure 2.1.1. 5HT synthesis pathway………………………………………………………………………………… 3

Figure 2.2.1. Serotonergic gastrointestinal-bone endocrine axis……………………………………………… 8

Figure 2.2.2. Serotonergic signaling in osteoclasts………………………………………………………………. 9

Figure 2.2.3. Central nervous system serotonergic signaling promotes craniofacial bone formation……………………………………………………………………………… 10

Figure 2.2.4. The contrasting roles of serotonin in bone homeostasis……………………………………… 13

Figure 2.3.1. Distribution percentage of primary canine osteosarcoma lesions by anatomic location……………………………………………………………………………….. 15

Figure 4.1.1. There is equivalent *htr1b* transcription and 5HTR1B expression between canine osteosarcoma cells, COS, and normal canine osteoblasts, CnOb …………….. 32

Figure 4.1.2. The 5HTR1B antagonist SB224289 decreases viability in malignant and normal osteoblasts while the receptor agonist anpirtoline has no effect on osteoblast viability……………………………………………………………………… 33

Figure 4.1.3. 5HTR1B antagonism promotes increased caspase-3/7 activity in osteosarcoma cells………………………………………………………………………………………… 34

Figure 4.1.4. Discrepant patterns in CREB and ERK phosphorylation are observed between normal and malignant osteoblasts following treatment with serotonin, anpirtoline, and SB224289……………………………………………………… 35

Figure 4.2.1. Increased *htr2a* transcription and 5HTR2A expression is present in canine osteosarcoma cells relative to normal canine osteoblasts…………………… 38

Figure 4.2.2. The 5HTR2A antagonist ritanserin decreases viability in malignant and normal osteoblasts…………………………………………………………………………………….. 39

Figure 4.2.3. 5HTR2A antagonism promotes increased caspase-3/7 activity in malignant canine osteoblasts cells………………………………………………………………. 40

Figure 4.2.4. Discrepant patterns in phosphorylated CREB and ERK are observed between normal and malignant osteoblasts following treatment with serotonin and ritanserin…………………………………………….. 41

LIST OF TABLES

Table Page

Table 2.1.1. The seven 5HTR families, with their associated signal potential, prototypical signaling cascade, and secondary messaging molecule…………………… 5

Table 2.3.1. Distribution of histologic subtypes in 132 primary osteosarcoma canine tumors……… 16

Table 2.3.2. Histologic grading system for primary appendicular and axial canine osteosarcoma tumors presented by Straw *et al.* …………………………………… 16

Table 2.3.3. Histologic grading system for canine osteosarcoma presented by Kirpensteijn *et al.*……………………………………………………………………… 17

Table 3.2.1. Nucleotide sequences for sequencing primers and qRT PCR primers for *htr1b, htr2a, and ywhaz*………………………………………………………………………. 25

LIST OF APPENDIX FIGURES

Figure Page

Figure A.1. Alignment of COS *htr1b* sequence and published canine *htr1b* reference sequences, NCBI accessions NM_001006948.1 (mRNA), AY323909.1 (genomic DNA), and AY204572.1 (genomic DNA)……………………………. 75

Figure A.2. Alignment of COS *htr2a* sequence and published canine *htr2a* reference sequence, NCBI accessions NM_001005869.1 (mRNA)………………………………………. 76

LIST OF APPENDIX TABLES

Figure Page

Table A.1. The anatomic distribution, action, mechanism of signal propagation, and prototypical G-protein cascade for select individual 5HTR and 5HTR families………………………………………………………………………………………………… 62

Table A.2. Comparison of similarities and differences between canine and human osteosarcoma with regards to clinical disease and tumor biology…………. 64

Table A.3. List of product manufacturers..……… 66

Table A.4. Summary of statistical conclusions from all 5HTR1B experiments...................………... 67

Table A.5. Summary of statistical conclusions from all 5HTR2A experiments..................……….... 72

DEDICATION

I would like to dedicate these efforts to animal owners and their furry companions - past, present, and future. Thank you for giving me a purpose in life.

The Expression, Function, and Potential Therapeutic Targeting of Serotonin Receptors 1B and 2A in a Canine Osteosarcoma Cell Line

1. INTRODUCTION

Osteosarcoma is the most common primary skeletal malignancy of the dog.[1] Canine osteosarcoma has long been considered a natural analog of human juvenile osteosarcoma, with the two neoplasms sharing numerous biological and clinical similarities.[2] For both species, standard of care entails surgical resection of the primary tumor coupled with adjunct chemotherapy or radiotherapy. Even with aggressive treatment, prognosis remains poor due to a high propensity for development of metastases.[2-4] Further investigation into the biology of osteosarcoma is warranted and is necessary to identify new translational therapeutic strategies.

The serotonergic system is a recently recognized principal regulator of osteogenesis and mature bone homeostasis.[5-8] This cellular communication system is comprised of the transmitter serotonin, also termed 5-hydroxytryptamine (5HT), which transduces signal through seven subfamilies of 5HT receptors (5HTR) present on the surface of target cells.[9] Cellular production of serotonin is controlled by regulating expression of tryptophan hydroxylase (Tph), the rate limiting enzyme in the 5HT synthesis pathway.[9] Termination of signaling is accomplished by rapid cellular internalization of 5HT, which depletes serotonin from the extracellular space. Cells internalize the transmitter through the 5HT transporter (5HTT) present in the plasma membrane.[9] These components of serotonin signaling have all been identified in osteoblasts, osteocytes, and osteoclasts.[7, 10-13] The serotonergic system is now known to modulate many physiologic processes in bone, including bone cell differentiation, maturation, proliferation, and trafficking.[6-8, 14]

The effect of serotonin on bone is dichotomized by the location of its production. While serotonergic communication is used in many physiologic processes, there are just two principal anatomic sites of 5HT synthesis.[6] In the central nervous system, serotonin is produced by neurons and chiefly used as a neurotransmitter. Neuronal serotonergic signaling supports bone formation by promoting osteogenesis and suppressing bone resorption.[6] Additionally select 5HTR – such as receptors 2A (5HTR2A) and 2B (5HTR2B) – promote maturation of osteoblasts.[14-16] Conversely, serotonin present in blood inhibits bone formation through a gastrointestinal-bone endocrine axis.[7] The majority of serotonin in peripheral tissue is synthesized by duodenal enterochromaffin cells (ECs). Gut-origin serotonin may function as a local paracrine factor or enter systemic circulation.[5] Neuronal derived serotonin does not enter vascular circulation as the blood-brain-barrier is impermeable to 5HT. Most circulating serotonin is rapidly sequestered by platelets, leaving a minority of 5HT freely soluble in plasma. Elevated blood levels of

5HT act to disrupt bone density maintenance by decreasing bone formation and increasing bone resorption. This effect is chiefly imparted through 5HTR 1B (5HTR1B) expressed by osteoblasts, with 5HTR1B preventing osteoblast survivability.[7] Simultaneously peripheral serotonin promotes bone resorption by stimulating osteoclast proliferation and maturation.[12]

Despite the major regulatory role of serotonin in bone physiology, there is currently minimal reported investigation of the serotonergic system with regards to osteosarcoma. Two recent studies demonstrate that pharmacologic inhibition of 5HTT in human osteosarcoma cells attenuates neoplastic cell viability through apoptosis.[17, 18] These findings indicate the presence of functional aspects of the serotonergic system in malignant osteoblasts and suggest that this system may be a viable treatment target.

The central intent of our study was to investigate the presence of functional 5HTR in canine osteosarcoma cells and explore the therapeutic potential of pharmacologic targeting of these receptors. Specifically, we examined the expression of 5HTR1B and 5HTR2A in a canine osteosarcoma cell line and normal canine osteoblasts. We then evaluated the effects of receptor agonism and antagonism on osteoblast viability and activation of the receptors' prototypical signaling pathways. Given the reported pro-proliferative activity of 5HTR2A and anti-proliferative action of 5HTR1B, we hypothesized that canine osteosarcoma cells would have higher 5HTR2A and lower 5HTR1B expression relative to normal osteoblasts. We expected 5HTR2A antagonism to decrease the viability of normal and malignant cells. Conversely, 5HTR1B blockade was anticipated to enhance cell viability while receptor agonism would have a suppressive effect. Treatment with serotonin was expected to enhance proliferation of both normal and malignant cells. We also predicted that 5HTR1B and 5HTR2A signaling would be abnormal in the osteosarcoma cells relative to normal osteoblasts.

2. LITERATURE REVIEW

2.1 The serotonergic signaling system

Serotonergic communication is one of the principal intercellular signaling systems. Serotonin is ubiquitously found throughout the animal kingdom, highlighting the conserved, yet varied, nature of its utilization. In mammals, serotonergic communication is employed in numerous processes, including gastrointestinal motility, peripheral vascular tone, airway bronchial resistance, hemostasis, reproduction, and central nervous system relays.[9, 19, 20]

This signaling system centers on the biogenic monoamine serotonin. Serotonin shares many structural similarities to other signaling monoamines, such as dopamine, epinephrine, and norepinephrine.[9] Cellular synthesis of 5HT involves a two-step cytosolic process beginning with the amino acid L-tryptophan. Tryptophan is converted to 5-hydroxtryptophan by Tph, with 5-hydroxytryptophan undergoing a decarboxylation reaction by L-aromatic amino acid decarboxylase to become 5HT (**Figure 2.1.1**).[9] Tph is the rate-limiting enzyme for the synthesis of 5HT and thus serves as the principal regulatory point for serotonin production. The distribution of Tph is highly restricted within the body. Tph expression, and consequently 5HT synthesis, was originally thought to only occur in neuronal and gastrointestinal tissues.[9, 19, 21] Two genes encoding Tph isoenzymes are transcribed in a tissue-dependent manner. *Tph1* encodes Tph1 and *tph2* encodes Tph2. Tph1 is primarily expressed by duodenal ECs while Tph2 is expressed in neurons.[6, 22] Recent studies have highlighted expression of *tph1* in other tissue types as well, including thyroid, pancreas, bone, and lung.[12, 23, 24] Synthesis of serotonin in these later tissues is suspected but not confirmed.

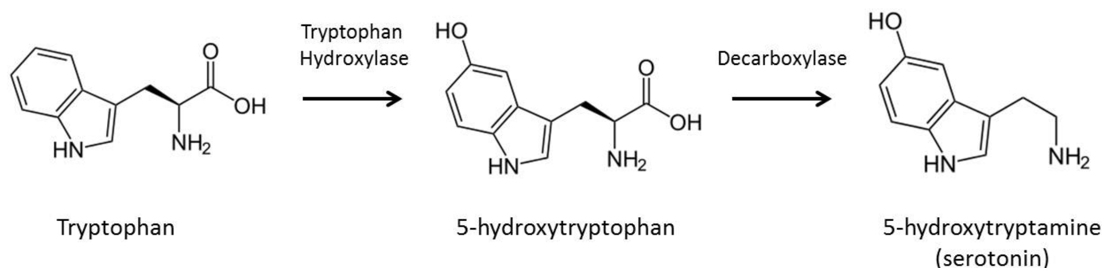

Figure 2.1.1. 5HT synthesis pathway. Tryptophan is converted to 5-hydroxytryptophan by the rate limiting enzyme in serotonin production, tryptophan hydroxylase. 5-hydroxytryptophan subsequently undergoes a decarboxylation reaction, facilitated by L-aromatic amino acid decarboxylase, to form 5HT. Adapted from Pytliak M *et al.*[25]

5HT synthesized by neurons is stored within neurosecretory vesicles localized within pre-synaptic axonal termini.[9, 26] For synaptic communication, these vesicles fuse with the presynaptic axonal membrane and release free 5HT into the synaptic cleft. Synaptic release may occur in response to presynaptic action potentials, presynaptic 5HT receptors, or neurotransmitter binding.[9, 19, 26, 27] Similarly, duodenal ECs store 5HT within neurosecretory vesicles present along the basal cell surface. Release of 5HT into the subepithelial lamina propria is controlled by sensory receptors present along the ECs luminal surface. These receptors detect a myriad of nutrients and functionally serve to provide a sampling of luminal contents to ECs.[24, 28] 5HT released into the lamina propria activates nerve endings of primary afferent neurons, which propagate signal to myenteric plexuses. Myenteric plexuses subsequently coordinate the gastrointestinal neuronal response, initiating such processes as motility. Through an unidentified mechanism, a small proportion of this serotonin enters peripheral blood. Approximately 95% of this 5HT is rapidly sequestered by platelets, which store serotonin within dense granules.[6, 9, 29, 30] Following platelet activation, 5HT is released from the dense granules and promotes further platelet activation, inflammatory cell chemotaxis, and endothelium activation.[6, 19, 30, 31] Only 5% of total blood 5HT is soluble in plasma.[6]

Recognition of serotonin on target cells occurs through binding of 5HT to plasma membrane 5HTR. Seven 5HTR families have been identified, with such receptor diversity thought responsible for the varied effects of serotonin. Signal transduction occurs through G-protein coupled cascades in six receptor families; one receptor family is ligand-gate voltage channels.[25, 32] The structure of the G-protein coupled receptors is highly conserved, with the proteins having 3 extracellular loops, 7 transmembrane hydrophobic domains, and 3 intracellular loops.[33] Receptors have either inhibitory or excitatory effects based upon their modulation of intracellular secondary messenger molecules.[25, 32] Although receptor families are coupled to a prototypical G-protein system, there is marked receptor promiscuity for other G-protein cascades.[32, 33] Use of alternate signaling systems likely results from RNA-splice variants and post translational modification of receptor intracellular domains.[33] Please see **Table 2.1.1** for an abbreviated summary of the receptor family characteristics; a more detailed list of receptor information is presented in **Appendix Table A.1**.

Family	Potential	Type	Mechanism of Action
5-HT_1	Inhibitory	G_i/G_0-protein coupled	Decreases intracellular [cAMP]
5-HT_2	Excitatory	G_{q11}-protein coupled	Increases intracellular [IP3] and [DAG]
5-HT_3	Excitatory	Ligand-gated Na^+/K^+ channel	Plasma membrane depolarization
5-HT_4	Excitatory	G_s-protein coupled	Increases intracellular [cAMP]
5-HT_5	Inhibitory	G_i/G_0-protein coupled	Decreases intracellular [cAMP]
5-HT_6	Excitatory	G_s-protein coupled	Increases intracellular [cAMP]
5-HT_7	Excitatory	G_s-protein coupled	Increases intracellular [cAMP]

Table 2.1.1. The seven 5HTR families, with their associated signal potential in neurons, prototypical signaling cascade, and secondary messaging molecule. Abbreviations: cAMP: cyclic AMP, IP3: inositol trisphosphate, DAG: diacylglycerol. Adapted from Pytliak M *et al.*[25]

Termination of serotonin signaling occurs by removal of 5HT from the extracellular space, thus reducing the quantity of free ligand available for receptor binding. Serotonin is actively internalized by cells expressing the membranous 5HTT.[9] Unlike the highly restricted distribution of Tph, 5HTT is expressed in numerous organs, including cardiac, pulmonary, vascular, brain, renal, ovarian, and bone tissue.[9, 11, 19, 25, 34] Depending upon the cell type, internalized 5HT can either be recycled into neurosecretory vesicles or metabolized by monoamine oxidase into 5-hydroxyindoleacetic acid (5HIAA). 5HIAA is actively secreted by cells and ultimately enters plasma, where it is passively filtered through the kidneys.[9] A minor amount of 5HT metabolism may occur by other cellular pathways, including glucuronidation or sulfation in the kidney, lung, liver, or brain.[9] Specifically in neuronal synaptic clefts, 5HT autoreceptors present on the presynaptic membrane can bind synaptic 5HT. This binding induces a negative feedback signal to reduce presynaptic release of 5HT vesicles. Thus, this scenario serves as ancillary method of extracellular 5HT depletion in addition to the 5HTT mechanism.[9]

Given the breadth of tissues employing serotonergic communication, disruption of this signaling system likely has a substantial role in the development and progression of certain pathologies. Indeed, there is a growing body of evidence incriminating aberrations in serotonergic signaling in both human and animal diseases. Alterations in 5HT or 5HTR production in central nervous tissue is associated with a number of human cognitive disorders, including attention deficit disorder, Alzheimer’s disease,

depression, and schizophrenia.[35-38] Chronic activation of 5HT signaling pathways contributes to progression of degenerative cardiac valve disease in people and dogs.[39-41] Similarly, overexpression of serotonin receptor 2B in cardiac myocytes may contribute to the pathology of canine dilated cardiomyopathy.[42] Pathologic induction of Tph1 and 5HTR1B expression in pulmonary endothelial cells induces marked pulmonary smooth muscle hyperplasia.[43] This scenario is now thought to be the principal pathophysiologic mechanism of pulmonary arterial hypertension.[43] Disruption of serotonin signaling contributes to motor and secretory abnormalities observed in human inflammatory bowel disease.[44] Dogs with inflammatory bowel disease have elevated gastrointestinal production of 5HT, suggesting a similar pathologic association in canids.[45] Recently, investigation of aberrant 5HT production by ECs and resultant bone mass deficiencies in mice lead to the discovery of a novel serotonergic gastrointestinal-bone endocrine axis.[7]

2.2 The role of the serotonergic system in bone physiology

Evidence suggesting a link between serotonin and bone originally arose from observational studies of the long term complications of chronic antidepressant therapy. Two classes of drugs that inhibit 5HTT, tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs), became widely used for depression treatment in the late 1980s and early 1990s.[46] In 1991, Ray *et al.* first observed that people treated with TCAs had increased risk for hip fracture relative to individuals receiving other antidepressant medication.[47] Increased incidence of osteoporosis was also observed in persons receiving these drugs.[46] Further investigations into these phenomena ultimately demonstrated that chronic TCAs and SSRIs therapy decreases bone mineral density.[46, 48-51] This attenuated bone density is suspected to increase the chance of fracture following bone trauma.[46] Although the mechanism linking 5HTT blockade to attenuated osteodensity was not yet established, these reports hinted to the presence of functional aspects of the serotonergic system in mature bone.

The full scope of the significance of serotonergic communication in bone physiology was realized from investigations into human genetic diseases associated with abnormal bone mass. The conditions osteoporosis pseudoglioma and type-1 autosomal dominant osteopetrosis arise from mutations in the gene (*lrp5*) encoding LDL receptor related protein 5 (Lrp5).[7, 8, 52] Loss-of-function mutations in *lrp5* cause a low bone mass phenotype in osteoporosis pseudoglioma. Conversely, *lrp5* gain-of-function mutations induce a high bone mass phenotype in type-1 autosomal dominant osteopetrosis. During their

evaluation of these genetic conditions in murine models, Yadav *et al.* discovered the presence of a serotonin-based gastrointestinal-bone endocrine axis.[7]

In this system, Lrp5 controls expression of Tph1 in duodenal ECs. Although the natural ligand for Lrp5 in the gut remains unknown, constitutive activation of Lrp5 attenuates *tph1* transcription in ECs.[7] Consequently, ablation of Lrp5 leads to uncontrolled up regulation of Tph1 in ECs. In this latter scenario, there is increased synthesis and secretion of 5HT by ECs into peripheral circulation.[7] This serotonin binds and activates 5HTR1B expressed by osteoblasts of mature bone. Downstream signaling of 5HTR1B blocks phosphorylation of the pro-survival transcription factor cAMP response element binding protein (CREB). In the absence of this transcriptional regulator there is decreased synthesis of cyclin D1, D2, and E1 in osteoblasts.[7] Hence, elevated blood concentrations of serotonin effectively preclude cell cycle advancement in osteoblasts of the mature bone. This situation ultimately results in attenuated bone biomass as osteoblasts are unable to proliferate to maintain bone density.[7] Osteoporosis associated with SSRI therapy is suspected to partially occur through this mechanism, as decreased serotonin internalization permits buildup of extracellular 5HT and continued 5HTR1B signaling.[8, 48, 50, 53]

In an opposing pattern, constitutively activated Lrp5 reduces *tph1* expression and thus decreases 5HT synthesis and secretion by ECs. With decreased 5HTR1B signaling at osteoblasts there is increased cell cycle progression, leading to increased osteoblast proliferation.[7] This situation explains the high bone mass phenotype observed with activating mutations in Lrp5. The key concepts identified by Yadav *et al.* are that peripheral serotonin is a major regulator of mature bone homeostasis and that circulating 5HT has an inhibitory effect on mature osteoblasts (**Figure 2.2.1**).[5, 7, 8]

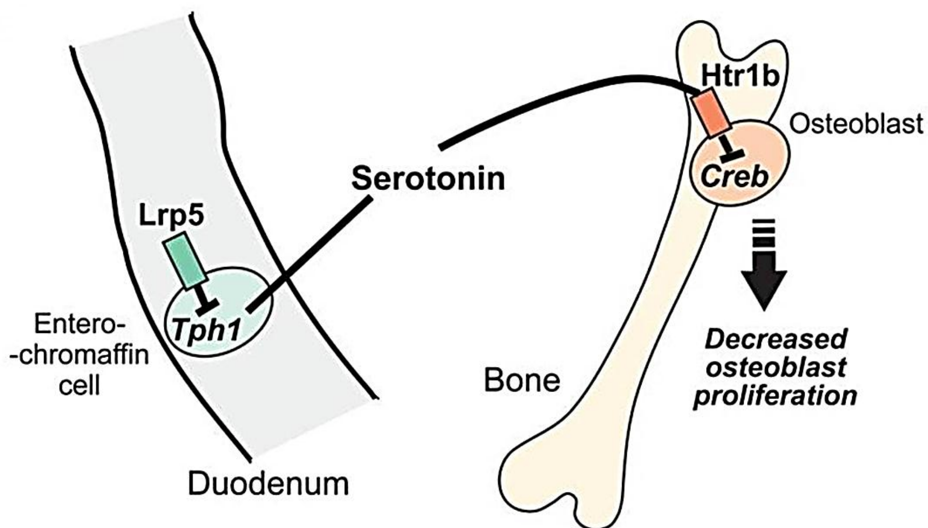

Figure 2.2.1. Serotonergic gastrointestinal-bone endocrine axis. In this endocrine axis, serotonin synthesized by duodenal enterochromaffin cells governs bone density homeostasis. In ECs, activated Lrp5 precludes expression of tph1. When inhibition of *tph1* transcription is removed, as with Lrp5 loss-of-function mutations, there is increased Tph1 expression which enhances synthesis and secretion of 5HT by ECs. This serotonin acts through the 5HTR1B receptor on osteoblasts to decrease phosphorylative activation of cAMP response element binding protein (CREB), a pro-proliferative transcription factor. With attenuated levels of activated CREB there is decreased synthesis of cyclins and less cell cycle progression. Thus, peripheral serotonin has an inhibitory effect on osteoblast proliferation. Figure from Yadav *et al.*[7]

Serotonin simultaneously has a stimulatory effect on bone osteoclasts (**Figure 2.2.2**). Signaling through either 5HTR1B or 5HTR2A promotes immature osteoclast proliferation and maturation.[12] This serotonin may be derived from peripheral circulation or synthesized directly by osteoclasts. Binding of the receptor activator for NF-KB ligand (RANKL) to the receptor activator of NF-KB (RANK) induces transcription of *tph1* in osteoclast.[12] Osteoclasts can theoretically secrete 5HT in the regional environment, effectively producing a 5HT autocrine loop. 5HT could then be actively recycled into osteoclasts through 5HTT, where it is kept in secretory granules for reuse.[12] Serotonin reutilization may be particularly important for osteoclast maturation as pharmacologic interference of 5HTT ablates osteoclast proliferation.[13, 54] At the tissue level, serotonergic osteoclast stimulation promotes increased bone resorption in mature bone.[12] As such, peripheral serotonin signaling can decrease bone density by both precluding osteoblast proliferation and enhancing osteoclastic resorption.

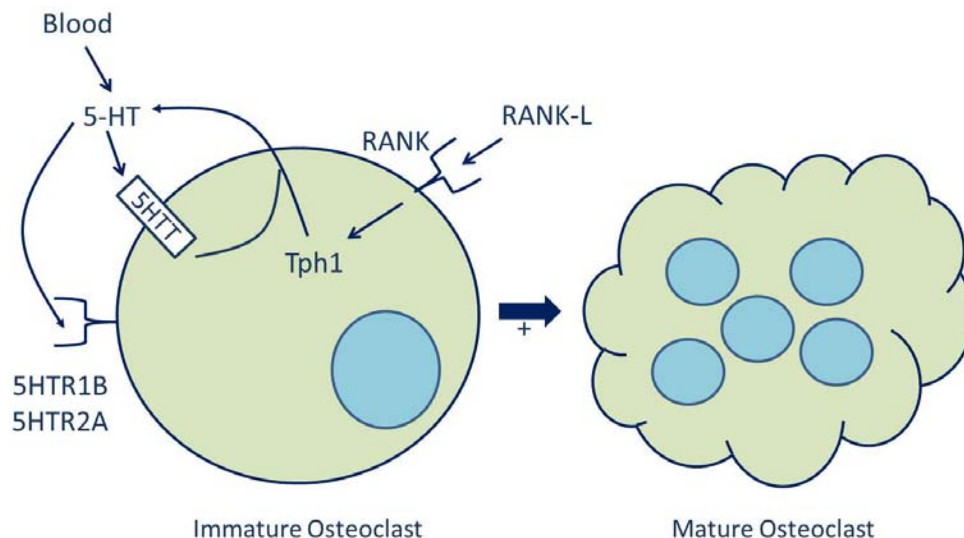

Figure 2.2.2. Serotonergic signaling in osteoclasts. Osteoclasts express multiple aspects of the serotonergic signaling system, including Tph1, 5HTT, 5HTR1B, and 5HTR2A. Serotonin promotes immature osteoclast proliferation and differentiation through 5HTR1B and 5HTR2A. Osteoclasts may respond to either circulating 5HT or actively synthesize serotonin. Activation of RANK induces transcription of *tph1*, thus possibly promoting 5HT synthesis. Once secreted, osteoclasts can reclaim 5HT for later utilization through 5HTT. Figure adapted from Chabbi-Achengli *et al.*[12]

Subsequent studies examining the effects of tissue specific ablation of 5HTR on bone demonstrated that central nervous system serotonergic signaling also has a major role in bone development.[6] *Tph2* knock-out mice have decreased cranial bone formation and enhanced bone resorption relative to wild-type mice, indicating a role for 5HT in cranial development.[55] Yadav *et al.* observed that serotonergic communication in specific neuronal foci of the ventromedial hypothalamus governs craniofacial bone development in mice.[55] Inter-neuronal signaling through the 2C serotonin receptor (5HTR2C) is centrally important in this process, as *htr2c* deficient mice have markedly decrease craniofacial bone mass.[55] Disruption of neuron-to-neuron serotonergic communication accentuates neuron-to-osteoblast sympathetic signaling through the β2 adrenergic receptor (Adrβ2).[55] Stimulated Adrβ2 provides an inhibitory signal to osteoblasts, attenuating cell proliferation, maturation, and migration.[55-57] The Adrβ2 signaling cascade ultimately decreases cyclin D1 expression in osteoblasts.[56] Simultaneously, adrenergic stimulation increases synthesis and secretion of RANKL into the regional environment which stimulates nearby osteoclasts to resorb bone.[6, 56] The net effect of Adrβ2 activation in craniofacial bones is decreased bone formation and enhanced resorption. Consequently, neuronal serotonergic communication in the hypothalamus promotes bone formation by blocking inhibitory sympathetic signals to osteoblasts (**Figure 2.2.3**).

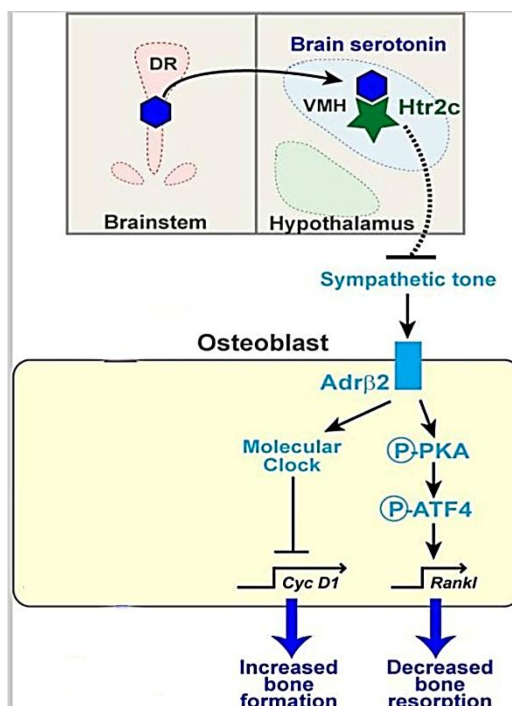

Figure 2.2.3. Central nervous system serotonergic signaling promotes craniofacial bone formation. Bone accrual in the craniofacial bones is partially governed by serotonergic neurons in the ventromedial hypothalamus (VMH). Activation of 5HTR2C in these neurons attenuates sympathetic signaling to the β2 adrenergic receptor (Adrβ2) on osteoblasts. In the absence of Adrβ2 signals, osteoblastic proliferation can progress due to increased cyclin transcription. Additionally, there is less osteoclastic resorptive activity because of decreased RANKL synthesis by osteoblasts. The net effect of VMH serotonergic signaling is enhanced bone formation and attenuated resorption. Figure adapted from Ducy *et al.*[6]

Further evidence supporting a pro-developmental role for serotonin signaling in bone formation arises from studies of 5HTT. In 1991, Shuey *et al.* showed murine embryos incubated with SSRIs develop craniofacial malformations in early embryogenesis.[58] SSRIs were also found to cause aberrations in dentition. [59] The role of 5HTT in osteogenesis was reaffirmed by Warden *et al.,* who observed that *5htt* null mice develop low bone-mass phenotypes in both axial and appendicular bone.[51] Inhibition of 5HT uptake appears to interfere with early epithelial-mesenchymal cell interactions important in craniofacial morphogenesis.[58, 59] Expression of 5HTT has been confirmed in all three major bone cells, osteocytes, osteoblasts, and osteoclasts.[11, 13, 54, 60] Through an unidentified mechanism, blockade or removal of 5HTT causes decreased bone cell proliferation and maturation.[11, 54, 58, 61] SSRIs also promote osteoblast and osteoclast apoptosis even in the absence of 5HT.[13, 17] Interestingly, there is equivocal human epidemiologic evidence that SSRI therapy during gestation causes minor fetal bone growth defects.[62, 63] The presence of functional 5HTT is now recognized as having an integral part in bone development, with a degree of temporal and species-specific variability to its role in osteogenesis.

Select 5HTR also exhibit pro-developmental activity in bone cells. The two receptors having the strongest mitogenic potential in osteoblasts are 5HTR2A and 5HTR2B.[6, 50, 64] This activity is in contrast to the reported anti-proliferative behavior of 5HTR1B. 5HTR2A expression has been identified *in vivo* and *in vitro* in murine and chicken osteoblasts and osteocytes.[11, 15, 60, 65] Challenging osteoblasts *in vivo* with specific 5HTR2A agonists induces osteoblast proliferation.[15, 61] The downstream signaling cascade associated with this proliferative potential is the extracellular signal-regulated kinase 1/2 (ERK) pathway, which undergoes phosphorylation following 5HTR2A stimulation.[15] Conversely, there is attenuated osteoblast viability when these cells are challenged with specific 5HTR2A antagonists.[15, 61] 5HTR2B has a similar expression profile to 5HTR2A in osteoblasts and osteocytes. [11, 14, 16, 60, 65, 66] *In vivo* models using *htr2b* deficient mice show marked, diffuse osteopenia in these animals.[14] 5HTR2B ablation decreases appendicular osteoblast recruitment, attenuates osteoblast proliferation and differentiation *ex vivo*, and mitigates osteoblast mineralization activity.[14, 16] In addition to ERK, another downstream pathway activated by this receptor is the nuclear peroxisome proliferator activated receptor ß/δ (PARR ß/δ).[16, 66] A key concept highlighted by these studies is that the net effect of serotonin on osteoblasts may be dependent upon the type and relative amount of 5HTR expressed by these bone cells.

Expression of other 5HTR has been confirmed in bone cells, although the functional activity of some receptors remains uncharacterized. Chicken osteoblasts express the 2A, 2B, and 2C receptors in a maturation dependent manner.[10] Gene transcription and/or receptor expression of 5HTR 1A, 1B, 1D, 1F, 2A, 2B, 3B, 6, and 7 have been confirmed in a myriad of murine osteoblast and osteocyte cell lines and primary osteoblasts.[11, 15, 60, 61, 65] Temporal receptor expression in murine osteoblasts was observed by Hirai *et al*., who found 5HTR2A in anaplastic and mature osteoblasts but 5HTR2B and 5HTT in mature osteoblasts only.[65] Differential expression was also observed between primary differentiated murine osteoblasts and an immortalized embryonic osteoblast line.[15] Few studies have evaluated 5HTR expression in human primary osteoblasts or established osteoblastic lines. Gustaffson *et al.* identified receptors 1A and 2A in human mesenchymal progenitor cells but only 5HTR2A in primary osteoblasts.[61] Additionally, non-mineralizing human osteoblasts express receptors 5HTR1B, 5HTR2A, and 5HTR2B while more differentiated, mineralizing variants lack 5HTR2B expression.[13] The expression of distinct 5HTR profiles at different phases of cell maturity suggests these receptors are major regulators of bone cell development. Temporal 5HTR expression may also explain how serotonin has a dichotomized effect on osteoblast vitality.

While most 5HT acting on bone is thought to enter the osseous environment through neuronal or hematogenous routes, there is growing evidence for endogenous serotonin synthesis in bone. As previously mentioned, increased RANK signaling in osteoclasts induces expression of Tph1.[12, 61] Tph1 expression has also been observed in murine osteoblasts *in vitro*.[61] Although suspected, actual synthesis and secretion of serotonin by these bone cells has not yet been reported. Should 5HT production actually occur by bone cells, this serotonin could plausibly have autocrine and paracrine activity within the osseous environment.

Based upon the current body of research, the picture emerges of serotonin simultaneously having stimulatory and inhibitory roles in bone biology. In an oversimplified model, centrally derived serotonin promotes osteogenesis while peripherally derived serotonin antagonizes bone maintenance (**Figure 2.2.4**). There are studies which partially challenge this model. For example, humans with carcinoid syndrome chronically overproduce peripheral serotonin but do not develop attenuated bone density.[67] Additionally, LRP5 has been shown to modulate osteoblast development directly in bone and independent of the peripheral serotonin reservoir.[52] These observations demonstrate that our understanding of the serotonin-bone relationship is grossly incomplete. Which specific serotonin receptors are involved in this process, and where those receptors localize developmentally and anatomically, is only partially characterized. Likewise, the functional importance of 5HTT and Tph1 in this system is only marginally known. Despite these knowledge gaps, the serotonergic system undoubtedly has a principal role in osteophysiology.

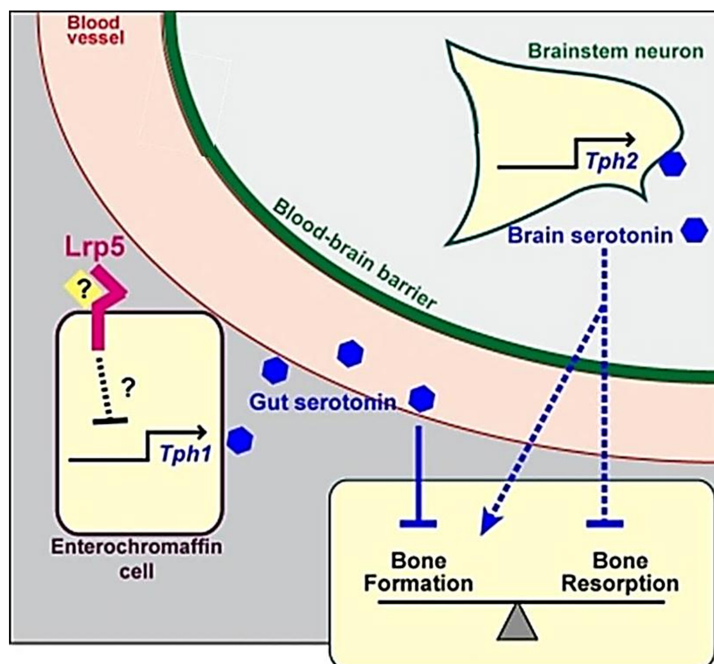

Figure 2.2.4. The contrasting roles of serotonin in bone homeostasis. The current model of serotonergic governance of bone remodeling indicates a dichotomized role for serotonin. Gastrointestinal derived serotonin attenuates bone formation, leading to decreased osteodensity. Conversely, central nervous system serotonergic signaling promotes bone formation and abates bone resorption. Adapted from Ducy *et al.*[6]

2.3 Canine osteosarcoma

Osteosarcoma is an osteoid-producing mesenchymal malignancy which arises from either osteoblasts or non-hematopoietic mesenchymal stem cells in bone.[68] In veterinary medicine, osteosarcoma occurs most frequently in the dog and second most frequently in the cat.[69, 70] Cases have been reported in most other domestic species.[1, 71] Osteosarcoma accounts for 85-95% of all primary canine bone tumors, with an estimated annual incidence of 10,000 cases in the United States.[2, 69, 70] Osteosarcoma is also the most common primary skeletal neoplasm of people. Human osteosarcoma is primarily a tumor of adolescents, with 800-1500 cases reported annually.[72-75] The dog and human neoplasms share numerous biological and clinical similarities, making canine osteosarcoma an excellent natural research model for the human disease.[2, 76, 77]

Canine osteosarcoma most frequently occurs in large- and giant-breed sized dogs, most commonly in the appendicular skeleton. Reportedly <5% of cases of appendicular osteosarcoma arise in dogs smaller than 15kg.[2, 69] Small breed dogs may be more likely to develop axial tumors than large breed dogs.[78] Breeds with the highest risk for tumor development are the Saint Bernard, Irish Setter, Great Dane, Doberman Pinscher, Rottweiler, German Shepherd, Golden Retriever, Afghan Hound, and Irish

Wolfhound.[69, 79, 80] Male dogs may be slightly more predisposed to developing appendicular osteosarcoma than female dogs.[2, 69, 76] However, female dogs have a two-fold higher risk for acquiring axial osteosarcoma relative to males.[81] There is contradictory evidence concerning the patient's sexual status and risk of tumor development. Ru *et al.* observed that intact male dogs had a 2-fold higher risk relative to castrated dogs.[80] Conversely, a lifelong observational study of 683 Rottweiler dogs found an approximately 3-fold increased risk for developing osteosarcoma in dogs gonadectomized at < 1 year of age.[82] The tumor exhibits a bimodal distribution for age of presentation, with cases clustering in dogs aged 18-24 months and a principal peak in dogs aged 7-9 years.[2, 69, 70]

In approximately 65-75% of canine osteosarcoma cases the primary tumor is located in the appendicular skeleton.[1, 69, 83, 84] Lesions occur twice as frequently in the forelimbs relative to the hindlimbs.[2, 83] Osteosarcoma typically arises in the metaphyseal regions of the long bones, with the most common sites being: distal radius, proximal humerus, distal femur, proximal tibia, and distal tibia (**Figure 2.3.1**).[2, 69, 83, 84] As for axial skeletal tumors, nearly 70% arise in the mandibular, maxillary, nasal, and cranium flat bones of the skull.[78, 81, 85] Tumors of the spine, ribs, and pelvis account for 15%, 10%, and 5% of axial osteosarcoma, respectively.[81] Extraosseous primary osteosarcoma is rarely reported, but documented cases include tumors of the os penis, spleen, small intestine, kidney, testicle, vagina, eye, meninges, lung, and adrenal gland.[69] Tumors arising from surgical items, such as gauze or metallic implants, are also reported.[86] At presentation, between 5-10% of dogs have radiographically detectable pulmonary metastases.[2] However, the majority of dogs likely have micrometastatic pulmonary involvement at the time of initial diagnosis.[1, 2, 69, 76] Bony metastases and regional lymph node metastases are reported in up to 8-10% and 4-9% of dogs, respectively.[2] Clinical signs at presentation usually reflect pathology from the primary tumor, with single limb lameness being the most common complaint for appendicular osteosarcoma.[1, 2, 69, 70] Painful bony masses are typically identified on physical examination.[69] Dogs rarely initially present with signs referable to metastatic lesions, like neurologic or respiratory disease.[69]

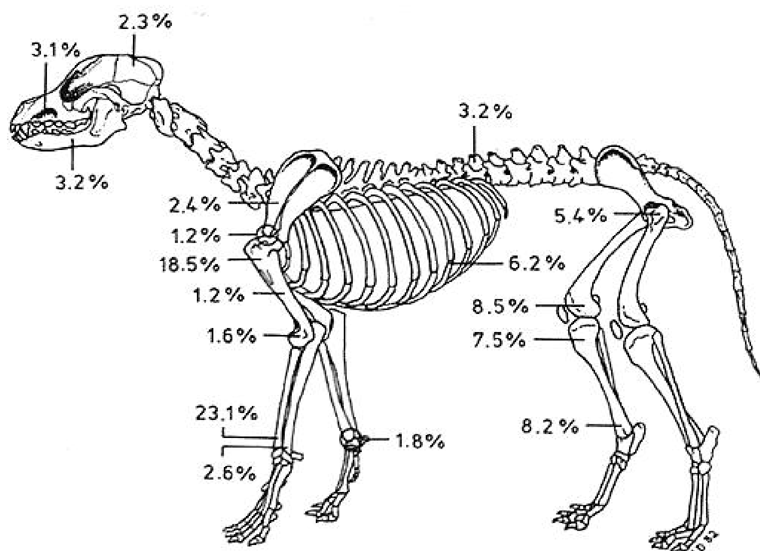

Figure 2.3.1. Distribution percentage of primary canine osteosarcoma lesions by anatomic location. Appendicular osteosarcoma accounts for 65-75% cases of canine osteosarcoma, with axial tumors comprising most remaining cases. Figure adapted from Kistler *et al.*[84]

The hallmark histologic feature of osteosarcoma is production of osteoid matrix by the malignant mesenchymal population.[69] Tumors are frequently histologically dynamic and produce other matrix variants, such as chondroid or collagen.[87] The neoplasm is often characterized by extensive destruction of medullary and cortical bone along with adjacent soft tissue structures.[1] Normal parenchyma is replaced by bundles of neoplastic cells associated with randomly arranged matrix.[1, 87] Multiple histologic subtypes of osteosarcoma are defined. One mode of histologic classification is based upon the predominant type of matrix present – the three categories include: osteoblastic, chondroblastic, and fibroblastic osteosarcoma.[1, 87] Osteoblastic osteosarcoma may be further subdivided into productive (abundant osteoid formation) or nonproductive (minimal osteoid formation) subtypes.[1] Other subtypes include: telangiectatic osteosarcoma (minimal osteoid production with abundant intratumoral vascular spaces), giant cell type osteosarcoma (numerous neoplastic multinucleate giant cells), and poorly differentiated osteosarcoma (anaplastic appearance to neoplastic spindle cells with minimal osteoid production). Osteoblastic osteosarcoma is the most common histologic subtype, accounting for approximately 70% of cases (**Table 2.3.1**).[88] A three-tiered histologic grading system was introduced in 1996 by Straw *et al.* which grades tumors by the degree of nuclear pleomorphism, mitotic index, and degree of tumor necrosis (**Table 2.3.2**).[88, 89] An updated histologic grading system was presented by Kirpensteijn *et al.* in 2002 (**Table 2.3.3**).[90] Most appendicular and axial osteosarcomas are Straw Grade II or III (72% of cases), with a greater proportion of high grade tumors present in appendicular sites than

axial sites.[88] Both histologic subtype and tumor grade are associated with clinical behavior and prognosis (discussed below).[1, 2, 69, 88]

Osteosarcoma Histologic Subtype	Number of cases (n = 132)
Osteoblastic productive	73
Osteoblastic minimally productive	18
Chondroblastic	17
Telangiectatic	9
Fibroblastic	7
Giant cell rich	3
Multipatterned	3
Poorly differentiated	2

Table 2.3.1. Distribution of histologic subtypes in 132 primary osteosarcoma canine tumors. Productive osteoblastic osteosarcoma is the most common histologic variant. Table from Loukopoulos *et al.*[88]

Osteosarcoma histologic scoring system from Straw *et al.*		
Parameter	**Description**	**Value**
Nuclear pleomorphism	None	0
	Mild	1
	Moderate	2
	Marked	3
Mitotic index (number of mitoses in 10 fields at x400)	1 – 10	1
	11 – 20	2
	21 – 30	3
	>30	4
Degress of necrosis (% volume)	None	0
	<15%	1
	15 – 50%	2
	>50%	3

Osteosarcoma grading system from Straw *et al.* (Cumulative score from histologic scoring system)	
Histologic score	Grade
1 – 5	I
6 – 7	II
8 - 10	III

Table 2.3.2. Histologic scoring and histologic grading system for primary appendicular and axial canine osteosarcoma tumors presented by by Straw *et al.* Tumors are scored on the degree of nuclear pleomorphism, mitotic index, and degree of necrosis. The cumulative score is used to establish tumor grade. Tables adapted from Loukopoulos *et al.* and Straw *et al.*[88, 89]

Osteosarcoma histologic grading system presented by Kirpensteijn *et al.*

Tumor grade	Nuclear pleomorphism	Mitotic index	Tumor matrix (% volume)	Tumor cells (% volume)	Necrosis (% volume)
I	0 – 1 (<25%)	<10	1 (>50%)	1 (<25%)	0-1 (<25%)
II	2 (25 - 50%)	10-20	2 (25-50%)	2 (25-50%)	2 (25-50%)
III	3 – 4 (> 50%)	>20	3 (<25%)	3-4 (>50%)	3-4 (>50%)

Table 2.3.3. Histologic grading system for canine osteosarcoma presented by Kirpensteijn *et al.* Tumor grade is determined by degree of nuclear pleomorphism, mitotic figure, percentage of tumor occupied by matrix, percentage of tumor occupied by cells, and percentage of tumor necrosis. Additionally, if neoplastic cells are present within vasculature tumors are qualified as grade III. Table adapted from Kirpensteijn *et al.*[90]

As previously suggested, canine osteosarcoma has a relatively rapid course of disease progression. Primary tumors eventually cause significant regional osteolysis with secondary periosteal proliferation. These changes substantially disrupt the normal load-bearing architecture of cortical bone in the appendicular skeleton, predisposing to pathologic fractures.[69] There is a high propensity for development of pulmonary metastases, with 72.5-100% of dogs having pulmonary involvement at necropsy.[1] Axial osteosarcoma has a lower pulmonary metastases rate of 35-46%.[78] Cancer cells are suspected to disseminate to the lungs through a hematogenous route.[69] The molecular mechanisms which promote preferential tumor localization to pulmonary parenchyma over other tissue types are only partially characterized. Overexpression of cytoskeletal regulatory proteins, including Cdc42-interacting protein 4 and centromere protein A, and chemotaxis receptors, like CXCR4, appear to confer a pro-metastatic phenotype in tumor cells.[91-93] The second most common site for metastatic lesions is other bones, with lesions observed in vertebral bodies, vertebral processes, ribs, pelvis, and sternum.[1] Spread to regional lymph nodes is rare, occurring in 4-9% of dogs.[2] Natural death or euthanasia occurs secondary to pulmonary dysfunction in nearly 75% of cases. The median survival time (MST) for untreated dogs, or dogs treated with primary tumor excision alone, is just 133 days for appendicular osteosarcoma.[1] Untreated or surgically treated axial osteosarcoma has a similar MST of 154 days, although mandibular osteosarcoma appears to have a better MST (525 days with surgery alone).[81, 94]

Osteosarcoma should be clinically suspected for large breed dogs presenting for the complaint of acute onset limb lameness. Following lesion localization, radiographs are advisable and may provide strong supporting evidence for osteosarcoma. Classic radiographic changes include focal cortical osteolysis associated with periosteal proliferation and swelling of adjacent soft tissue structures.[69] The relative severity of these changes can be variable between cases.[69] For preparation of limb sparing procedures, advanced imaging modalities, such as computed tomography (CT) or magnetic resonance imaging (MRI), may help with surgical planning. Wallach *et al.* found that MRI was superior to CT or digital radiography for characterizing the extent of medullary involvement.[95] However, Davis *et al.* found no difference between these three imaging modalities for identifying the extent of tumor invasiveness.[96] Nuclear scintigraphy can overestimate region of neoplastic involvement, and thus lead to accidental resection of non-diseased bone.[95, 97] Thoracic radiography is warranted to characterize tumor stage, although metastatic lesions must be >7mm in diameter to be detected.[98] Thoracic CT is superior to digital radiography for detecting pulmonary lesions, with the capacity to identify tumors of 1mm in diameter.[98] Survey radiographs to identify metastatic bone lesions may be pursued, but nuclear scintigraphy is diagnostically superior.[69]

A tentative diagnosis of osteosarcoma may be reached with cytologic evaluation of affected tissue, but definitive diagnosis requires lesion histology. The diagnostic yield of both needle aspirate samples and core excisional biopsies can be increased with ultrasonographic, radiographic, or fluoroscopic guidance.[2, 69] Cytology samples acquired by a fine-needle aspiration technique have an 85% concordance with histology for diagnosis osteosarcoma.[99] Concordance with the histologic diagnosis can be improved to 95% using a core aspirate technique.[99] Application of an alkaline phosphatase substrate to cytology preparations is useful in differentiating osteosarcoma from other primary or metastatic bone sarcomas. Barger *et al.* observed that alkaline phosphatase positivity was 100% sensitive and 89% specific for osteosarcoma; Neihaus *et al.* observed 100% sensitivity and 100% specificity.[99, 100] Three techniques have been described for bone biopsy. An open surgical approach yields the largest amount of tissue for histology but is associated with many postsurgical complications, such as local tumor seeding and pathologic fracture.[69] Samples acquired through trephination have a 94% diagnostic accuracy, but the procedure is also associated with pathologic fracture development.[69] The technique associated with the least frequency of complications is Jamshidi needle biopsy.[69, 101] Jamshidi needle biopsy has a diagnostic accuracy of 92% for neoplastic bone lesions and provided enough tissue for histologic subclassification

in 62% of the osteosarcoma cases.[101] If surgically excised, the primary tumor should always be submitted for histologic assessment to confirm the cytology or biopsy results.

Treatment of canine osteosarcoma may be approached from either curative- or palliative-intent perspectives. The staple of any curative or palliative approach is surgical excision of the primary tumor. In appendicular osteosarcoma, this excision is usually achieved by limb amputation.[2, 69] As an alternative to limb amputation, numerous surgical limb-sparing procedures have been developed. In these limb sparing procedures, sections of diseased long bone are replaced with frozen cortical allograft, endoprosthesis, normal bone autograft, or sterilized diseased bone autograft.[2, 69] These limbs are then stabilized using internal or external bone fixation devices. Both procedures serve to remove the primary tumor focus and to alleviate tumor-associated pain. Limb sparing procedures do not confer a survival advantage over amputation procedures.[2] With the exception of mandibular osteosarcoma, excision of the primary tumor alone also does not appear to improve survival; most studies report MST of 103-175 days with stand-alone surgery.[2, 89]

Adjunct chemotherapy following primary tumor excision improves survival in canine osteosarcoma. A number of chemotherapy protocols have been investigated, the majority using a combination of anthracycline and platinum-based agents.[2, 69, 102] Single or multiple agent protocols using cisplatin, carboplatin, lobaplatin, and doxorubicin confer MSTs between 290-450 days for appendicular and axial osteosarcoma.[2, 94, 102] Recently, Selmic *et al.* compared five different single- and multi-drug protocols and found no difference in MST or disease free interval between the protocols.[102] For dogs with mandibular osteosarcoma undergoing hemimandibulectomy, adjunct chemotherapy nearly doubles the MST from 525 days to 1023 days.[94] Chemotherapy appears less efficacious when pulmonary metastases are radiographically detectable at presentation.[2] Aminobisphosphonates therapy to inhibit osteoclastic bone resorption have become more recently utilized. Aminobisphosphonate therapy does not improve survival and there is equivocal evidence for reduction in bone pain.[69, 103] As such, they may be used palliatively when primary tumor excision cannot be pursued.

Radiotherapy is another therapeutic modality available for canine osteosarcoma. Radiotherapy is typically approached from a palliative perspective for cases where the primary tumor cannot be surgically removed. In these cases, the main therapeutic benefit is marked alleviation of tumor pain.[2, 69, 104] Near complete resolution of bone pain occurs in 50-93% of treated dogs, although the pain-free

duration is quite variable (53 days to 130 days).[2, 69] Most published palliative-intent protocols use 2-4 fractional treatments of 6-10 Gy with cumulative doses <32 Gy.[69, 104] Few studies investigating curative intent radiotherapy for the primary long bone tumor have been reported.[104] In a study of 14 dogs receiving a curative protocol (nineteen 3 Gy fractions for cumulative 57 Gy dose) with or without adjunct chemotherapy, Walter *et al.* reported a MST of 209 days.[105] Oblak *et al.* reported a similar MST of 178 days and also observed that adjunct chemotherapy increased MST to 307 days.[106] Curiously, concurrent utilization of aminobisphosphonates with radiotherapy dramatically reduces survival.[106] Although no studies have directly compared standard radiotherapy alone to surgery alone, there appears to be minimal difference in reported MST.

More advanced variants of radiotherapy appear to yield better survival times in dogs with appendicular osteosarcoma. Intra-operative extracorporeal radiation therapy is a technique coupled to limb sparing procedures. In this protocol, the diseased aspects of exposed bone are subjected to focused, extremely high levels of radiation (70 Gy).[104] This level of radiation is tumoricidal to the malignant osteoblasts, but mostly spares normal bone, joint, and limb tissues due to the inherent resistance of these tissues to radiation induced necrosis and the focused delivery of radiation. The reported MST for this procedure is 298 days.[104] Non-invasive stereotactic radiosurgery, in which a computer-guided focused radiation beam is precisely delivered to the primary tumor, has a reported MST of 363 days.[104]

Less frequently employed treatment strategies include radiopharmaceutical and immunomodulatory strategies. With the radiopharmaceutical approach, patients are administered radioactive isotypes with high affinity for bony tissue. Given the increased metabolic activity of malignant cells, these radioisotopes preferentially cluster in areas of the malignancy. The most investigated radioactive isotopes for treatment of canine osteosarcoma are samarium-153-lexidronam and radium 223 chloride.[2, 69, 104] Barnard *et al.* reported that samarium-153-lexidronam therapy is associated with minimal adverse side effects in dogs and significantly decrease bone-associated pain. [107] However, a MST of just 100 days was observed.[107] Few studies utilizing immunomodulatory agents have been reported. In a small cohort of dogs, serial administration of the immunostimulant L-MTP-PE after amputation yielded a MST of 222 days versus a MST of 77 days for dogs treated with amputation alone.[108] Recently, an anti-cancer vaccine utilizing *Listeria monocytogenes* modified to express the osteosarcoma tumor antigen Her2 prevented development of lung metastases in 5 out of 6 dogs at one year post-amputation.[109, 110]

A number of clinical, clinicopathologic, histologic, and genetic factors with prognostic potential have been identified in canine osteosarcoma. Both increased age and body mass are negatively correlated with survival.[2-4, 111] Dogs with humeral tumors have a shorter MST relative to other appendicular lesions.[3, 4] For flat bone tumors, dogs with scapular tumors have a 2.8-fold increased hazard ratio for death relative to dogs with osteosarcoma in other flat bones.[85] Additionally, mandibular osteosarcoma has the highest reported MSTs for any anatomic location.[78, 94] Tumor size may also correlate with prognosis, as large primary tumors have a higher incidence of pulmonary metastases at presentation.[4, 112] The presence of pulmonary or lymphatic metastases at presentation is a negative prognostic indicator.[4, 113] The only clinicopathologic abnormalities routinely found to have prognostic significance are elevated total- and bone-fraction serum alkaline phosphatase (ALP) activity.[3, 4, 114, 115] Garzotoo *et al.* found dogs with normal total- and bone-fraction serum ALP activity have MST of 12.5 months and 16.6 months, respectively.[114] In contrast, dogs with elevated total- and bone-fraction serum ALP activity have survival times of just 5.5 months and 9.5 months.[114] A failure of serum ALP activity to normalize following primary tumor excision is associated with a decreased response to chemotherapy and shorter survival time.[115] Histologically, a high mitotic index or high percentage of tumor necrosis are associated with higher tumor grade and decreased survival.[88, 90] High grade tumors, and tumors of the osteoblastic minimally productive subtype, are also negative prognostic features.[88, 90] Genetically, tumors having aberrant expression of p53, vascular endothelial growth factor, vascular endothelial growth factor receptor, mesenchymal-epithelial transition factor, cyclooxygenase 2, ezrin, matrix metalloproteinase-2, matrix metalloproteinase-9, HER2, and PTEN are associated with either increased risk for metastases or decreased survival time.[4, 75, 76] p53 mutations are present in 40-84% of canine osteosarcoma cases and dogs harboring this mutation have shorter survival times.[4, 76, 116] From a treatment perspective, incomplete surgical margins on surgically resected flat-bone tumors carry a worse prognosis.[2, 4, 117]

Few underlying etiologies contributing to the development of osteosarcoma in dogs have been elucidated. Muggenburg *et al*. evaluated the effects of plutonium radioisotope inhalation in a colony of beagles.[118] In the experiment, all dogs developed disseminated osteosarcoma within three years of exposure. There are many published reports of dogs developing appendicular osteosarcoma as a late-onset complication of prior radiotherapy.[2] These observations reflect numerous human studies linking radiation to bony neoplasia, supporting the notion that single high dose or chronic low dose radiation exposure contributes to tumorigenesis.[2, 76, 119] Chronic bone microtrauma or inflammation, such as that

arising from repeat fractures, unstable repaired fractures, metallic surgical implants, or osteomyelitis, may predispose tumor development.[1, 2, 69, 120, 121] Numerous cases detailing osteosarcoma arising at the site of tibial plateau leveling osteotomy are reported.[2, 69] However, Murphy *et al.* argue the occurrence of tumors at these sites may be secondary to surgically induced bone fracture rather than the implant devices.[120] Altered expression of tumor suppressors of p53, Rb, and PTEN are found in primary tumors and osteosarcoma cell lines from people and dogs.[2, 75, 76] Increased expression of select oncogenes, including hepatocyte growth factor, c-MET, insulin like growth factor 1, insulin like growth factor 1 receptor, c-MYC, and c-FOS, has been observed *in vitro* and *in vivo*.[76] Malignant canine osteoblasts may also upregulate expression of anti-apoptotic pathways *in vivo*.[122] As with other neoplasia, these genetic aberrations likely play a central role in malignant transformation of osteoblasts.

Canine and human juvenile osteosarcoma share many similarities with regards to tumor biology and behavior. Human osteosarcoma most frequently arises in metaphyseal sections of long bones, with males more predisposed to tumor development than females.[2, 76] Primary tumors are typically high grade. The clinical course of disease is nearly identical; <20% of people having detectable pulmonary metastases at diagnosis but nearly 90% have pulmonary involvement at death.[2, 76] Limb-sparing procedures with adjunct chemotherapy or radiotherapy are the preferred treatment modalities for people. Canine and human osteosarcoma share nearly identical negative prognostic indicators.[76] Additionally, many of the same genetic irregularities first identified in malignant human osteoblasts have been observed in canine osteosarcoma.[2] A key difference between the two tumor types is the age of onset. The median age at diagnosis for people is 16 years, with cases rarely diagnosed in humans <10 years or >20 years of age.[2, 76] This contrasts with the bimodal age distribution of canine osteosarcoma, in which most affected dogs cluster in 7-9 years age range. Additionally, greater survival times are reached in people; the 5 year survival rate in people is 60% overall.[2, 76] This increased survival rate likely reflects more aggressive chemotherapeutic protocols used in human medicine.[2, 76] Similarities between canine and human osteosarcoma are summarized in **Appendix Table A.2**.[2]

2.4 The serotonergic signaling system in osteosarcoma

Given the major regulatory role of serotonin in bone physiology, the serotonergic system may have significance for osteosarcoma tumorigenesis or treatment. Surprisingly, very few published studies have explored the relationship between 5HT and this bony neoplasm. Increased expression of LRP5 in a human osteosarcoma cell line promotes a more aggressive, pro-metastatic phenotype in a murine

xenograft model.[123] Additionally, LRP5 is a biomarker for high grade osteosarcoma for people *in vivo* and predicts development of pulmonary metastases.[124] Guo *et al.* showed that LRP5 signaling is important for mesenchymal-to-epithelial transition, and thus facilitates neoplastic cell dissemination beyond the primary tumor.[125] Expression of 5HTT in human osteosarcoma cell lines is suspected, as incubation with the SSRIs sertraline or paroxetine attenuates viability and promotes apoptosis in malignant osteoblasts.[17, 18] To our knowledge, there currently are no published reports describing the expression of any 5HTR in human or canine osteosarcoma cells. Increased expression of serotonergic system components have been documented in other neoplasms. For example, increased 5HTR expression in human prostatic adenocarcinoma contributes to neoplastic cell proliferation.[126] Overall, the serotonergic system remains a relatively unexplored aspect of tumor biology.

3. EXPERIMENTAL DESIGN AND METHODOLOGY

3.1 Experimental design

To assess 5HTR1B and 5HTR2A expression, function, and therapeutic potential of receptor drug targeting in canine osteosarcoma we designed an *in vitro* study utilizing a canine osteosarcoma cell line and primary normal canine osteoblasts. We examined receptor gene transcriptionof malignant and normal osteoblasts through quantitative real-time reverse transcription PCR (qRT-PCR) and protein expression by immunoblot. Looking for possible genomic mutations, we then sequenced the osteosarcoma cells *htr1b* and *htr2a* genes. Osteoblasts were pharmacologically challenged with the following drugs: serotonin, anpirtoline (5HTR1B agonist), SB224289 (5HTR1B antagonist), and ritanserin (5HTR2A antagonist). Challenge with a 5HTR2A agonist was not performed, as the prototypical, highly selective 5HTR2A agonist DOI is a controlled pharmaceutical and our laboratory lacks the proper controlled drug licensure. We then examined effect of drug challenge on 1) the viability of malignant and normal osteoblasts and 2) the phosphorylation statuses of CREB and ERK. Additinoally, we evaluated for malignant cells the effect of drug incubation on caspase activity, a proxy for apoptosis.

3.2 Methodology

Product manufacturer is denoted by alphabetic superscript. Manufacturer information is presented in **Appendix Table A.3.**

Cell Lines

The previously characterized canine osteosarcoma cell line, COS, and undifferentiated, non-malignant canine osteoblast primary cells, CnOb[A], were utilized in our study.[127, 128] Additionally, the human mammary carcinoma cell line MCF7 and immortalized rat suprachiasmatic nuclei cell line SCN2.2 were used to provide protein immunoblot controls.[129, 130] COS, MCF7, and SCN2.2 were maintained in light-treated, serum-free, RPMI 1640[B] medium supplemented with sodium pyruvate, HEPES buffer, penicillin, streptomycin, and L-glutamine. CnOb was cultured in light-treated, proprietary medium provided by the manufacturer. All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Gene Sequencing

To sequence *htr1b* in COS, genomic DNA was extracted from confluent 10.0 cm cell plates with the QiaAmp DNA Mini Kit[C] according to the manufacturer's protocol. A cDNA template was used for sequencing of COS *htr2a* (see *Receptor Expression and Quantification* section for mRNA extraction and cDNA conversion protocols). Standard PCR was performed on the purified DNA utilizing the following conditions in a 25 µL reaction: 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.1U Platinum Taq DNA Polymerase[B], 1× PCR buffer[B], 0.4µM primer mix, and 1 µL purified DNA. The thermocycling profile was 1) 94°C for 2 min, 2) 40 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, and 3) 72°C for 2 min. Reactions were performed for each of four different primer pairs for *htr1b* and three primer pairs for *htr2a* (**Table 3.2.1**). Amplification products were visualized through electrophoresis on 1.5% agarose gel containing SYBR Safe[B]. Amplicon bands of predicted size were purified from the gel with the QIAquick Gel Extraction Kit[C]. Sequencing was conducted by the Center for Genome Research and Biocomputing (Oregon State University, Corvallis, OR, USA). COS *htr1b* sequence was compared to reference canine genomic and mRNA *htr1b* sequences, National Center of Biotechnology Information (NCBI) accessions AY204572.1 (genomic DNA), AY323909.1 (genomic DNA), and NM_001006948.1 (mRNA). COS *htr2a* sequence was compared to the reference canine genomic and mRNA *htr2a* sequences, NCBI accessions NC_006604.3 (genomic DNA) and NM_001005869.1 (mRNA).

Receptor Expression and Quantification

To quantitatively assess *htr1b* and *htr2a* transcription in COS and CnOb, confluent cells from 10.0 cm plates were collected in 1mL TRIzol®[B] and RNA was extracted according to the manufacturer's directions with an additional glycogen reprecipitation step to increase purity. RNA quantity and quality were evaluated by spectrophotometry using the Nanodrop-1000 Spectrophotometer[D].[131] One microgram of RNA was converted to cDNA with the High Capacity RNA-to-cDNA™ Kit[B] according to the manufacturer's protocol. Real-time quantitative reverse transcription PCR (qRT-PCR) was performed in triplicate using Power SYBR® Green Master Mix[B] in a MicroAmp® Optical 96-well reaction plate[B] with the Step One Plus qRT-PCR System[B]. Reaction volume was 10 µL, containing: 2.5µL cDNA previously diluted 1:6 with H_2O, 0.8 µL 100µM stock primer mix, 1.7 µL H_2O, and 5.0 µL Power SYBR®. The thermocycling profile was 1) 95°C for 10 min, 2) 40 cycles of 95°C for 5 sec, 3) 59°C for 30 sec, and 4) 70°C for 35 sec. Relative *htr1b* and *htr2a* expression in COS was determined with the $2^{-\Delta\Delta CT}$ method in relation to endogenous expression of the maintenance gene *ywhaz* (NCBI accession XM003639646) and CnOb.[132] Presence of a single amplification product in each reaction was confirmed by observing a single melt curve peak at the correct temperature for each primer pair (**Table 3.2.1**). The experiment was performed in triplicate.

Primer	Sequence (5'-3')	Location relative to start codon
htr1b sequencing primers		
F1	CCCTTGACCTCTCTGGGTTC	-102
R2	TCGGAGACCCGCACTTTGAC	884
F3	CCTGGAAAGTGCTCCTGG	137
R4	TCGGAGACCCGCACTTTGAC	884
F5	GAAACAGACGCCCAACAGGA	717
R6	CTACCCCATTGCAACTGACAA	1172
F7	GCTTTAAGTGCGCAGGTTGA	1151
R8	CCCCAAACACAGGGTATC	1622
htr2a sequencing primers		
F1	GCTTCCGTGTGACAGAGACA	-103
R2	CCCAGCAGCATATCAGCTA	529
F3	CGGTCGTGATTATTCTGACCA	245
R4	CCCTGTGGATTGATCGTTG	1062
F5	GGGCTACAGGATGATTCCAA	640
R6	CCCCCCAGATAGGTGAAAA	1475
htr1b qRT PCR primers		
F	ACCTGCTCGTCTCCATCCT	
R	TCCGACGACAGCCACAAGTC	
htr2a qRT PCR primers		
F	CCCATTCTTCATCACGAACAT	

R	GGAGAGGTAACCGATCCAGAC
ywhaz qRT PCR primers	
F	AGCCTGCTCTCTTGCAAAGAC
R	GGGTATCCGATGTCCACAATG

Table 3.2.1. Nucleotide sequences for sequencing primers and qRT PCR primers for *htr1b, htr2a, and ywhaz.*

For detection of 5HTR1B and 5HTR2A by immunoblot in COS and CnOb, confluent cells in 10cm plates were washed with phosphate buffered saline (PBS) and incubated for 10 min in 500 µL RIPA Lysis Buffer[E] with Protease Inhibitor Cocktail[D] and Halt Phosphatase Inhibitor Cocktail[D]. As a positive control for 5HTR1B, protein lysate from confluent SCN2.2 cells was similarly collected.[133, 134] Protein lysate from MCF7 was used as a 5HTR2A positive control.[135] RIPA solutions were centrifuged at 8,300 × g at 4°C for 15 min and lysate collected. Protein concentration was determined with the Pierce® BCA Protein Assay[D]. Lysate volumes containing 20 µg were mixed at 4:1 ratio with a solution of 10 mM Tris-HCL buffer (pH 6.8) containing 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol, and 10% glycerol. Samples were denatured by heating for 5 min at 100°C. Ten microliters of Odyssey™ Protein Molecular Weight Marker[F] were used as a protein ladder. Samples and ladder were electrophoresed through a SDS-PAGE gel at a constant current of 10 mA and transferred to a nitrocellulose membrane. The membrane was blocked with 1% bovine-serum albumin (BSA) dissolved in tris-buffered saline with Tween 20 (TBST) [150 mM NaCl, 20 mM Tris-HCl titrated with HCl to pH 74, and 0.1% Tween-20] for 1 hour and then incubated overnight at 4°C with primary goat polyclonal anti-5HTR1B IgG (ab52025[G]) diluted 1:200 or polyclonal anti-5HTR2A IgG (sc-32538[H]) diluted 1:200. Primary antibody was detected with secondary donkey anti-goat IgG conjugated with IR800 (926-32214[F]) diluted 1:5000. The membrane was subsequently stripped with Restore™ PLUS Western Blot Stripping Buffer[D] and incubated with primary rabbit polyclonal anti-α tubulin IgG (sc-12462-R[H]) diluted 1:1000 and secondary goat anti-rabbit IgG conjugated with IR800 (926-32211[F]) diluted 1:5000. Membranes were imaged in an Odyssey Imaging System[F].

Cell Viability

To assess the effect of HTR1B activation, 5HTR1B blockade, and 5HTR2A blockade on osteoblast viability, proliferation experiments were performed on COS and CnOb utilizing the CellTiter 96® Aqueous One Solution MTS assay[I]. Experiments were performed in three biological replicates, with three technical replicates per experiment. Cells were seeded in 96-well culture plates at a density of 20,000 cells per well and incubated in serum-free media for 24 hours. Thereafter the cells were incubated for 24 hours

with fresh media containing the following drugs [concentrations]: serotonin[J] [3.125, 6.25, 12.5, 25, and 50 µM], the 5HTR1B selective agonist anpirtoline[J] [3.125, 6.25, 12.5, 25, and 50 µM], the 5HTR1B selective antagonist SB224289[J] [3.125, 6.25, 12.5, 25, and 50 µM, and the 5HTR2A selective antagonist ritanserin[J] [SB224289 [3.125, 6.25, 12.5, 25, and 50 µM]. Additionally, COS was challenged with anpirtoline [3.125, 6.25, 12.5, 25, and 50 µM] and serotonin [12.5 µM], SB2224289 [3.125, 6.25, 12.5, 25, and 50 µM] and serotonin [12.5 µM], and ritanserin [3.125, 6.25, 12.5, 25, and 50 µM] and serotonin [12.5 µM]. Drugs were dissolved in DMSO; the final concentration of DMSO on cells was 0.1%. For a vehicle control, cells were incubated with 0.1% DMSO in media. The MTS assay was performed according to manufacturer's protocol and absorbance measured at 490nm on the microplate reader Multiskan Go[D]. Background absorbance, from media without cells, was subtracted from each sample absorbance and the viability index was then determined by the formula: (mean absorbance of treated cells) / (mean absorbance of control cells) × 100.

Apoptosis Assay

To determine if apoptosis was induced by pharmacologic manipulation of 5HTR1B and 5HTR2A in malignant cells, caspase-3/7 activity was quantified in COS. Caspase activity was assessed by the Caspase-Glo® 3/7 luminogenic caspase 3/7 substrate assay (Promega). Cells were seeded in 96-well culture plates at a density of 10,000 cells/well with serum free RPMI media for 24 hours. The media was then replaced with fresh media containing: serotonin [3.125, 6.25, 12.5, 25, and 50 µM], anpirtoline [3.125, 6.25, 12.5, 25, and 50 µM], SB224289 [3.125, 6.25, 12.5, 25, and 50 µM], ritanserin [3.125, 6.25, 12.5, 25, and 50 µM], anpirtoline [3.125, 6.25, 12.5, 25, and 50 µM] and serotonin [12.5 µM], SB224289 [3.125, 6.25, 12.5, 25, and 50 µM] and serotonin [12.5 µM], or ritanserin [3.125, 6.25, 12.5, 25, and 50 µM] and serotonin [12.5 µM]. Additionally, cells were pre-incubated with the caspase inhibitor Z-VAD-FMK[L] [25 µM] for 60 min before being challenged with fresh media containing SB2242489 [6.25 µM] or ritanersin [12.5 µM]. For an apoptosis positive control, cells were incubated with taurolidine[K] [125 µM], an amino-acid derivative previously shown to cause apoptosis in COS.[136] Drugs were dissolved in DMSO, with cells exposed to a final vehicle concentration of 0.1%. For a vehicle control, cells were incubated with 0.1% DMSO in media. All experiments were performed in triplicate. Cells were challenged for 12 hours after which the caspase activity assay performed according to the manufacturer's protocol. Cumulative luminescence over 1 sec was measured using a GloMax° 96 Microplate Luminometer[I]. Relative caspase activity was calculated using the formula: relative caspase activity = (mean luminescence of treated cells) / (mean luminescence of vehicle control) × 100.

Receptor Signaling

To evaluate activation of the classical signaling systems associated 5HTR1B and 5HTR2A, the relative phosphorylation statuses of CREB and ERK following drug treatment were examined by immunoblot. Confluent COS and CnOb cells were incubated with serum-free media for 24 hours, which was replaced with fresh media containing the following: serotonin [3.125, 12.5, and 50 µM], anpirtoline [3.125, 12.5, and 50 µM], SB224289 [3.125, 6.25, and 25 µM], and ritanserin [3.125, 12.5, and 50 µM]. Drugs were dissolved in DMSO at a final vehicle concentration of 0.1%. Cells were also incubated with 0.1% DMSO in media as the vehicle control. Cells were challenged for 10 min; protein lysates were then collected and protein concentrations quantified. As a positive control for phosphorylated ERK (pERK), ERK, phosphorylated CREB (pCREB), and CREB, protein lysate from confluent MCF7 cells treated with 100 nM estrogen (Tocris) for 10 min was collected and pERK, ERK, pCREB, and CREB expression confirmed against A431 (Santa Cruz) and SK-N-MC (Cell Signaling, Beverly, MA USA) whole cell lysates.[137, 138] For some immunoblots A431 and SK-N-MC protein lysates were used for pERK/ERK and pCREB/CREB controls, respectively. For separation, 10 µg protein aliquots were prepared for SDS-PAGE, electrophoresed, and transferred to a PVDF membrane. Ten microliters of NOVEX® Sharp Pre-stained Protein Standard (Life Technologies Corporation) was used as a molecular weight standard. For pERK, membranes were incubated with primary mouse monoclonal anti-pERK IgG (SC-7383; Santa Cruz) followed by secondary HRP-conjugated goat anti-mouse IgG (SC-2005, Santa Cruz). The membrane was stripped and incubated with primary rabbit polyclonal anti-ERK IgG (SC-93; Santa Cruz) with secondary HRP-conjugated goat anti-rabbit IgG (SC-2004; Santa Cruz). The membrane was stripped again and incubated with primary rabbit polyclonal anti-α tubulin IgG with secondary HRP-conjugated goat anti-rabbit IgG. With an identical protocol, fresh membranes were probed for pCREB, with a primary rabbit monoclonal anti-pCREB IgG (9189; Cell Signaling, Beverly, MA USA), and CREB, with a primary rabbit polyclonal anti-CREB IgG (SC-186; Santa Cruz). All primary antibodies were diluted 1:5000 and secondary antibodies diluted 1:50,000. These antibodies have previously been validated for use in the dog.[139] Membranes were developed using the chemiluminescent substrate Amersham™ ECL Select™ (GE Healthcare, Uppsala, Sweden) with detection in the ImageQuant LAS 4000 (GE Healthcare).

Statistical Analysis

Statistical comparisons were performed with GraphPad Prism software[M]. Relative expression of *htr1b* between COS and CnOb as determined by qRT-PCR was evaluated using a two-tailed, unpaired T test with Welch's correction. An identical comparison was performed for relative expression of *htr2a*

between COS and CnOb. For evaluation of cell viability, one-way ANOVA with Dunnett's correction was used to assess the viability index of each concentration of serotonin, anpirtoline, SB224289, and ritanserin against the vehicle control. Additionally, one-way ANOVA with Sidak's correction was used to compare the viability index of each concentration of anpirtoline, SB224289, and ritanserin against the equivalent concentration of anpirtoline and serotonin, SB224289 and serotonin, and ritanserin and serotonin, respectively. The 50% inhibitor concentration of SB224289 and ritanserin was calculated using a non-linear regression of the log of the inhibitor versus a variable slope response equation, with constraints set at 100% for the upper and 0% for the lower baseline.[136] To examine caspase activity in COS, one-way ANOVA with Dunnett's correction was used to assess the relative caspase activity of each concentration of serotonin, anpirtoline, SB224289, and ritanserin against the vehicle control. One-way ANOVA with Sidak's correction was used to evaluate the relative caspase activity of each concentration of anpirtoline, SB224289, and ritanserin against the equivalent concentration of anpirtoline and serotonin, SB224289 and serotonin, and ritanserin and serotonin, respectively. A two-tailed, unpaired T test with Welch's correction was used to examine the relative caspase activity between SB224289 and ritanserin treated cells with and without pretreatment with a caspase inhibitor. Statistical significance was set at $p = 0.05$.

4. RESULTS

4.1 Evaluation of 5HTR1B

Expression of 5HTR1B in normal and malignant canine osteoblasts

Transcription of *htr1b* was observed in COS and CnOb, with no difference in relative transcript level present ($p = 0.15$) (**Figure 4.1.1**). No genomic sequence mutations were identified between COS *htr1b* and the reference sequences (**Appendix Figure A.1**). The 5HRT1B immunoblot identified an approximately 50 kDa band in lysates from COS, CnOb, and SCN2.2, consistent with canine 5HTR1B (predicted molecular weight 44-48 kDa) and documented rat 5HTR1B.[133, 134, 140] No difference in 5HTR1B signal intensity was observed between COS and CnOb (**Figure 4.1.1**). All statistical information in provided in **Appendix Table A.4**.

Effects of 5HTR1B agonism and antagonism on viability of normal and malignant canine osteoblasts

There was no change in viability observed in COS or CnOb when treated with any concentration of anpirtoline (**Figure 4.1.2**). Incubation of COS with the 5HTR1B antagonist SB224289 induced a >70% decreased viability at ≥ 6.25 µM ($p < 0.01$) (**Figure 4.1.2**). SB224289 treatment exhibited a biphasic effect on viability in CnOb. Concentrations ≤ 6.25 µM marginally increased viability and concentrations ≥ 12.5 µM decreased viability, with the maximal change (-53.3 ± 9.3%, $p < 0.01$) observed at 50.0 µM. Serotonin increased viability in COS at all tested concentrations. The greatest increases in viability are present between 3.125 – 12.5 µM serotonin, with 25.0 - 50.0 µM treatments yielding less change. Conversely, serotonin induced no change in viability in CnOb at 3.125 – 25.0 µM and caused a small decrease in viability at 50.0 µM. The inhibitory concentration 50 (IC50) of SB224289 for COS was 4.21 µM and could not be established for CnOb at the concentrations tested.

No additive or synergistic effect on viability was observed when COS was concurrently incubated with serotonin (12.5 µM) and escalating concentrations of anpirtoline (**Figure 4.1.2**). A mild protective effect was observed in COS treated at 6.25 µM SB224289 and 12.5 µM serotonin, but at no other concentration of SB224289 (**Figure 4.1.2**). All statistical information is summarized in **Appendix Table A.4**.

Effects of 5HTR1B agonism and antagonism on apoptosis in malignant canine osteoblasts

No change in caspase 3/7 activity was observed in COS treated with any concentration of serotonin (**Figure 4.1.3**). Marked elevations in caspase activity were observed in COS treated with any

concentration of SB224289, with the maximal increase present at 12.5 µM (572.7 ± 111.3%; $p < 0.01$). No attenuation of caspase activity was observed in cells concurrently incubated with 12.5 µM serotonin and any concentration of SB224289 (**Figure 4.1.3**). However, COS pre-incubated with the caspase inhibitor Z-VAD-FMK and then challenged with SB224289 had dramatically reduced caspase activity ($p < 0.01$) (**Figure 4.1.3**). Mild increases in caspase activity were also identified in cells incubated with 3.125, 25.0, and 50 µM anpirtoline ($p < 0.01$). When concurrently incubated with 12.5 µM serotonin, caspase activity returned to levels equivalent to the activity observed in vehicle-treated cells for all concentrations of anpirtoline (**Figure 4.1.3**). All statistical information in provided in **Appendix Table A.4**.

Effects of 5HTR1B agonism and antagonism on phosphorylation of CREB and ERK

COS endogenously expressed phosphorylated CREB and phosphorylated ERK while CnOb only expressed endogenous pERK. Negligible change in the level of pCREB expression was observed in malignant cells treated with any concentrations of serotonin, anpirtoline, or SB224289 (**Figure 4.1.4**). No drug treatment induced CREB expression or phosphorylation in CnOb. A marginal increase in pERK expression was observed in serotonin treated COS. Anpirtoline mildly decreased pERK expression at higher concentrations in malignant cells and SB224289 caused no change (**Figure 4.1.4**). In CnOb a mild reduction in pERK expression was observed with escalating doses of serotonin. Anpirtoline did not change the expression level of pERK; SB224289 treatment mildly enhanced pERK in the normal osteoblasts (**Figure 4.1.4**).

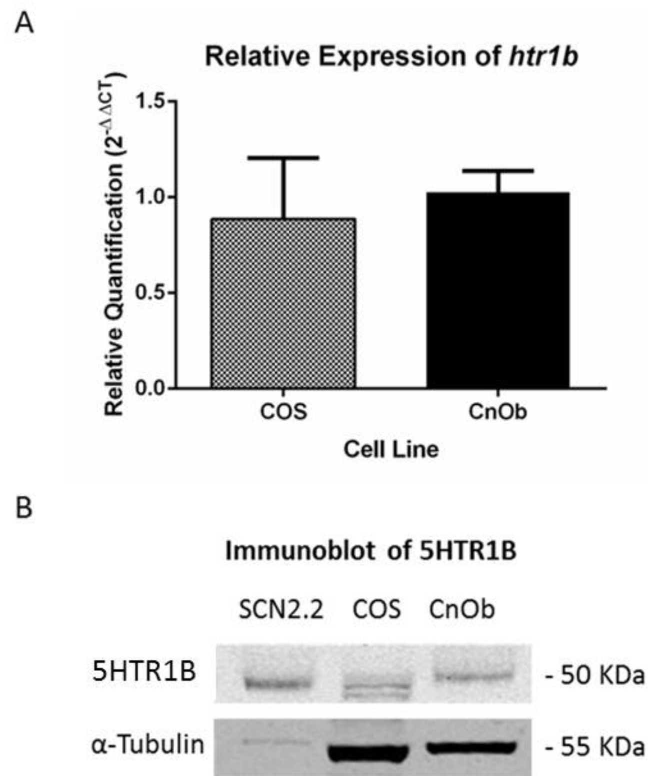

Figure 4.1.1. There is equivalent *htr1b* transcription and 5HTR1B expression between canine osteosarcoma cells, COS, and normal canine osteoblasts, CnOb. (A) *htr1b* mRNA levels in COS and CnOb normalized to CnOb expression, as assessed by qRT-PCR. Graphed bars are mean relative quantification ± 95% confidence interval of mean. (B) Immunoblot demonstrating expression of 5HTR1B in COS, CnOb, and the positive control SCN2.2, an immortalized rat suprachiasmatic nuclei cell line.

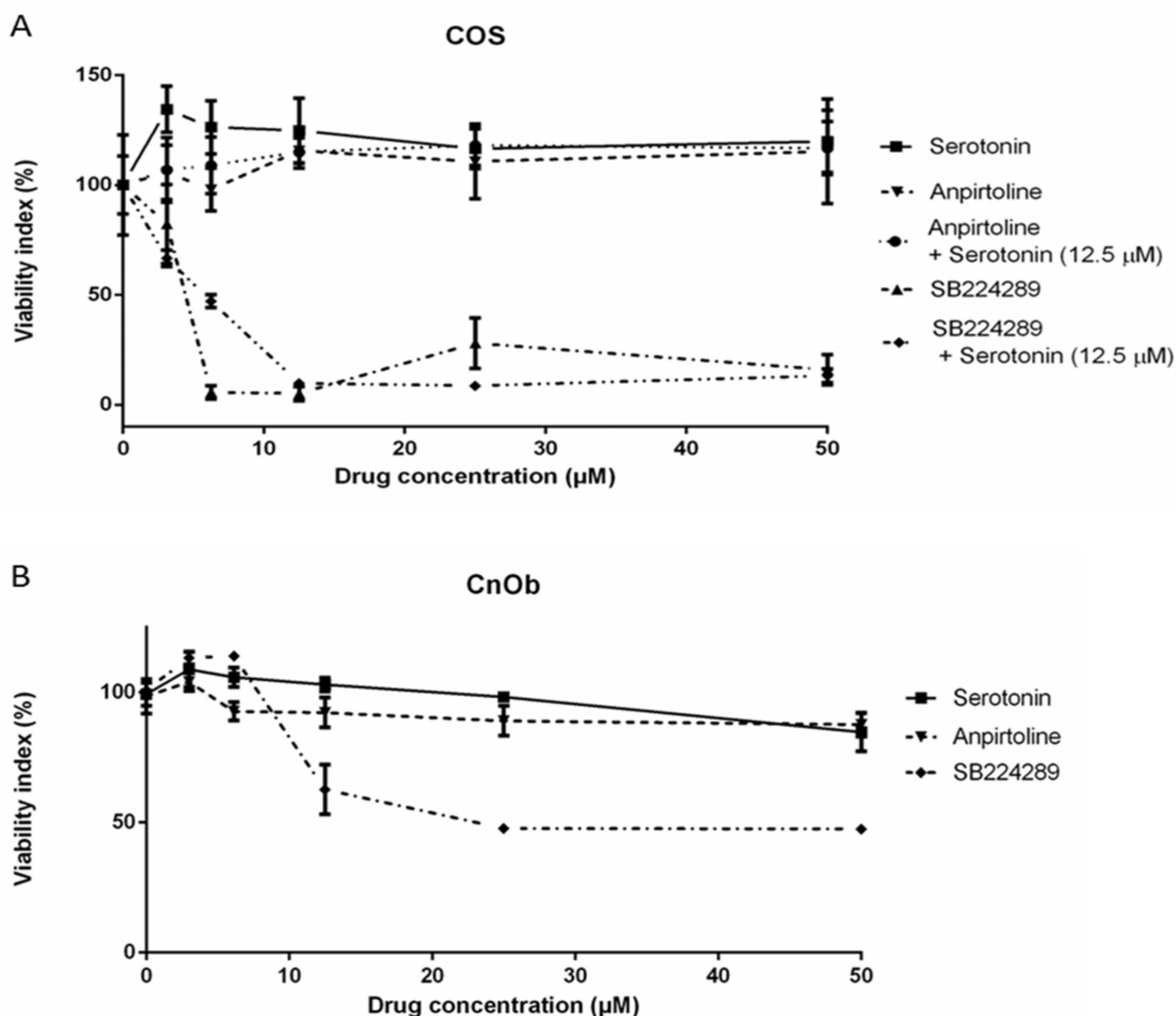

Figure 4.1.2. The 5HTR1B antagonist SB224289 decreases viability in malignant and normal osteoblasts while the receptor agonist anpirtoline has no effect on osteoblast viability. (A) Effects of serotonin, anpirtoline, SB224289, anpirtoline and 12.5 µM serotonin, and SB224289 and12.5 µM serotonin treatment on the viability of COS. SB224289 treatment decreases viability at all tested concentrations and concurrent incubation with 12.5 µM serotonin attenuates this decrease at 6.25 µM SB224289 only ($p < 0.01$). Anpirtoline in the presence or absence of serotonin does not influence COS viability. Serotonin enhanced viability at all concentrations tested ($p < 0.01$). Plotted values are mean viability index ± 95% confidence interval of mean. (B) Effects of serotonin, anpirtoline, and SB224289 treatment on the viability of CnOb. SB224289 treatment mildly enhances viability at 3.125 µM ($p = 0.01$) and 6.25 µM ($p < 0.01$) but decreases the viability index at ≥ 12.5 µM ($p < 0.01$). No change in viability is appreciated at any concentration of anpirtoline or serotonin, except for a mild decrease at 50.0 µM serotonin ($p = 0.01$). Plotted values are mean viability index ± 95% confidence interval of mean.

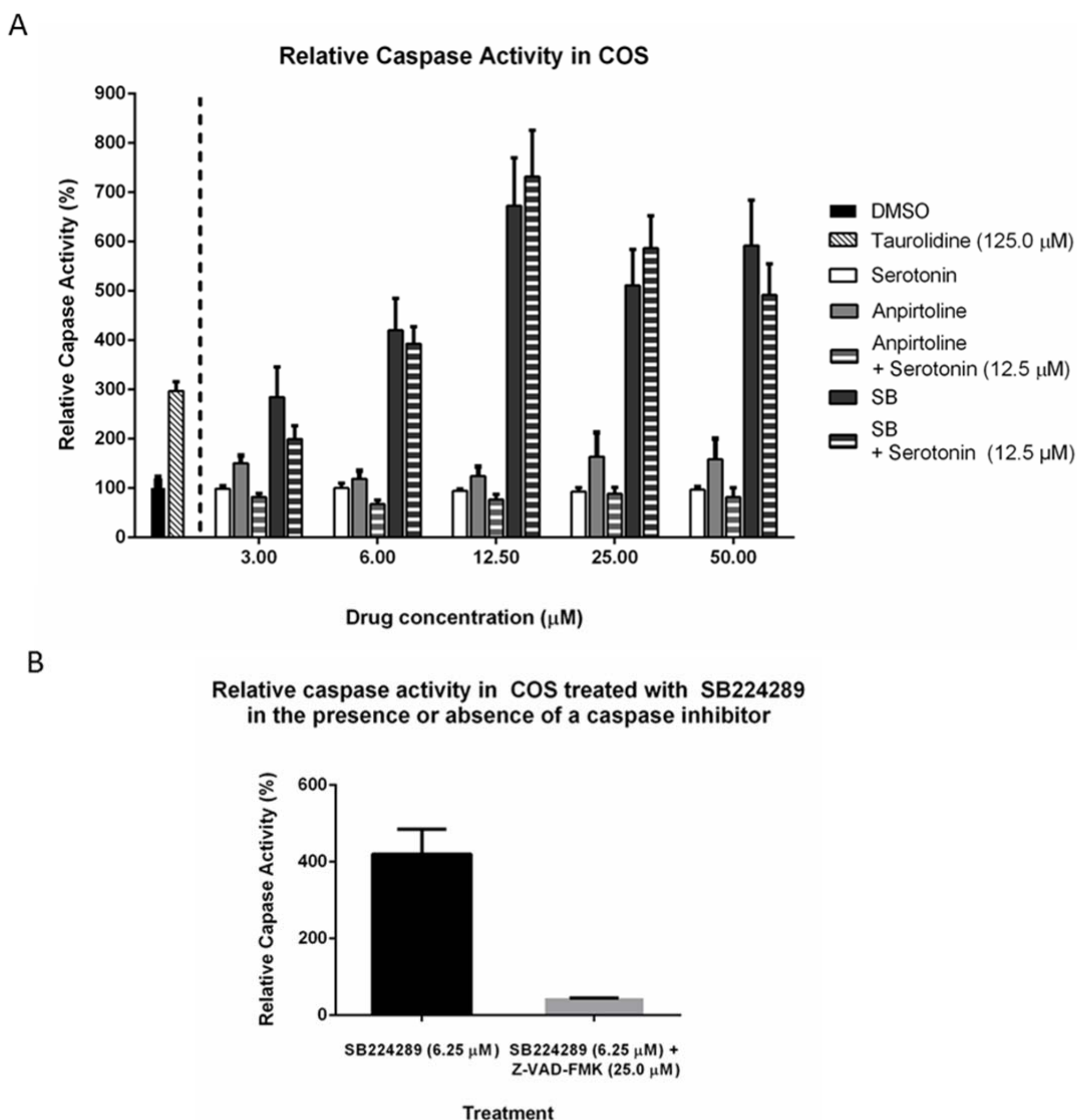

Figure 4.1.3. 5HTR1B antagonism promotes increased caspase-3/7 activity in osteosarcoma cells. (A) Relative caspase activity in COS following treatment with serotonin, anpirtoline, SB224289, anpirtoline and 12.5 μM serotonin, and SB224289 and12.5 μM serotonin. SB224289 increases caspase activity at all tested concentrations and concurrent treatment with serotonin does not mitigate this effect ($p < 0.01$). Mild increases in caspase activity are observed with 3.125, 25.0, and 50.0 μM anpirtoline ($p < 0.01$). However, concurrent incubation with serotonin normalizes caspase activity at all concentrations of anpirtoline. No change in caspase activity relative to DMSO-treated cells is observed at any concentration of serotonin. The positive control, taurolidine, increases caspase activity in COS ($p < 0.01$). (B) Relative caspase activity in COS cells treated with either 6.25 μM SB224289 or pre-incubated with the caspase inhibitor Z-VAD-FMK and then treated with 6.25 μM SB224289. Pretreatment with a caspase inhibitor prevents the SB224289 induced increase in caspase activity ($p < 0.01$). Plotted values are mean relative caspase activity ± 95% confidence interval of mean.

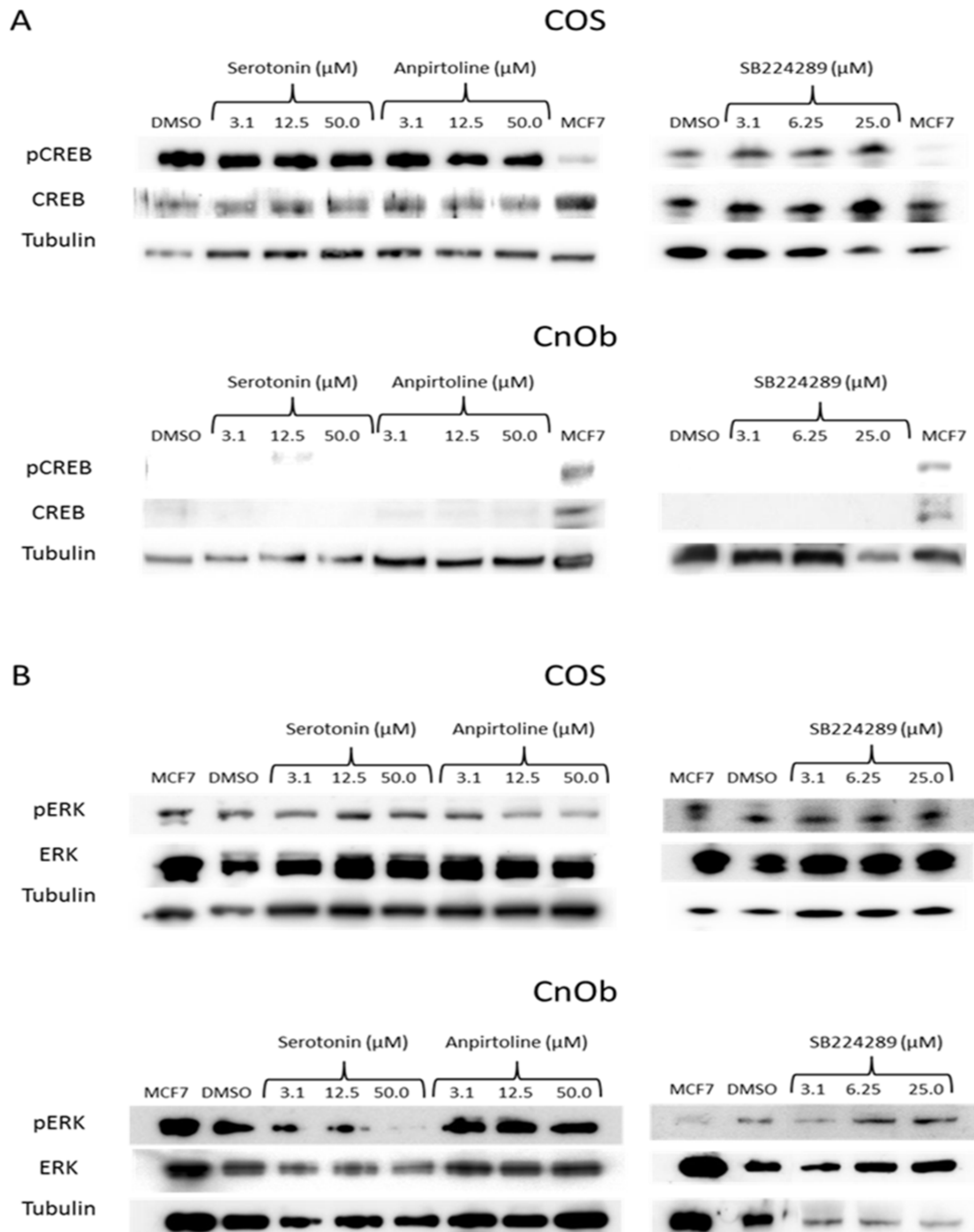

Figure 4.1.4. Discrepant patterns in CREB and ERK phosphorylation are observed between normal and malignant osteoblasts following treatment with serotonin, anpirtoline, and SB224289. (A) pCREB and CREB immunoblots from COS and CnOb following treatment with serotonin, anpirtoline, and SB224289. The level of CREB phosphorylation does not appreciably change with serotonin, anpirtoline, nor SB224289 treatment in COS. Endogenous CREB expression is not found in CnOb and was not induced with drug treatments. (B) pERK and ERK immunoblots from COS and CnOb following treatment with serotonin, anpirtoline, and SB224289. At higher concentrations anpirtoline decreases pERK in malignant cells, but SB224289 treatment induces no change in ERK phosphorylation. Conversely, escalating concentrations of serotonin marginally enhance levels of pERK in COS. In normal osteoblasts, anpirtoline has no effect on ERK phosphorylation but SB224289 mildly enhances phosphorylation. Serotonin ablates ERK phosphorylation at high concentrations in normal osteoblasts. Immunoblots also demonstrate lysates from vehicle control, DMSO-treated COS and the positive control MCF7.

4.2 Evaluation of 5HTR2A

Expression of 5HTR2 in normal and malignant canine osteoblasts

Htr2a transcription was observed in normal and malignant osteoblasts, with COS exhibiting a 5.6 ± 1.2 fold increased in transcription relative to CnOb (p = 0.04) (**Figure 4.2.1**). The 5HTR2A immunoblot revealed a 50 kDa band in lysates from COS, CnOb, and MCF7, consistent with the documented human 5HTR2A and predicated molecular weight of canine 5HTR2A (50-50 kDa).[135] The relatively increased *htr2a* transcription in COS was reflected in the immunoblot, with COS producing stronger signal intensity for the receptor than CnOb (**Figure 4.2.1**). No mutations were identified in COS *htr2a* sequence relative to the reference sequences (**Appendix Figure A.2**). All statistical information in provided in **Appendix Table A.5**.

Effect of 5HTR2A antagonism on viability of normal and malignant canine osteoblasts

Ritanserin treatment induced a moderate decrease in COS viability at 6.25 µM, with >80% decreases in viability observed at 12.5, 25.0, and 50.0 µM (p < 0.01) (**Figure 4.2.2**). When concurrently incubated with 12.5 µM serotonin, a higher viability index was observed at 6.25 µM and 12.5 µM ritanserin compared to similar doses of ritanserin alone (p < 0.01). For CnOb, ritanserin induced a moderate decrease in viability at 50.0 µM only (**Figure 4.2.2**). Similar to our observations in the 5HTR1B viability experiment, serotonin enhanced viability in COS at 3.125, 6.25, and 12.5 µM. However, cell viability returned to levels equivalent to non-treated cells at 25.0 and 50.0 µM. Serotonin failed to enhance viability and CnOb and elicited a small decrease in viability at 50.0 µM. The calculated IC50 of ritanserin for COS was 6.3 µM and could not be established for CnOb at the concentrations tested. All statistical information in provided in **Appendix Table A.5**.

Effect of 5HTR2A antagonism on apoptosis in malignant canine osteoblasts

Mild increases in caspase activity were observed in COS treated with 3.125 µM (p = 0.02) and 12.5 µM (p = 0.02) ritanserin, with >2-fold increased activity present at 25.0 and 50.0 µM (p < 0.01) (**Figure 2.4.3**). Concurrent incubation with 12.5 µM serotonin attenuated the increase in caspase activity at 3.125 µM ritanserin only (p = 0.049). No treatment of serotonin enhanced caspase activity (**Figure 2.4.3**). COS cells pre-incubated a caspase inhibitor and then challenged with 12.5 µM ritanserin had markedly reduced caspase activity (p < 0.01) (**Figure 2.4.3**). All statistical information in provided in **Appendix Table A.5**.

Effect of 5HTR2A antagonism on phosphorylation of ERK and CREB

Similar to our 5HTR1B experiment, serotonin treatment mildly enhanced ERK phosphorylation in COS and moderately reduced the level of pERK in CnOb (**Figure 2.4.4**). Neoplastic osteoblasts challenged with escalating concentrations of ritanserin actually showed increased ERK phosphorylation (**Figure 2.4.4**). Conversely, ritanserin treatment ablated phosphorylated ERK in CnOb. Concerning CREB, serotonin caused equivocal change in the amount of phosphorylated CREB in COS while ritanersin induced a marked increase in pCREB (**Figure 2.4.4**). CnOb did not endogenously express CREB, and pCREB/CREB expression was not induced by treatment with serotonin or ritanserin.

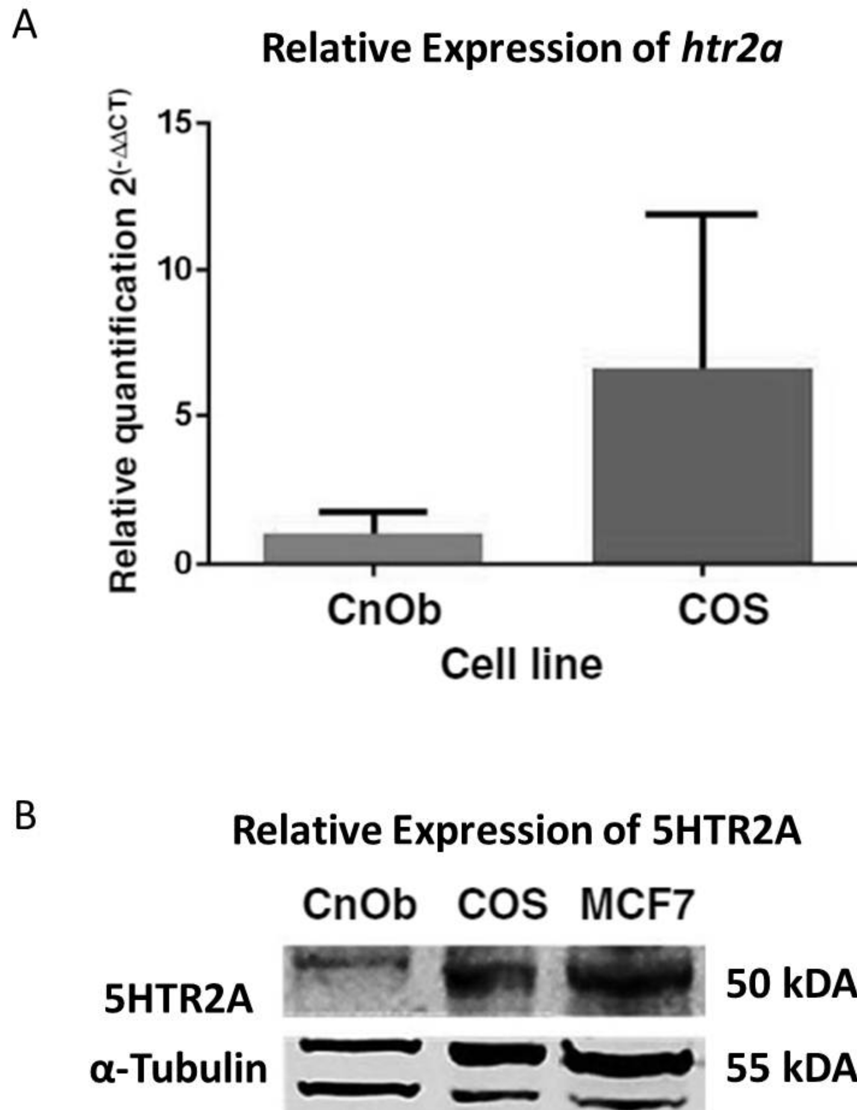

Figure 4.2.1. Increased *htr2a* transcription and 5HTRA expression is present in canine osteosarcoma cells relative to normal canine osteoblasts. (A) *htr2a* mRNA levels in COS and CnOb normalized to CnOb expression, as assessed by qRT-PCR. *Htr2a* expression is 5.6 ± 1.2 fold greater in malignant COS relative to normal CnOb (p = 0.04). Graphed bars are mean relative quantification ± 95% confidence interval of mean. (B) Immunoblot demonstrating increased expression of 5HTR2A in COS relative to CnOb. 5HTR2A expression is confirmed in the positive control MCF7, a human mammary carcinoma cell line.

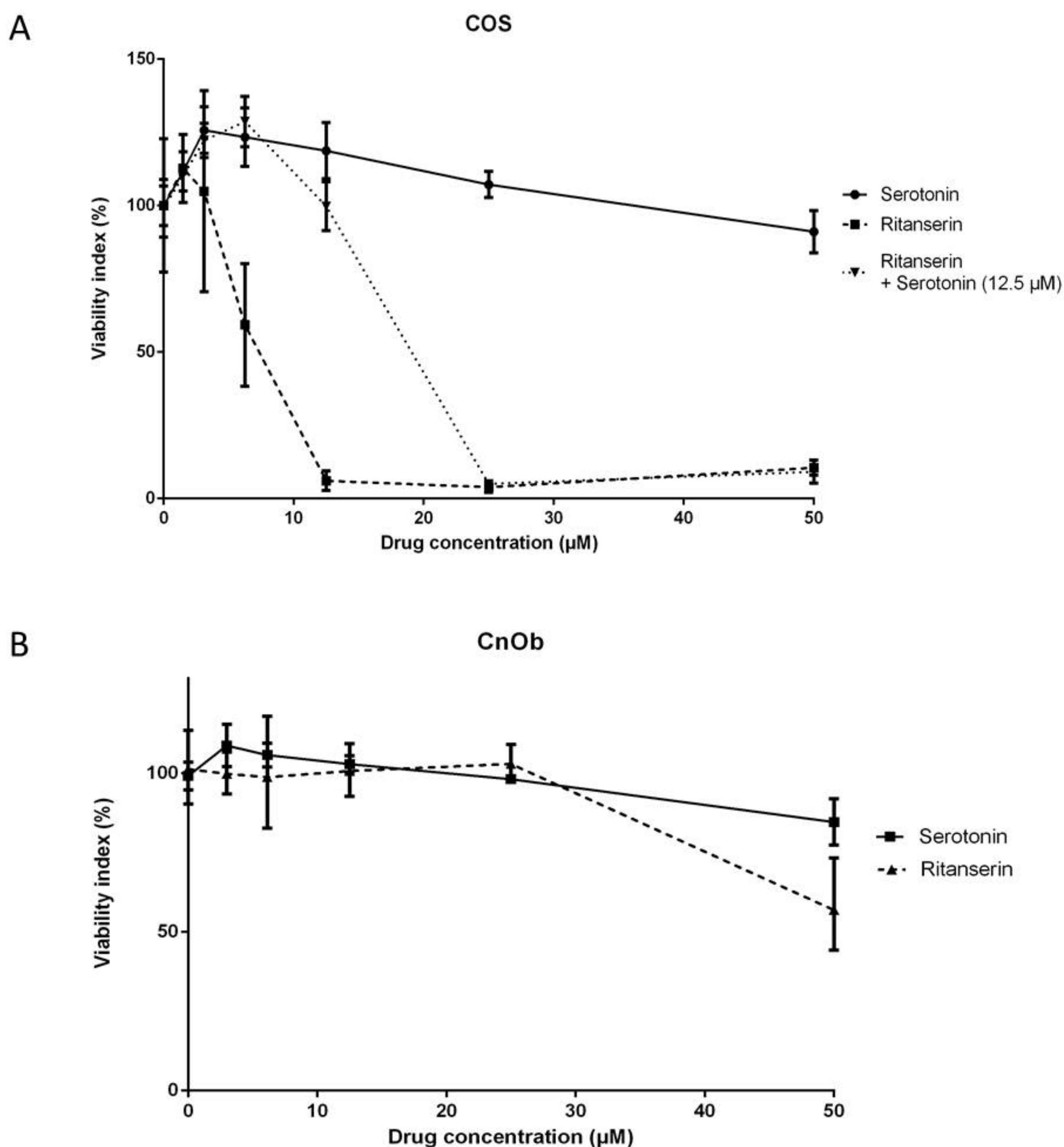

Figure 4.2.2. The 5HTRA antagonist ritanserin decreases viability in malignant and normal osteoblasts. (A) Effects of serotonin, ritanserin, and ritanserin and and12.5 μM serotonin treatment on the viability of COS. Ritanserin decreases viability at concentrations > 6.25 μM and concurrent incubation with 12.5 μM serotonin attenuates this decrease at 6.25 μM and 12.5 μM ritanserin ($p < 0.01$). Serotonin enhances viability between 3.125 – 12.5 μM ($p < 0.014$), with no change in viability observed at 25.0 μM and 50.0 μM. Plotted values are mean viability index ± 95% confidence interval of mean. (B) Effects of serotonin and ritanserin treatment on the viability of CnOb. Both serotonin and ritanserin caused decreased viability at 50.0 μM only ($p = 0.01$). Plotted values are mean viability index ± 95% confidence interval of mean.

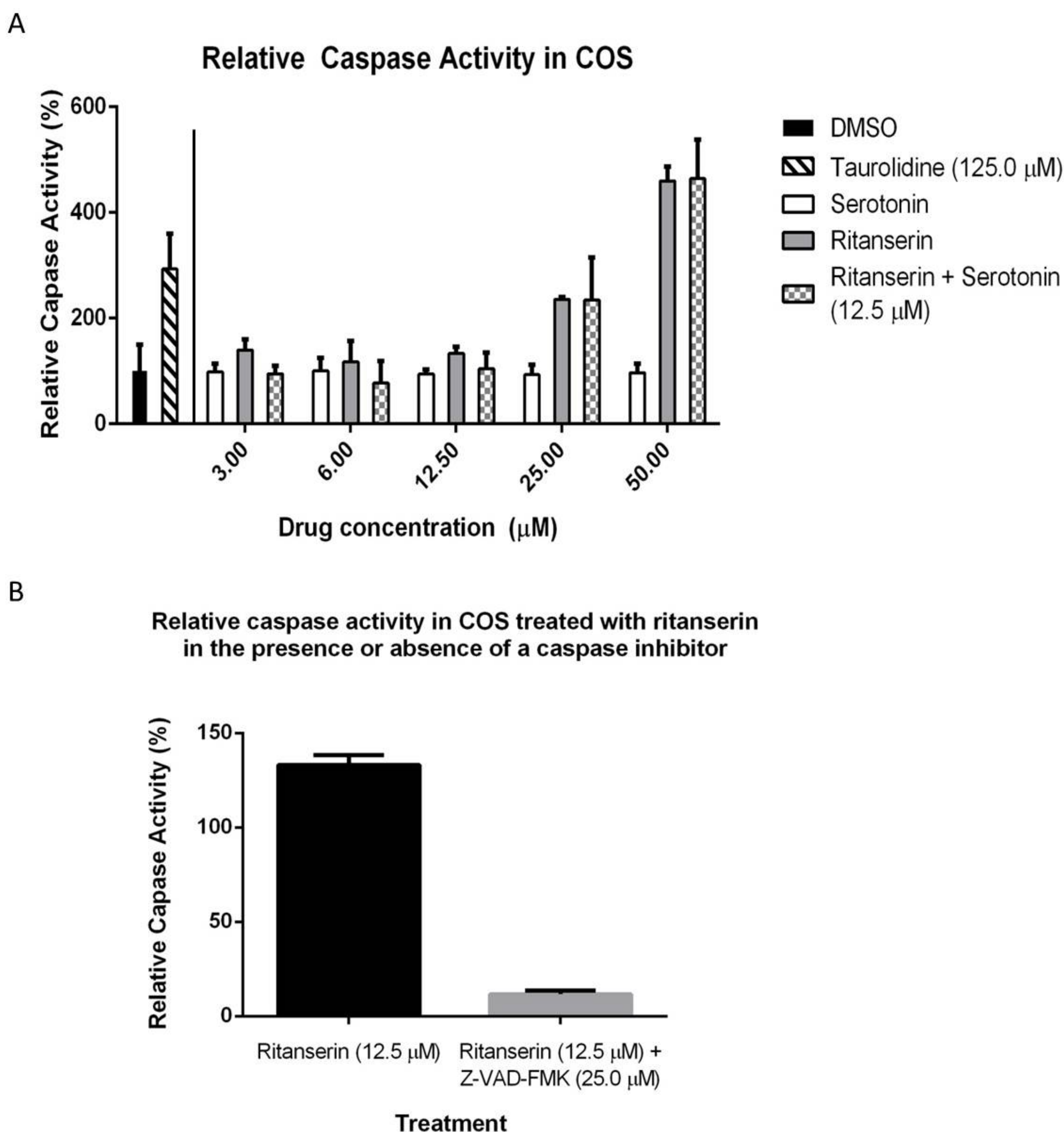

Figure 4.2.3. 5HTR2A antagonism promotes increased caspase-3/7 activity in malignant canine osteoblasts cells. (A) Relative caspase activity in COS following treatment with serotonin, ritanserin, and ritanserin and 12.5 μM serotonin. Ritanserin induces mild increases caspase activity at 3.125 μM and 12.5 μM ($p = 0.02$, $p = 0.025$, respectively), with markedly increased activity evident at 25.0 μM and 50.0 μM ($p < 0.01$). Concurrent incubation with 12.5 μM serotonin normalizes caspase activity at 3.125 μM ritanserin only. No change in caspase activity relative to DMSO-treated cells is observed at any concentration of serotonin. The positive control, taurolidine, increases caspase activity in COS ($p < 0.01$). (B) Relative caspase activity in COS cells treated with either 12.5 μM ritanserin or pre-incubated with the caspase inhibitor Z-VAD-FMK and then treated with 12.5 μM ritanserin. Pretreatment with a caspase inhibitor abolishes the ritanserin induced increase in caspase activity ($p < 0.01$). Plotted values are mean relative caspase activity ± 95% confidence interval of mean.

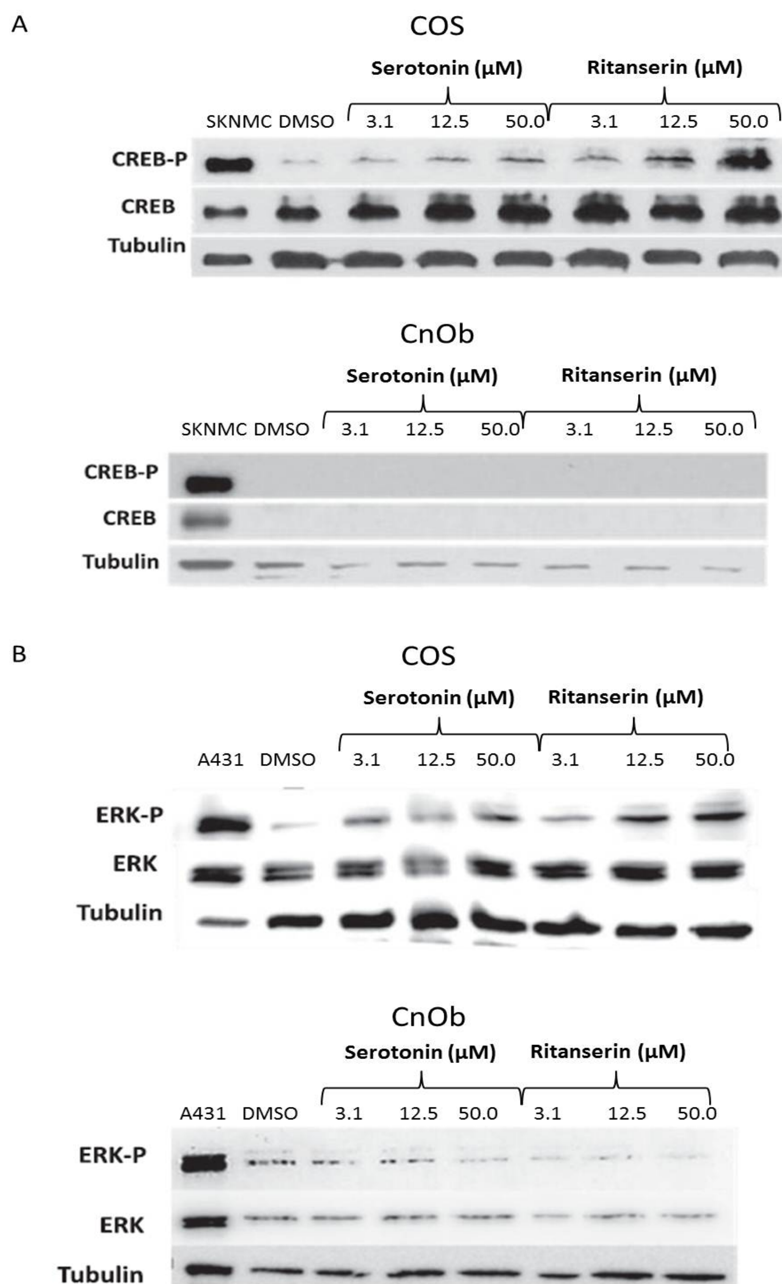

Figure 4.2.4. Discrepant patterns in phosphorylated CREB and ERK are observed between normal and malignant osteoblasts following treatment with serotonin and ritanserin. (A) pCREB and CREB immunoblots from COS and CnOb following treatment with serotonin and ritanserin. In COS, the level of CREB phosphorylation did not appreciably change with serotonin. However, ritanserin induced a marked increase in pCREB. Endogenous CREB expression is not found in CnOb and was not induced with serotonin or ritanserin treatments. (B) pERK and ERK immunoblots from COS and CnOb following treatment with serotonin and ritanserin. Escalating concentrations of serotonin enhanced ERK phosphorylation in the malignant cells. In CnOb, serotonin ablates phosphorylated ERK. Ritanserin increases the level of pERK in COS and attenuated ERK phosphorylation in CnOb. Immunoblots also demonstrate lysates from vehicle control, DMSO-treated COS and the positive controls SKNMC (for pCREB/CREB) and A431 (for pERK/ERK).

5. DISCUSSION

4.1 5HTR1B in malignant and normal canine osteoblasts

In our study we identified the expression of 5HTR1B in normal canine osteoblasts and a canine osteosarcoma cell line. No difference in the level of receptor expression was observed between malignant and normal cells, contrary to our hypothesis. This finding suggests that receptor abundance is not responsible for the variation in viability found between COS and CnOb following pharmacologic receptor manipulation. Interestingly, 5HTR1B antagonism decreased osteoblast viability whereas agonist binding had no effect, a pattern different than previously reported for this receptor.[7] Canine osteosarcoma cells were more sensitive to this inhibitory effect than normal osteoblasts and this attenuated viability in malignant cells likely occurs through induction of apoptosis, as demonstrated by increases in caspase 3/7 activity. These effects of 5HTR1B on canine osteoblasts appeared independent from changes in activation of the receptor's prototypical downstream transcriptional regulator, CREB. Serotonin had a positive effect on malignant canine osteoblast viability *in vitro*.

Yadav *et al.* first established an inhibitory role for 5HTR1B in bone with identification of a serotonergic gastrointestinal-bone endocrine axis.[7] In this endocrine axis Lrp5 controls expression of tph1, the rate limiting enzyme in serotonin synthesis, in duodenal ECs. Loss-of-function mutations in Lrp5 result in constitutive expression of Tph1 that cause aberrantly elevated blood levels of 5HT. This peripheral serotonin signals through 5HTR1B to suppress osteoblast proliferation by decreasing phosphorylative activation of CREB and downstream transcription of cyclins.[7] 5HTR1B may also promote concurrent bone resorption by inducing osteoclast differentiation and maturation.[12]

In this context, we were surprised to observe that 5HTR1B antagonism – not agonism – attenuated viability in both normal and malignant canine osteoblasts. An explanation for this finding in the undifferentiated normal canine osteoblasts may relate to temporal expression of serotonin receptors in osteoblasts. Prior studies have identified differential expression and function of 5HTR during the developmental stages of bone cells.[10, 12, 65] While 5HTR1B is preferentially expressed by mature osteoblasts of appendicular bone relative to osteoblasts of neuronal-associated bone[7], the expression and function of 5HTR1B in undifferentiated osteoblasts has not been explored. In opposition to the receptor's suppressive effects on mature osteoblasts, 5HTR1B activation by 5HT could possibly support the vitality of progenitor cells and immature osteoblasts like CnOb. Concerning COS, the receptor signaling cascade appears aberrant and may no longer be coupled to an inhibitory pathway. In these

malignant canine osteoblasts receptor agonism did not attenuate viability, or CREB phosphorylation, as previously described in mature murine osteoblasts.[7] Osteosarcoma cells may have co-opted 5HTR1B to facilitate a survival signal, given how receptor blockade decreased COS viability. 5HTR1B is known to promote survival of neoplastic cells, and is being explored as a treatment target, in human prostatic, transitional cell, and colonic adenocarcinoma.[126, 141, 142]

5HTR1B antagonism is a potent inducer of apoptosis in osteosarcoma cells. Even the lowest tested concentration of SB224289, 3.125 µM, elicited a near three-fold increase in caspase activity relative to vehicle treated cells. Caspase activation and resultant apoptosis likely account for the dramatic reduction in cell viability observed with SB224289 treatment. *In vitro* blockade of 5HTR1B is also reported to induce apoptosis in other tumor types, such as a human colorectal cancer cell line.[142] Interestingly, COS treated with higher concentrations of anpirtoline exhibited an approximately 50% increase in caspase activity. This finding is more in line with the traditional anti-proliferative activity of 5HTR1B in mature osteoblasts. However, the relatively mild increase in caspase activity may be insufficient to overcome anti-apoptotic systems over-expressed in canine osteosarcoma, and thus not translate into decreased viability.[122, 143] Although not with equal magnitude, both 5HTR1B agonism and antagonism did increase caspase activity in COS. A potential explanation for this behavior may relate to intrinsic 5HTR1B signaling. 5HTR1B is a G-protein coupled receptor and exhibits a basal level of intrinsic intracellular signaling even without ligand binding.[144] SB224289 is a highly selective 5HTR1B antagonist with potent negative intrinsic activity and ablates the receptor's intrinsic signaling upon binding.[144] In COS, the basal signal from 5HTR1B may be important in sustaining cell vitality and signal removal may promote cell death.

Our assessment of the two transcriptional regulators commonly coupled with 5HTR signaling in osteoblasts revealed different phosphorylation patterns in normal and malignant canine osteoblasts. Endogenous CREB expression was not identified in CnOb; this finding is in opposition to innate CREB expression in murine osteoblasts and may reflect differences in cell maturity or species.[7] Neither anpirtoline nor SB224289 treatment elicited an appreciable change in the phosphorylation status of CREB in COS, suggesting that 5HTR1B may be uncoupled from this transcriptional regulator. 5HTR, including the 5HTR 1 subfamily receptors, have the capacity to signal through multiple pathways.[33] Thus, in malignant osteoblasts 5HTR1B signal transduction may occur through other 5HTR associated G-protein cascades or utilize alternative secondary cytosolic messenger molecules. This idea is supported

by the finding that a) anpirtoline decreased pERK in COS but not in CnOb and b) SB224289 treatment enhanced pERK in normal osteoblasts but not in COS. Cumulatively, these discrepant phosphorylation profiles suggest that 5HTR1B signaling is aberrant in the neoplastic cells relative to normal canine osteoblasts. A more comprehensive examination of other 5HTR associated signaling cascades is necessary to fully characterize 5HTR1B signaling in this canine osteosarcoma cell line.

Although we identified apoptosis as a mechanism for decreased viability in malignant osteoblasts challenged with a 5HTR1B antagonist, we did not fully elaborate the cellular mechanisms linking receptor blockade and caspase activation. While alterations in the phosphorylation status of CREB and ERK have been linked to apoptosis, the mild changes in phosphorylation we observed in these transcriptional regulators are likely insufficient to explain our observations.[145, 146] Chou *et al.* found that activation of p38 MAP kinase induced the caspase 3 pathway in human osteosarcoma cells treated with an SSRI.[17] 5HTR2B is also known to signal through PARR ß/δ in osteoblasts and suppression of PARR ß/δ promotes apoptosis.[66, 147] The 5HTR1B antagonist induced caspase activation observed in COS could occur through one of these alternative apoptosis initiation pathways, especially if receptor signaling is anomalous.

Serotonin marginally increased pERK in COS. Enhanced ERK phosphorylation is associated with increased osteoblasts viability in murine osteoblasts and likely explains the increase in COS viability with serotonin treatment.[15] 5HTR2A and 5HTR2B appear to be the most mitogenic 5HTR in osteoblasts and 5HTR2A is expressed in COS.[14-16, 65, 66, 139] Given the markedly increased expression of 5HTR2A in the osteosarcoma cells relative to normal osteoblasts, 5HTR2A signaling may be responsible for the serotonin-induced proliferation in COS. As serotonin binds all 5HTR, the overall effect on cell viability likely reflects the sum of positive and negative survival signals from the receptor subtypes expressed on the osteoblast. A pro-viability effect of serotonin was not observed in CnOb. These normal canine osteoblasts may not express 5HTR2B, or adequate levels of 5HTR2A, to confer a proliferative signal from serotonin binding. Our results demonstrate that canine osteosarcoma cells have the potential to utilize endogenous serotonin as a growth factor.

In summary, we found that there is equivalent expression of 5HTR1B between normal canine osteoblasts and a canine osteosarcoma cell line. Antagonism of this receptor induced decreased cell viability in canine osteoblasts, with malignant cells more sensitive to this inhibitory effect. This

attenuated viability in osteosarcoma cells appears to occur through induction of apoptosis. Differential 5HTR1B signaling was present between normal and malignant cells, suggesting altered receptor transduction in canine osteosarcoma. Serotonin promoted proliferation of only osteosarcoma cells. Additional studies exploring pharmacologic targeting of 5HTR1B as a treatment strategy for canine osteosarcoma appear warranted.

4.2 5HTR2A in malignant and normal canine osteoblasts

We observed expression of 5HTR2A in both malignant and normal canine osteoblasts. The osteosarcoma cells exhibited nearly five-fold increased receptor expression relative to normal osteoblasts. As predicted, 5HTR2A antagonism attenuated viability in both osteoblast types. COS cells experienced reduced viability at lower concentrations of ritanserin than did CnOb. Additionally, malignant cells appeared to experience greater decreases in cell viability with receptor antagonism than normal osteoblasts. Antagonism induced apoptosis appears responsible for viability reduction in neoplastic osteoblasts, given the increased caspase activity after receptor blockade. Discordant patterns of CREB and ERK phosphorylation were found between malignant and normal osteoblasts following 5HTR2A pharmacologic manipulation, suggesting aberrant receptor signaling in osteosarcoma cells.

Serotonin may promote or abate osteoblast proliferation based upon the type and relative amount of 5HT expressed by the cell. The two receptors known to have strong mitogenic activity in osteoblasts are 5HTR2A and 5HTR2B.[14-16, 66] Agonistic stimulation of these receptors promotes phosphorylative activation of ERK, which subsequently activates pro-proliferative other transcriptional regulators.[15] 5HTR2B may also utilize other intracellular signaling pathways to regulate cell viability, such as PARR ß/δ.[66] Upregulated 5HTR2A expression in canine osteosarcoma cells may provide neoplastic cells with a survival or proliferation signal from endogenous 5HT. This scenario reflects our observations that serotonin treatment enhances COS viability while concurrently enhancing ERK phosphorylation. As previously discussed, CnOb may have inadequate of 5HTR2A – or lack 5HTR2B expression – and thus be unable to perceive a proliferation signal from serotonin.

Pharmacologic 5HTR2A blockade attenuates viability in both malignant and normal canine osteoblasts. Osteosarcoma cells are more dramatically affected by ritanserin than normal osteoblasts, highlighting the possible therapeutic potential of 5HTR2A. These observations are consistent with the receptor having intrinsic mitogenic potential in canine osteosarcoma and further suggest that basal receptor

signaling may be necessary for cell survival. The mechanism of cell death in osteosarcoma cells appears to be induction of apoptosis rather than drug toxicity, given the marked elevations in caspase 3/7 activity following ritanserin challenge. As with our investigation of 5HTR1B, we did not fully establish the signaling link between 5HTR2A and caspase activation. Unlike antagonism of 5HTR1B by SB224289, ritanserin induces moderate-marked increases in the levels of phosphorylated CREB and ERK. Although increased CREB and ERK phosphorylation are classically associated with promoting osteoblast viability, activated forms of these transcriptional regulators have been shown to induce apoptosis.[145, 146, 148] In context of manipulation of 5HTR2A, the possibility of pCREB or pERK contributing to cell death in COS cannot be excluded. However, induction of apoptosis could occur through other signaling cascades, such as PARR ß/δ.[147] In this later scenario, increased CREB and ERK phosphorylation may reflect attempts of the malignant osteoblasts to survive and thus not actually promote apoptosis.

Similar to our examination of 5HTR1B signaling, pharmacologic manipulation of 5HTR2A yields different patterns of transcriptional regulator phosphorylation between COS and CnOb. Receptor blockade attenuates ERK phosphorylation in normal canine osteoblasts, consistent with the receptor's signaling behavior in murine osteoblasts.[15] Serotonin also decreases the amount of pERK in normal osteoblasts, which reflects the lack of increased cell viability following challenge with 5HT. Being the non-specific natural 5HTR ligand, serotonin could also be working through other serotonin receptors expressed by CnOb to attenuate ERK phosphorylation. Unlike normal canine osteoblasts, the osteosarcoma cells exhibit increased ERK phosphorylation when incubated with ritanserin. This response is in opposition to the prototypical signaling behavior of 5HTR2A, suggesting 2Areceptor signaling is abnormal in COS.[15] Ritanserin also affects the phosphorylation status of CREB, showing 5HTR2A may also modulate this transcriptional regulator in neoplastic cells. This specific observation does not necessarily imply abnormal receptor behavior, as 5HTR2A may couple with multiple different G-protein cascades and affects CREB activation in other tissue types.[33, 149-151] To our knowledge, there are no published studies exploring the relationship between 5HTR2A and CREB in osteoblasts. Taken together, these findings suggest altered ERK pathway signaling in this canine osteosarcoma cell line. As with 5HTR1B, further evaluation of other signaling cascades is needed to define the signaling behavior of 5HTR2A in COS.

In conclusion, we identified overexpression of 5HTR2A in canine osteosarcoma cells relative to normal canine osteoblasts. Malignant cells challenged with the receptor antagonist ritanserin had markedly decreased viability, implying 5HTR2A supports neoplastic cell survival. Conversely, normal osteoblasts

appeared less sensitive to ritanserin. The attenuated viability observed in canine osteosarcoma cells following 5HTR2A blockade likely results from cell death by apoptosis. Receptor pharmacologic manipulations produce different patterns of ERK phosphorylation between malignant and normal osteoblasts, suggesting abnormal 5HTR2A signaling in neoplastic cells. As ritanserin increased CREB and ERK phosphorylation in osteosarcoma cells while simultaneously decreasing viability, activated forms of these proteins could be coupled with initiation of apoptosis in neoplastic cells. Pharmacologic inhibition of 5HTR2A represents a novel therapeutic approach for the treatment of canine osteosarcoma, with future studies needed to determine its *in vivo* practicality.

6. CONCLUSION

Serotonergic communication is critically important in the development and maintenance of bone. Nearly all aspects of the serotonergic signaling system, including 5HTR, 5HTT, and Tph, are found in osteoblasts, osteocytes, and osteoclasts. Serotonin may have a stimulatory or inhibitory effect on bone depending upon anatomic site of 5HT production and the 5HTR variants present on targeted bone cells. Serotonin generated in peripheral tissue by duodenal ECs inhibits osteoblast proliferation through activity on 5HTR1B. Conversely, central nervous system serotonergic signaling promotes osteoblast proliferation and maturation. Select 5HTR, like 5HTR2A, also have mitogenic activity in bone cells.

Osteosarcoma is an aggressive primary bone tumor that primarily affects dogs and adolescent humans. The biology of osteosarcoma is very similar between canids and people, as is the clinical course of disease. Even with aggressive intervention, entailing primary tumor excision with adjunct chemotherapy or radiotherapy, most patients succumb to complications from tumor progression.
Despite the major regulatory role of serotonin in bone physiology, comparatively little information is known about the role of serotonergic signaling in canine osteosarcoma.

Our investigation sought to elucidate the expression profile, functionality, and therapeutic potential of 5HTR1B and 5HTR2A in canine osteosarcoma cells and normal canine osteoblasts. We hypothesized that both receptors would be expressed in normal and neoplastic osteoblasts, but that osteosarcoma cells would have comparatively lower 5HTR1B and higher 5HTR2A expression. Exposure to serotonin was expected to enhance viability on both cell types. We anticipated 5HTR1B agonism would attenuate viability while antagonism would enhance viability. Conversely, 5HTR2A antagonism was expected to decrease osteoblast viability. We suspected that different receptor signaling patterns – as assessed by changes in phosphorylated CREB and ERK following receptor pharmacologic manipulation – would be observed between osteosarcoma cell and normal osteoblasts.

We observed that 5HTR1B and 5HTR2A are indeed expressed in normal and malignant osteoblasts, and that 5HTR2A is relatively overexpressed in osteosarcoma cells. Serotonin enhanced viability of malignant cells but not in normal osteoblasts, suggesting osteosarcoma cells may use 5HT as a growth factor. Contrary to our predictions, 5HTR1B antagonism attenuated osteoblast viability while 5HTR1B agonism had no effect on viability. 5HTR2A antagonism also decreased cell viability, as expected. Neoplastic cells appeared more sensitive to the inhibitory effects of 5HTR1B blockade and 5HTR2A

blockade than normal cells. This attenuated viability in osteosarcoma cells appeared to result from induction of apoptosis following receptor antagonism. Aberrant 5HTR1B and 5HTR2A signaling was observed in osteosarcoma cells relative to normal osteoblasts, and for 5HTR2A these changes in signaling could be associated with initiation of apoptosis. Both receptors appear plausible therapeutic targets for the treatment of osteosarcoma, although more investigation into their clinical utility is necessary.

To our knowledge, this if the first study demonstrating that functional aspects of the serotonergic signaling system are present in canine osteosarcoma cells. Given our knowledge of serotonergic communication with regards to normal murine and human osteoblasts, we expect other components of this endocrine system are present in malignant osteoblasts. As such, much work is warranted to better characterize the full function of serotonin in osteosarcoma. Further elaboration of this system will undoubtedly expand our knowledge of osteosarcoma biology and may help discover novel treatments for this tumor in dogs and humans alike.

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8. APPENDIX

Table A.1. The anatomic distribution, action, mechanism of signal propagation, and prototypical G-protein cascade for select individual 5HTR and 5HTR families. Abbreviations: AC: adenylate cyclase, PLC: phospholipase c. Adapted from Pytliak M, *et al.*, Barnes M, *et al.*, and Tocris.[25, 152, 153]

Receptor Subtype	Localization	Function	Transduction Mechanism and G Protein Cascade
5-HT1A	Limbic system (hippocampus, lateral septum, cortical areas), mesencephalic raphe nuclei	Hyperpolarization, modulation of neurotransmitter release, anxiolysis, hypothermia, hyperphagia	↓AC (Gi/o)
5-HT1B	Basal ganglia, striatum, amygdala, trigeminal ganglion, vascular smooth muscle	Autoreceptor, locomotion, hypophagia, hypothermia, modulation of neurotransmitter release, vasoconstriction	↓AC (Gi/o)
5-HT1D	Basal ganglia, hippocampus, cortex, spinal cord, vascular smooth muscle	Autoreceptor, modulation of neurotransmitter release	↓AC (Gi/o)
5-HT1E	Cortex, caudate putamen, claustrum, hippocampus, amygdala	Unknown	↓AC (Gi/o)
5-HT1F	Hippocampus, cortex, dorsal raphe nucleus, uterus	Speculative role in visual and cognitive function	↓AC (Gi/o)
5-HT2A	Forebrain, caudate nucleus, nucleus accumbens, hippocampus, olfactory tubercle, vascular smooth muscle, blood platelets	Neuronal depolarization, head twitch, hyperthermia, modulation of neurotransmitter release smooth muscle contraction, platelet activation	↑ PLC
5-HT2B	Brain, stomach fundus (rat), gut, heart, kidney, lung	Contraction of the stomach fundus, anxiety	↑ PLC
5-HT2C	Choroid plexus, cortex, limbic system, basal ganglia	Hypolocomotion, hypophagia, penile erection, hyperthermia, anxiety, ↓ noradrenalin and dopamine release	↑ PLC

5-HT3 (3A, 3B)	Dorsal vagal complex, hippocampus, amygdala, caudate, cerebral cortex, heart, intestines	Anxiety, cognition, pain , reward/withdrawal, vomiting reflex, vasodilation, intestinal tone and secretion	Ion channel (Na+, K+, Ca2+)
5-HT4 (4A-H)	Cerebral cortex, limbic areas, hippocampus, colliculus, intestines	Learning and memory, visual perception, anxiety, motor coordination, arousal, smooth muscle relaxation, modulation of neurotransmitter release	↑ AC (Gs)
5-HT5	Amygdala, hippocampus, caudate nucleus, cerebellum, hypothalamus, thalamus, substantia nigra, spinal cord	Modulation of exploratory behavior and locomotion	↓ AC (Gi/o)
5-HT6	Striatum, olfactory tubercles, nucleus accumbens, hippocampus, stomach, adrenal glands	Memory and learning, modulation of neurotransmitter release	↑ AC (Gs)
5-HT7	Thalamus, hypothalamus, hippocampus, cerebral cortex, amygdala, GI and vascular smooth muscle, heart	Circadian rhythms, smooth muscle relaxation, nociception, hypotension, modulation of REM sleep, learning and memory, LH release	↑ AC (Gs)

Table A.2. Comparison of similarities and differences between canine and human osteosarcoma with regards to clinical disease and tumor biology. Table from Morello *et al.*[2]

Similarities and differences between human and canine appendicular osteosarcomas.

Variables	Dog	Human
Incidence in USA	>8000 cases/year	600 cases/year
Median age	Middle-aged to older dogs	Adolescent disease
	Peak of incidence 7-9 years	Peak of incidence at 10-20 years
	Second small peak at 18-24 months	Median peak age at 16 years
Median peak age at 7 years		
Race/breed	Large/giant breeds	None
	Familiar pattern in Saint Bernard<comma> Rottweiler and Scottish Deerhound	
Sex	Males slightly more than females: Ratio 1.1-1:5:1	Males more than females: Ratio 1.6:1
Site	75% appendicular skeleton, metaphysis of long bones, mainly distal radius, proximal humerus, distal femur and proximal and distal tibia	Metaphysis or diaphysis of long bones (80-90%)
		Bones of the knee joint (50%)
		Proximal humerus (25%)
Aetiology	Not completely known	Not completely known
Histopathological grade	High grade	High grade
Molecular and genetic alterations	p53: Mutated	p53: Mutated
	IGF-1/IGF-1R: Over-expressed; Poor clinical outcome	IGF-1/IGF-1R: Over-expressed; Poor clinical outcome
	HGF/c-Met: Over-expressed; Contributes to malignant phenotype	HGF/C-Met: Over-expressed; Contributes to malignant phenotype
	ErbB-2/HER-2: Over-expressed; Poor clinical outcome	ErbB-2/HER-2: Over-expressed; Poor clinical outcome
	PTEN: Mutated or down-regulated	PTEN: Mutated or down-regulated
	Ezrin: Detected; Contributes to malignant	Ezrin: Detected; Contributes to malignant phenotype

	phenotype	
	Matrix metalloproteinases: Expressed	Matrix metalloproteinases: Expressed
	PDGF-Î²: Expressed	PDGF-Î²: Expressed
	VEGF: Expressed	VEGF: Expressed
	P-gp: Expressed	P-gp: Expressed
Clinical signs	Pain	Pain
	Swelling	Leg swelling
	Hard painful mass	Hard painful mass
	Uncommon pathological fracture (3%)	Uncommon pathological fracture
Metastatic site	10% of cases with metastasis at diagnosis: Lung, bone (7.4%)	20% of cases with metastasis at diagnosis: Lung, bone
	Regional lymph node metastasis (4.4-9.0%)	Regional lymph node metastasis < 10%
Treatment	Amputation	Limb-sparing techniques (90% cases)
	Limb-sparing techniques	Amputation (rare)
	Adjuvant chemotherapy: Doxorubicin, platinum	Neoadjuvant chemotherapy: Doxorubicin, methotrexate, isofosfamide, platinum and adjuvant post-surgery
Survival	60% survival at 1 year with chemotherapy	70% survival at 5 years with chemotherapy
Negative prognostic indicators	Metastasis at diagnosis: Lungs, bones, lymph nodes	Metastasis at diagnosis: Lungs, bones, lymph nodes
	High serum ALP, LDH activities	High serum ALP, LDH activities
	Tumour volume	Tumour volume
	Tumour grade	Tumour grade
	Age: Young dogs	Age: Youngest affected
	Poor response to neoadjuvant chemotherapy: % tumour necrosis	
Positive prognostic indicators	Post-operative limb-sparing infection	Post-operative limb-sparing infection
	High percentage of tumor necrosis induced by chemotherapy or radiotherapy	High percentage of tumor necrosis induced by chemotherapy or radiotherapy

Table A.3. List of product manufacturers.

Superscript label	Company	Location
A	Cell Application Inc.	San Diego, CA, USA
B	Life Technologies Corp.	Carlsbad, CA, USA
C	Qiagen	Valencia, CA, USA
D	Thermo Fischer Scientific	Rockford, IL, USA
E	AMRESCO	Solon, OH, USA
F	LI-COR Biosciences	Lincoln, NE, USA
G	ABCAM	Cambridge, UK
H	Santa Fruz Biotechnology	Dallas, TX, USA
I	Promega	Madison, WI, USA
J	Tocris	Bristol, UK
K	TauroPharm GmbH	Würzburg, Germany
L	Calbiochem	San Diego, CA, USA
M	GraphPad Software	La Jolla, CA, USA

Table A.4. Summary of statistical conclusions from all 5HTR1B experiments.

***Htr1b* expression between COS and CnOb**					
Unpaired t test					
P value	< 0.0001				
P value summary	****				
Significantly different? (P < 0.05)	Yes				
One- or Two-tailed P value?	Two-tailed				
T, df	t=23.14 df=4				
How big is the difference?					
Mean ± SEM of column A	368.3 ± 13.98, n=3				
Mean ± SEM of column B	44.45 ± 0.4480, n=3				
Difference between means	-323.8 ± 13.99				
95% confidence interval	-362.7 to -285.0				
R squared	0.9926				
5HTR1B COS Viability					
[Serotonin] relative to control					
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 3.125	-34.53	-49.82 to -19.23	Yes	****	< 0.0001
0 vs. 6.25	-26.42	-41.72 to -11.12	Yes	***	0.0002
0 vs. 12.5	-24.89	-40.19 to -9.592	Yes	***	0.0005
0 vs. 25	-16.58	-31.88 to -1.278	Yes	*	0.0293
0 vs. 50	-19.87	-35.17 to -4.574	Yes	**	0.0065
[Anpirtoline] relative to control					
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 3.125	-5.787	-32.93 to 21.35	No	ns	0.972
0 vs. 6.25	2.081	-25.06 to 29.22	No	ns	0.9997
0 vs. 12.5	22.87	-1.405 to 47.14	No	ns	0.0708
0 vs. 25	-10.65	-34.93 to 13.62	No	ns	0.6733
0 vs. 50	-15.27	-40.29 to 9.753	No	ns	0.3739
[SB224289] relative to control					
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value

0 vs. 1.75	17.3	-1.828 to 36.43	No	ns	0.0903
0 vs. 3.125	17.72	-1.408 to 36.85	No	ns	0.0792
0 vs. 6.25	94.4	75.27 to 113.5	Yes	****	< 0.0001
0 vs. 12.5	95	75.87 to 114.1	Yes	****	< 0.0001
0 vs. 25	71.99	52.86 to 91.12	Yes	****	< 0.0001
0 vs. 50	84.11	64.40 to 103.8	Yes	****	< 0.0001
[Anpirtoline] relative to [Anpirtoline] + Serotonin (12.5μM)					
Sidak's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. AS 0	0.06522	-18.42 to 18.55	No	ns	> 0.9999
3.125 vs. AS 3.125	-1.095	-21.76 to 19.57	No	ns	> 0.9999
6.25 vs. AS 6	-10.98	-31.64 to 9.692	No	ns	0.6397
12.5 vs. AS 12	0.5431	-18.51 to 19.60	No	ns	> 0.9999
25 vs. AS 25	-7.401	-25.89 to 11.09	No	ns	0.8655
50 vs. AS 50	-1.505	-20.56 to 17.55	No	ns	> 0.9999
[SB224289] relative to [SB224289] + Serotonin (12.5μM)					
Sidak's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
3.125 vs. SBS 3	12.65	-0.965 to 25.34	No	ns	0.1235
6.25 vs. SBS 6	-41.51	-54.20 to -28.83	Yes	****	< 0.0001
12.5 vs. SBS 12	-4.756	-17.45 to 7.933	No	ns	0.8633
25 vs. SBS 25	12.42	-1.727 to 25.10	No	ns	0.0689
50 vs. SBS 50	2.654	-10.43 to 15.73	No	ns	0.9892
5HTR1B CnOb Viability					
[Serotonin] relative to control					
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 3	-9.577	-21.08 to 1.928	No	ns	0.1154
0 vs. 6	-6.587	-18.09 to 4.918	No	ns	0.3706
0 vs. 12	-3.733	-15.24 to 7.771	No	ns	0.8096
0 vs. 25	1.04	-10.46 to 12.54	No	ns	0.9986
0 vs. 50	14.55	3.045 to 26.05	Yes	*	0.0128

[Anpirtoline] relative to control					
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 3	-5.55	-15.65 to 4.545	No	ns	0.4324
0 vs. 6	5.78	-4.315 to 15.88	No	ns	0.396
0 vs. 12	6.243	-3.853 to 16.34	No	ns	0.3289
0 vs. 25	9.405	-0.6903 to 19.50	No	ns	0.0724
0 vs. 50	10.9	-0.007465 to 21.80	No	ns	0.0502
[SB224289] relative to control					
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 3	-12.44	-22.25 to -2.625	Yes	*	0.0127
0 vs. 6	-13.17	-22.99 to -3.361	Yes	**	0.0086
0 vs. 12	38.14	28.32 to 47.95	Yes	****	< 0.0001
0 vs. 25	53.09	43.28 to 62.91	Yes	****	< 0.0001
0 vs. 50	53.29	43.48 to 63.11	Yes	****	< 0.0001
5HTR1B COS Caspase Activity					
[Serotonin] relative to control					
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 3	1.209	-24.00 to 26.42	No	ns	0.9998
0 vs. 6	-0.225	-25.43 to 24.98	No	ns	> 0.9999
0 vs. 12	5.146	-20.06 to 30.36	No	ns	0.9662
0 vs. 25	6.83	-18.38 to 32.04	No	ns	0.9027
0 vs. 50	3.17	-22.04 to 28.38	No	ns	0.9958
[Anpirtoline] relative to control					
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 3	-50.49	-96.68 to -4.303	Yes	*	0.0283
0 vs. 6	-19	-65.19 to 27.19	No	ns	0.7125
0 vs. 12	-24.45	-70.64 to 21.74	No	ns	0.4967
0 vs. 25	-63.93	-110.1 to -17.75	Yes	**	0.0041
0 vs. 50	-58.53	-104.7 to -12.34	Yes	**	0.0091
[SB224289] relative to control					

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 3	-184.6	-295.9 to -73.33	Yes	***	0.0006
0 vs. 6	-320.4	-431.7 to -209.1	Yes	****	< 0.0001
0 vs. 12	-572.7	-684.0 to -461.4	Yes	****	< 0.0001
0 vs. 25	-411.4	-522.7 to -300.1	Yes	****	< 0.0001
0 vs. 50	-492.2	-603.5 to -380.9	Yes	****	< 0.0001
[Anpirtoline] relative to [Anpirtoline] + Serotonin (12.5μM)					
Sidak's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
3 vs. AS3	68.14	14.97 to 121.3	Yes	**	0.0068
6 vs. AS6	51.45	-1.722 to 104.6	No	ns	0.0618
12 vs. AS12	47.76	-5.412 to 100.9	No	ns	0.0958
25 vs. AS25	75.29	22.12 to 128.5	Yes	**	0.0024
50 vs. AS50	76.62	23.44 to 129.8	Yes	**	0.002
[SB224289] relative to [SB224289] + Serotonin (12.5μM)					
Sidak's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
3 vs. SB3	84.7	-58.25 to 227.6	No	ns	0.462
6 vs. Sb6	27.17	-115.8 to 170.1	No	ns	0.9909
12 vs. SB12	-59.19	-202.1 to 83.76	No	ns	0.7905
25 vs. SB25	-75.38	-218.3 to 67.57	No	ns	0.5843
50 vs. SB50	100.4	-42.58 to 243.3	No	ns	0.2846
SB224289 (6.25 μM) relative to SB224289 (6.25 μM) + Z-VAD-FMK					
Unpaired t test					
P value	< 0.0001				
P value summary	****				
Significantly different? (P < 0.05)	Yes				
One- or two-tailed P value?	Two-tailed				
t, df	t=23.14 df=4				
Mean ± SEM of column A	368.3 ± 13.98, n=3				
Mean ± SEM of column B	44.45 ± 0.4480, n=3				

Difference between means	-323.8 ± 13.99				
95% confidence interval	-362.7 to -285.0				
R squared	0.9926				

Table A.5. Summary of statistical conclusions from all 5HTR2A experiments.

***htr2A* expression between COS and CnOb**					
5HTR1B COS Viability					
Unpaired t test with Welch's correction					
P value	0.0434				
P value summary	*				
Significantly different? (P < 0.05)	Yes				
One- or two-tailed P value?	Two-tailed				
Welch-corrected t, df	t=4.486 df=2.069				
How big is the difference?					
Mean ± SEM of column A	6.603 ± 1.233, n=3				
Mean ± SEM of column B	1.027 ± 0.1618, n=3				
Difference between means	-5.577 ± 1.243				
95% confidence interval	-10.76 to -0.3953				
R squared	0.9068				
5HTR2A COS Viability					
[Serotonin] relative to control					
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 3.125	-25.76	-35.41 to -16.12	Yes	****	< 0.0001
0 vs. 6.25	-23.39	-33.34 to -13.45	Yes	****	< 0.0001
0 vs. 12.5	-18.74	-28.69 to -8.800	Yes	****	< 0.0001
0 vs. 25	-7.16	-17.10 to 2.783	No	ns	0.2319
0 vs. 50	8.898	-0.7483 to 18.54	No	ns	0.0796
[Ritanserin] relative to control					
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 3.125	-4.863	-28.12 to 18.40	No	ns	0.975
0 vs. 6.25	40.75	17.49 to 64.01	Yes	***	0.0002
0 vs. 12.5	93.97	70.71 to 117.2	Yes	****	< 0.0001
0 vs. 25	96.11	72.85 to 119.4	Yes	****	< 0.0001
0 vs. 50	89.49	66.23 to 112.7	Yes	****	< 0.0001

[Ritanserin] relative to [Ritanserin] + Serotonin (12.5µM)					
Sidak's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
R3.125 vs. SR3.125	-17.37	-35.08 to 0.3436	No	ns	0.0572
R6.25 vs. SR6.25	-69.42	-87.13 to -51.71	Yes	****	< 0.0001
R12.5 vs. SR12.5	-93.69	-111.4 to -75.98	Yes	****	< 0.0001
R25 vs. SR25	-1.106	-18.82 to 16.60	No	ns	> 0.9999
R50 vs. SR50	1.433	-16.28 to 19.14	No	ns	0.9999
5HTR2A CnOb Viability					
[Serotonin] relative to control					
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 3	-9.577	-21.08 to 1.928	No	ns	0.1154
0 vs. 6	-6.587	-18.09 to 4.918	No	ns	0.3706
0 vs. 12	-3.733	-15.24 to 7.771	No	ns	0.8096
0 vs. 25	1.04	-10.46 to 12.54	No	ns	0.9986
0 vs. 50	14.55	3.045 to 26.05	Yes	*	0.0128
[Ritanserin] relative to control					
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
cnob r 0 vs. cnob r 3	1.565	-14.67 to 17.80	No	ns	0.9996
cnob r 0 vs. cnob r 6	2.257	-13.97 to 18.49	No	ns	0.9975
cnob r 0 vs. cnob r 12.5	0.6675	-15.56 to 16.90	No	ns	> 0.9999
cnob r 0 vs. cnob r 25	-1.518	-17.75 to 14.71	No	ns	0.9996
cnob r 0 vs. cnob r 50	44.04	27.81 to 60.27	Yes	****	< 0.0001
5HTR1B COS Caspase Activity					
[Serotonin] relative to control					
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 3.125	1.209	-23.60 to 26.02	No	ns	0.9998
0 vs. 6.25	-0.2256	-25.03 to 24.58	No	ns	> 0.9999
0 vs. 12.5	5.146	-19.66 to 29.95	No	ns	0.9585

0 vs. 25	6.829	-17.98 to 31.64	No	ns	0.8854
0 vs. 50	3.169	-21.64 to 27.98	No	ns	0.9947
[Ritanserin] relative to control					
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 3.125	-39.53	-72.04 to -7.012	Yes	*	0.017
0 vs. 6.25	-17.97	-47.05 to 11.11	No	ns	0.3013
0 vs. 12.5	-33.18	-62.26 to -4.098	Yes	*	0.0248
0 vs. 25	-135.6	-164.7 to -106.5	Yes	****	< 0.0001
0 vs. 50	-359.8	-388.8 to -330.7	Yes	****	< 0.0001
[Ritanserin] relative to [Ritanserin] + Serotonin (12.5μM)					
Sidak's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
3.125 vs. SR3.125	44.58	0.02449 to 89.14	Yes	*	0.0498
6.25 vs. SR6.25	40.83	0.9753 to 80.69	Yes	*	0.043
12.5 vs. SR12.5	29.01	-10.85 to 68.86	No	ns	0.2334
25 vs. SR25	0.7584	-39.10 to 40.61	No	ns	> 0.9999
50 vs. SR50	-4.315	-44.17 to 35.54	No	ns	0.9992
Ritanserin (12.5 μM) relative to Ritanserin (12.5 μM) + Z-VAD-FMK					
Unpaired t test					
P value	< 0.0001				
P value summary	****				
Significantly different? (P < 0.05)	Yes				
One- or two-tailed P value?	Two-tailed				
t, df	t=37.93 df=4				
How big is the difference?					
Mean ± SEM of column A	133.2 ± 3.020, n=3				
Mean ± SEM of column B	11.65 ± 1.071, n=3				
Difference between means	-121.5 ± 3.204				
95% confidence interval	-130.4 to -112.6				
R squared	0.9972				

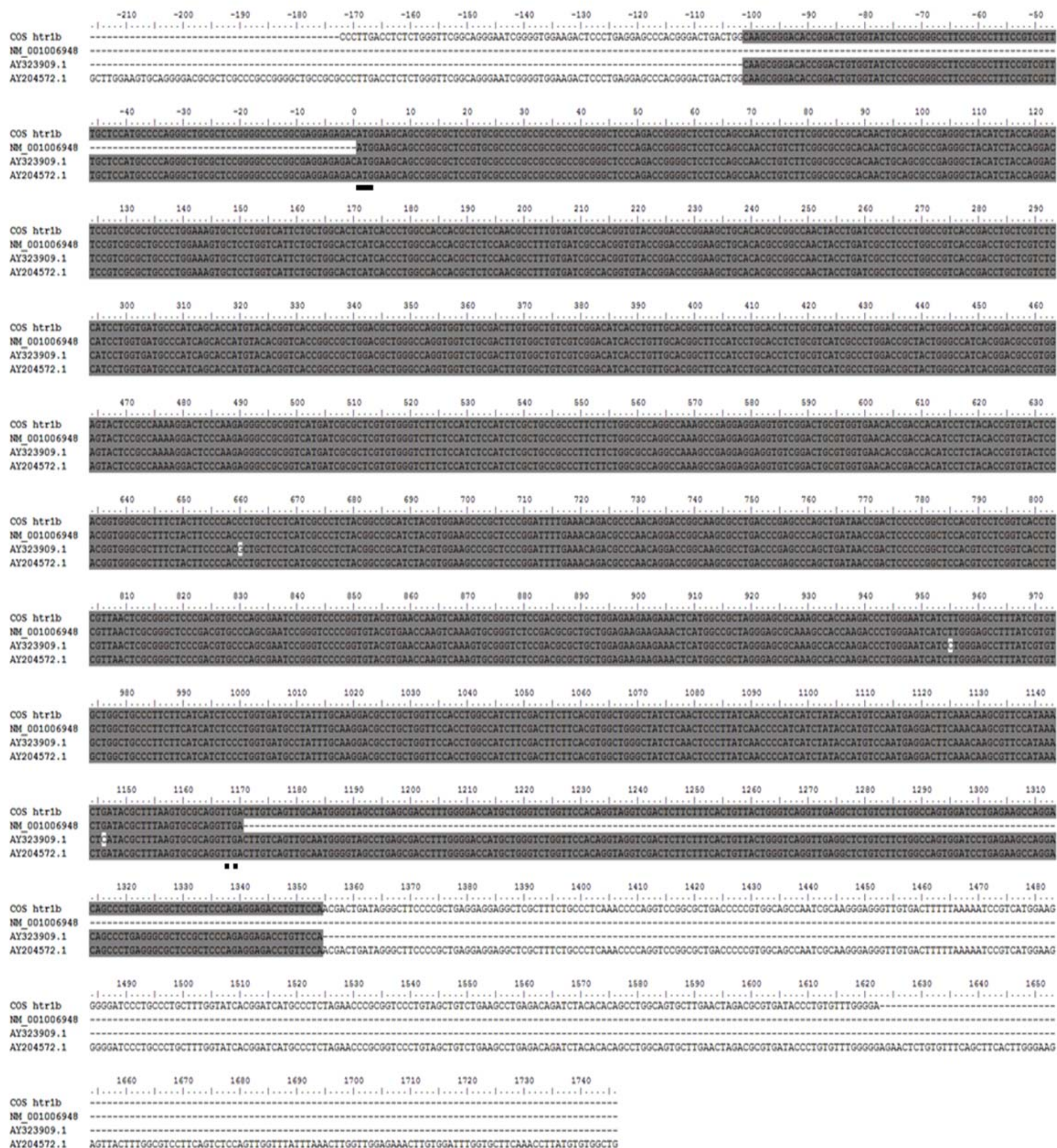

Figure A.1. Alignment of COS *htr1b* sequence and published canine *htr1b* reference sequences, NCBI accessions NM_001006948.1 (mRNA), AY323909.1 (genomic DNA), and AY204572.1 (genomic DNA). Start codon is denoted by solid line and stop codon highlighted by the dashed line. No sequence mutations are identified between COS *htr1b* and the reference sequences, with the possible exception of three single nucleotide polymorphisms present in AY323909.1 relative to the other sequences.

Figure A.2. Alignment of COS *htr2a* sequence and published canine *htr2a* reference sequences, NCBI accessions NM_001005869.1 (mRNA). Start codon is denoted by solid line and stop codon highlighted by the dashed line. No sequence differences are identified between COS *htr2a* and the *htr2a* mRNA reference sequence or exon sequences from genomic DNA, NCBI accession NC_006604.3 (comparison of COS *htr2a* to genomic DNA sequences not shown).