AN ABSTRACT OF THE THESIS OF

Candice Bailey for the degree of Master of Science in Veterinary Science presented on June 27, 2013
Title: Expression of Serotonin, Chromogranin-A, Serotonin Receptor-2B, Tryptophan hydroxylase-1, and Serotonin Reuptake Transporter in the Small Intestine of Dogs with Inflammatory Bowel Disease

Abstract approved:

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Craig G. Ruaux

The neuroendocrine system of the gut plays a significant role in regulating intestinal motor and sensory functions, as well as modulating inflammation in the intestine. The intestinal mucosa is the dominant site of serotonin (5HT) synthesis in mammals, with more than 95% of the intestinal 5HT being present in the enterochromaffin cells (ECC). When released from these cells, 5HT acts locally on the intestinal tract, affecting intestinal motility and nociception.

5HT excess is associated with autonomic hyperactivity of the intestinal tract and has been shown to be modulated in the gut of dogs with inflammatory bowel disease (IBD), a chronic inflammatory condition of the gastrointestinal (GI) tract of dogs that commonly causes chronic vomiting, diarrhea, weight loss, and/or anorexia. Because IBD is commonly diagnosed and treated in companion animals, continuing to identify avenues by which therapy can be enhanced is relevant. Therefore, the first objective of this study was to retrospectively compare the expression of 5HT and Chromogranin-A (CgA) in the intestine between dogs with IBD and a group of healthy dogs (CG1). A second objective was to prospectively quantify and compare the expression of serotonin receptor 2B
(5HT$_{2B}$), tryptophan hydroxylase-1 (THP-1), and serotonin transporter (SERT), in the intestine between IBD dogs and a group of healthy dogs (CG2).

For the retrospective aspect of the study, a search of the medical record database at Lois Bates Acheson Veterinary Teaching Hospital at Oregon State University College of Veterinary Medicine revealed nine client-owned dogs diagnosed with IBD that were included. Histologic grading of eleven specimens from the nine dogs was determined based on WSAVA guidelines and disease severity was determined via inflammatory bowel index scores. 5HT and CgA expression was determined by immunohistochemistry (IHC). CG1 included nine healthy control dogs from an unrelated project. For the second part of the study, GI samples were prospectively collected from seven dogs with a clinical history consistent with that of IBD with histopathologic evidence of mucosal inflammation consistent with IBD. 5HT$_{2B}$, THP-1, and SERT expression was determined by quantitative reverse transcriptase polymerase chain reaction (QRT-PCR), and compared to expression of 5HT$_{2B}$, THP-1, and SERT in CG2, which included eight healthy dogs.

Both the IBD group and CG1 showed enterocytes with strong staining for 5HT and CgA. The mean positive cells/hpf were significantly increased for both 5HT and CgA immunopositive cells in the IBD dogs compared to the control group (5HT mean 2.3 cells/hpf vs. 1.0 cells/hpf, p<0.01; CgA 2.7 cells/hpf vs. 1.7 cells/hpf, p<0.05). There was a significant correlation between 5HT and CgA positive cells/hpf across all dogs (Pearson $r^2$=0.2433, p=0.016). 5HT$_{2B}$ expression was significantly lower in the IBD dogs (p<0.05). The RQ values in the IBD group overall were approximately 0.5 times those of CG2
(mean relative target sequence expression value: IBD group 0.5; CG2 1.1). There was no significant difference in the amount of THP-1 expressed in the IBD dogs versus CG2 (p=0.7413). Although there was a trend towards a lower relative target sequence expression value for SERT in the dogs with IBD, there is no significant difference found (p=0.34).

Overall, in this study we were able to demonstrate that there was increased expression of 5HT and CgA in the small intestine of IBD dogs versus that of CG1, that correlated with previous studies in humans. There was a decrease in the relative target sequence expression of 5HT2B in the IBD dogs. We were unable to establish a significant difference in the expression of THP-1 and SERT between the two groups.
Expression of Serotonin, Chromogranin-A, Serotonin Receptor-2B, Tryptophan hydroxylase-1, and Serotonin Reuptake Transporter in the Small Intestine of Dogs with Inflammatory Bowel Disease

by
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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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Candice Bailey, Author
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DEDICATION

I would like to dedicate this work to my grandfather who taught me the true meaning of perseverance and hard work. I would like to thank my parents for their continual support and encouragement.
INTRODUCTION

The gastrointestinal (GI) tract is the only organ that manifests complex integrative behaviors and reflexes in the absence of input from the central nervous system, mainly due to the enteric nervous system. The neuroendocrine system of the gut plays a significant role in regulating intestinal motor and sensory functions, as well as modulating inflammation. A major contributor in this system is serotonin (5HT), a monoamine neurotransmitter produced in the central nervous system and enterochromaffin cells (ECC). The significance of 5HT in health and disease has been under recognized in veterinary medicine, which is in contrast to human medicine where it has been studied for over half a century. The intestinal mucosa is the dominant site of 5HT synthesis in mammals, with more than 95% of the intestinal 5HT present in the ECC. When released from ECCs, 5HT acts locally on the intestinal tract, participating in the regulation of intestinal motility, fluid secretion, and regional blood flow. 5HT excess is associated with autonomic hyperactivity of the intestinal tract. Although the role of 5HT varies in the pathophysiology of GI diseases, two of its most important roles are its influence on GI motility and modulation of the immune system. It has been previously shown how the immune system can influence 5HT-expressing ECC biology; however, 5HT can also influence the immune system, as there are many serotonergic receptors found on various immune cells.

Canine idiopathic inflammatory bowel disease (IBD) encompasses a group of chronic enteropathies characterized by persistent or recurrent clinical signs of GI disease of unidentified cause which is associated with histologic evidence of inflammatory
infiltration of the intestinal mucosa. It is not an uncommon disease, with long-term management being challenging in many cases. Continuing to explore possibilities to enhance therapy of this condition is exceedingly relevant. Because of its effects on the intestine, 5HT can be a therapeutic target to help control the clinical signs of dogs with IBD. Its intricate origin and metabolism allows for different ways to alter its expression. 5HT is produced from tryptophan in a 2-step process in which hydroxylation by tryptophan hydroxylase (THP-1) is the rate-limiting step. To facilitate the actions of 5HT and prevent receptor desensitization, intestinal 5HT transporter (SERT), causes rapid re-uptake of 5HT. There is evidence that supports downregulation of SERT in inflammatory or diarrheal disorders.

One aim of the study was to compare the expression of 5HT and Chromogranin-A (CgA) in the intestine between a group of dogs diagnosed with IBD, and a group of healthy dogs (CG1). Another aim was to compare the expression of 5HT$_{2B}$, THP-1, and SERT, in the intestine between a group of dogs diagnosed with IBD, and a second group of healthy dogs (CG2). We hypothesized that 5HT and CgA expression would be increased in the intestine of IBD dogs versus that of CG1, and that there would be an altered expression of 5HT$_{2B}$, THP-1, and SERT, between the IBD dog group versus CG2.
LITERATURE REVIEW

Gastrointestinal Physiology

The GI tract is a generously proportioned system that passes through the entire length of the body. It is composed of many parts, all of which have specific functions that include grasping and shearing of food in the oral cavity, passage of food through the esophagus, temporary storage in the stomach, digestion and absorption of nutrients in the small intestine, and absorption of water in the large intestine.

The motor functions of the GI tract are performed by the different layers of smooth muscle. From the outer surface inward the layers are the following: serosa, a longitudinal smooth muscle layer, a circular smooth muscle layer, the submucosa, and the mucosa. Although the smooth muscles of the GI tract share general characteristics and function of smooth muscle elsewhere in the body, there are specific characteristics of GI smooth muscle that make this organ very efficient and successful at its functions. Specifically, the specific characteristics involve its syncytial composition and the makeup of its electrical activity.  

The individual smooth muscle fibers in the GI tract are 200 to 500 micrometers in length and 2 to 10 micrometers in diameter, and they are arranged in bundles of as many as 1000 parallel fibers. Within each bundle, the muscle fibers are electrically connected with one another through large numbers of gap functions that allow for low-resistance movement of ions from one muscle cell to the next. This ultimately allows movement along the length of the bundle to be more rapid.
Although the GI tract is under the influence of the central nervous system, it can function in isolation because it has its own nervous system. GI absorption, secretion, motility, and sensation are regulated and coordinated by the enteric nervous system. This system lies within the wall of the GI and extends its entire length. There are two plexuses that compose the enteric nervous system. An outer plexus lies between the longitudinal and circular muscle layers, called the myenteric plexus (Auerbach’s plexus), and an inner plexus is called the submucosal plexus (Meissner’s plexus). Along with these plexuses there is an intricate network of intrinsic and extrinsic afferent neurons, interneurons, and motor neurons, which interact with longitudinal and circular smooth muscle and mucosal endocrine cells. Submucosal intrinsic primary afferent neurons, which innervate the secretory epithelium, are critical in the initiation of both secretory and peristaltic reflexes, while myenteric intrinsic primary afferent neurons may participate in the regulation of peristalsis through the initiation of giant migrating contractions. Myenteric motor neurons innervate smooth muscle via the interstitial cells of Cajal, also known as the gut pacemaker cells, and regulate mechanical activity.

**Serotonin and the Enterochromaffin Cell**

It is well established that normal secretory, absorptive, and motor functions of the GI tract are controlled by a complex combination of regulatory mechanisms that are chemically mediated. These regulatory chemical messengers, GI hormones and neurotransmitters, are usually either biogenic amines or polypeptides, and are normally present in the nerve terminals of the myenteric plexuses and in endocrine cells dispersed within the mucosal lining of the GI tract. The enterochromaffin cell (ECC) is the
enteroendocrine and neuroendocrine cell occurring in the GI tract. These cells are derived from the same stem cells as the rest of the epithelium, and are not derived from the migratory neural crest source that provides the enteric nervous system. The ECC population includes several different sub-populations, and morphological differences in shape, luminal endings and secretory granules suggest region-specific functions.

The main secretory product of ECC is 5HT, and as stated previously, more than 95% of the intestinal 5HT is present in the ECC. Of all the endogenous factors influencing GI function, 5HT is specifically pertinent. It is a hormone, a neurotransmitter, and a mitogen that has been shown to modulate GI motility, peripheral vascular tone, cerebral vascular tone, and platelet function. 5HT is produced in two steps. In the first step, the essential amino acid tryptophan is hydroxylated to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase. This enzyme occurs in two isoforms, tryptophan hydroxylase 1 (THP-1) and tryptophan hydroxylase 2, which are responsible for the non-neuronal and neuronal synthesis of serotonin, respectively. This is the rate limiting step. The cofactors oxygen and tetrahydropteridine are required for this reaction to occur. In a second step 5-HTP is in turn converted to 5HT via the enzyme L-amino acid decarboxylase.

Microvilli present on the apical end of the ECCs project into the lumen and function as sensors of luminal content, turning the physiochemical signals of the lumen into biochemical endocrine signals. The released 5HT from ECCs either enters the lumen or lamina propria where it can act upon enterocytes or cells of the enteric nervous system and initiate secretion and enteric pulsation patterns. The complex interaction
of the microbiota, diet, and the cells of the intestine all have an influence on 5HT synthesis, release, and degradation, and therefore all may be responsible for the altered 5HT function seen in many GI diseases.\textsuperscript{23}

It is critical to maintain optimal extracellular availability of 5HT in the gut to facilitate its physiological actions and prevent receptor desensitization.\textsuperscript{24} This is predominantly the responsibility of the intestinal serotonin transporter (SERT), that plays an important role in the clearance of 5HT by its rapid uptake through an apically localized, fluoxetine-sensitive NaCl-dependent transport process.\textsuperscript{25-27}

\textit{Chromogranin-A}

CgA is a 68-kDa protein comprising 439 amino acid residues, which was isolated for the first time from secretory granules of the bovine adrenal medulla.\textsuperscript{28,29} It is co-stored and co-released with monoamines and peptide hormones of the adrenal medulla, pituitary gland, parathyroid, thyroid C-cells, pancreatic islets, endocrine cells of the gastrointestinal tract and sympathetic nerves.\textsuperscript{28,30} Often, in the world of research, serum and tissue content of CgA serve as a marker for gut endocrine cells, including ECCs, and tumors such as pheochromocytoma. CgA has been considered the best general neuroendocrine serum or plasma marker available both for diagnosis and therapeutic evaluation and is increased in 50–100% of patients with various neuroendocrine tumors. Levels of CgA reflect tumor load, and it may be an independent marker of prognosis in patients with midgut carcinoids.\textsuperscript{31}
Canine Inflammatory Bowel Disease

Canine idiopathic IBD encompasses a group of chronic diseases of the intestinal tract characterized by histologic evidence of inflammatory infiltration that also involve continual or repeated GI clinical signs such as vomiting, diarrhea, anorexia, and/or weight loss. It is classified based on the predominant cell type found in the intestinal wall, and in the small intestine most commonly includes lymphocytic-plasmacytic enteritis and eosinophilic enteritis. In the large intestine, conditions include lymphocytic-plasmacytic colitis, eosinophilic colitis, and histiocytic ulcerative colitis. Along with the small and large intestine, the inflammation can involve the stomach and can include lymphocytic-plasmacytic gastroenteritis and eosinophilic gastroenteritis. Often, multiple locations of the gut are involved leading to a gastroenterocolitis or an enterocolitis. Because of the inflammation, normal physiology of the gut is interrupted and clinical signs ensue.

Although the etiology of IBD is not completely understood, based on veterinary literature, it appears to capture a multifactorial etiology, including genetic predisposition, dysbiosis or microbial imbalance in the gut, allergy to a dietary protein source, and mucosal inflammation. One or more than one of these causes may be identified or at least suspected in a patient. Because the gut is constantly exposed to foreign material, mainly food, there has to be a system in place to prevent constant triggering of the immune system. This homeostasis is maintained by tolerance. Tolerance is the equilibrium between the reactions to pathogens and to commensal bacteria or other inoffensive luminal antigens. The difference between tolerance and reaction has been shown to be
based on pattern recognition receptors (PRRs), which are proteins expressed by cells of the innate immune system. These PRRs are able to recognize microflora based on their pathogen-associated molecular patterns or microbe-associated molecular patterns. The difference between tolerance and reaction is also based on whether an antigen is presented to the immune system or not.

Toll-like receptors (TLRs) are a type of PRR that mediate the recognition of extracellular or endosomal pathogen-associated molecular patterns. When TLRs are quickened, they initiate pro-inflammatory activity through cytokine production and other co-stimulatory molecules. It has been shown that three TLRs (2, 4, 9) stimulated by bacteria are up-regulated in dogs with IBD. It has also been shown that IBD dogs have different small intestinal bacteria from those in healthy dogs. This supports the impression that there is a connection between microflora and IBD.

Currently, the main goal of treatment for IBD is to decrease the amount of inflammation present in the gut. Because of this, it becomes very important to be able to assess the amount of active inflammation present either prior to initiating therapy or once therapy has begun. This has proven to be extremely difficult. When used together, endoscopic evaluation with histopathologic evaluation of tissue samples is the gold standard for identifying and measuring intestinal inflammation. However, it tends to be difficult to arrive at a consensus on histopathologic evaluation of IBD in dogs. In order to standardize histopathologic evaluation of dogs with IBD, the World Small Animal Veterinary Association International Gastrointestinal Standardization Group has developed criteria to determine the type and amount of inflammatory cell infiltrate and
structural changes in tissue. In addition to histopathologic examination, there are other potential monitoring tools that have been utilized and include clinical scoring indices (clinical inflammatory bowel disease activity index and canine chronic enteropathy clinical activity index), abdominal ultrasound, serologic markers (C-reactive protein, albumin, cobalamin and folate), and fecal markers (calprotectin, S100A12, alpha 1-proteinase inhibitor, N-Methylhistamine).

As previously mentioned, treatment for idiopathic canine IBD involves controlling the inflammation that is present, thus controlling the clinical signs, and as a result improving quality of life. Treatment involves both dietary and pharmacologic interventions as well as therapeutic manipulation of the enteric microbiota through the use of antibiotics like metronidazole and tylosin, and soluble fiber supplements. Most dogs with advanced IBD are not controlled by dietary therapy alone. Thus, there are various medications utilized to control active inflammation in these patients; and because the inflammation is believed to be due to an overactive immune system, immunosuppressive medications are often used. Patients with mild to moderate disease may respond to prednisone or prednisolone therapy alone. Prednisone is a glucocorticoid that is commonly used because its side effects are well known, dogs tend to respond favorably, and it is affordable for owners. However, because of the side effects of glucocorticoids, including polyuria, polydipsia, polyphagia, weight gain, anxiety, and muscle wasting, or if severe disease is present, combination drug therapy can be used. Along with glucocorticoids, azathioprine, another immunosuppressive drug, is used for therapy. Azathioprine is a purine analog, and the accepted mechanism of action is at the
level of DNA. It becomes incorporated into replicating DNA and can also block the de novo pathway of purine synthesis. It is this action that is thought to contribute to its relative specificity to lymphocytes due to their lack of a salvage pathway. The combination of prednisolone and azathioprine has been described in dogs. Other rescue therapies for canine IBD include cyclosporine, methotrexate, and cyclophosphamide, which have been described in dogs with IBD as well. Cyclosporine has potent immunosuppressive properties, and has the ability to block the transcription of cytokine genes in activated T cells. It is well established that cyclosporin inhibits the phosphatase activity of calcineurin, a compound that activates T cells. Methotrexate, more commonly used in human medicine, has multiple mechanisms of action, including inhibition of purine and pyrimidine synthesis, suppression of transmethylation reactions with accumulation of polyamines, reduction of antigen-dependent T-cell proliferation, and promotion of adenosine release with adenosine-mediated suppression of inflammation. Cyclophosphamide is an alkylating agent that has been shown to suppress the immune response by attaching an alkyl group to the guanine base on DNA, that leads to irreversible cell death. Another alternative therapy for refractory IBD is another drug in the glucocorticoid family called budesonide. Although budesonide is a glucocorticoid, it allows attainment of maximal therapeutic effect of conventional steroids while minimizing deleterious systemic effects, due to a significant first pass hepatic metabolism. Thus this drug is an option for dogs who do not tolerate prednisone well.
The prognosis for IBD in dogs is good for controlling the clinical signs. Because there is no cure for IBD, this disease requires chronic management. Patients can have relapses of clinical signs that can affect overall quality of life, and in some cases lead to humane euthanasia. It is one of the most common chronic GI diseases diagnosed and treated in dogs. Understanding the pathophysiology along with treatment and monitoring strategies contribute to allowing further exploration into acquiring more knowledge about this disease. Also, it contributes to aiding veterinarians in providing the best quality of life possible for patients with IBD as well as for owners of dogs with IBD.
MATERIALS AND METHODS

Study population

For the retrospective aspect of the study, the medical database at the Lois Bates Acheson Veterinary Teaching Hospital at Oregon State University College of Veterinary Medicine was searched for dogs diagnosed with IBD from 2002-2011. Criteria for selection of dogs with IBD included histopathologic evidence of IBD and the presence of a complete medical record. Medical records were reviewed and information related to the following was obtained: signalment, physical examination findings, and history of clinical signs. Disease severity was determined by use of the canine inflammatory bowel disease activity index (CIBDAI), which included assessment of the following factors: attitude and activity, appetite, vomiting, fecal consistency, fecal frequency, and weight loss. The scores of these six factors were totaled to reveal an overall composite score, which indicated clinically unimportant (score, 0 to 3), mild (score, 4 to 5), moderate (score, 6 to 8), or severe (score, 9 or greater) clinical signs of IBD.51 For the prospective aspect of the study, mucosal biopsy specimens or full thickness biopsy specimens were taken from dogs presenting to Lois Bates Acheson Veterinary Teaching Hospital at Oregon State University College of Veterinary Medicine from September 2005-August 2012 with suspected IBD based on history and diagnostics. A diagnosis of IBD was based on histopathologic evidence of intestinal inflammatory cell infiltrates in the mucosa. Seventeen mixed-breed dogs owned by Texas A&M University College of Veterinary Medicine Gastrointestinal Lab, euthanized for an unrelated project, were included in the healthy control groups (CG1 and CG2). CG1 and CG2 were judged
healthy based on an unremarkable physical examination and the absence of clinical signs consistent with gastrointestinal disease.

**Sample collection**

For the retrospective aspect of the study, small intestinal biopsy specimens were obtained endoscopically or by way of laparotomy, from the duodenum, jejunum, and/or ileum for histologic examination as part of a standard esophagastroduodenoscopy procedure or abdominal exploratory surgery, performed at Oregon State University. Specimens were placed in 10% neutral-buffered formalin, routinely processed, and stained with hematoxylin and eosin (H&E) for histologic examination. For the prospective aspect of the study, dogs were prepared for gastroduodenoscopy by fasting for 12–18 hours. Four to six mucosal biopsy specimens were obtained from the proximal to middle segments of the duodenum of each dog. One to two mucosal biopsy specimens from the duodenum of each dog were placed in a commercial liquid RNA preservative immediately after collection and set aside at −80°C until isolation of RNA. The remaining specimens were placed in 10% neutral-buffered formalin, routinely processed, and stained with H&E for histologic examination. Ten small intestinal full thickness biopsy samples from nine control dogs were routinely processed, and stained with hematoxylin and eosin (H&E) for histologic examination. Small intestinal full thickness biopsy samples from eight control dogs were placed in a commercial liquid RNA preservative immediately after collection and set aside at −80°C until isolation of RNA.

All H&E stained sections of intestinal biopsy samples (IBD and CG1) were assessed and characterized by use of histopathologic guidelines for the evaluation of GI
inflammation set by the World Small Animal Veterinary Association (WSAVA) International GI Standardization Group standardized criteria\textsuperscript{40} to determine the type and amount of inflammatory cell infiltrate and structural changes in tissue. All intestinal mucosal biopsy specimen sections were evaluated by a board-certified veterinary pathologist (BV) and a small animal internal medicine resident (CB).

**Immunohistochemistry**

**Serotonin**

Tissues were sectioned at 4-5 \textmu{}m and sections were collected on charged slides and baked at 60°C for 1 hour. Slides were rehydrated through 2 changes of xylene, 2 changes of 100% ethanol, 1 change of 80% ethanol and water. Slides were placed on the Dako Autostainer and washed in TBST (Biocare Medical, TBW945M) followed by 3% H$_2$O$_2$ (Sigma) in TBST for 10 minutes. Samples were exposed to Proteinase K (Dako s3020) for 5 minutes, washed in TBST, blocked with Dako serum-free protein block (x0909) for 10 minutes and finally blown dry with air. The primary antibody (mouse) to 5HT (Abcam ab16007) was diluted 1:40 in Dako antibody diluent (s3022) and applied to the slide for 30 minutes at room temperature. After washing in TBST, Dako Mouse Universal Negative control (N1698) was used as the negative control. After washing in TBST, MaxPoly-One polymer HRP anti-mouse secondary antibody (MaxVision Biosciences) was applied to the slide for 10 minutes at room temperature and then the slide was washed in TBST. The chromogen Nova Red (Vector Laboratories, SK-4800) was applied to the slide for 5 minutes and the slide was washed in dH$_2$O. The samples
were stained with Dako hematoxylin (s3302) diluted 1:3 in dH2O for 5 minutes, rinsed in dH2O, rinsed in TBST to blue.

**Chromogranin-A**

Tissues were sectioned at 4-5 µm and sections were collected on charged slides and baked at 60°C for 1 hour. Slides were rehydrated through 2 changes of xylene, 2 changes of 100% ethanol, 1 change of 80% ethanol and water. High temperature antigen retrieval was performed in a microwave pressure cooker (Viking Tender cooker) using Dako Target Retrieval solution (s1699) for 10 minutes after pressure was reached. Pressure cooker was slowly vented and the container holding the slides was allowed to sit for 20 minutes at room temperature. Slides were placed on the Dako Autostainer and washed in TBST (Biocare Medical, TWB945M) followed by 3% H2O2 (Sigma) in TBST 10 minutes, Dako serum-free protein block (x0909) for 10 minutes and blown with air. The primary antibody (rabbit) to CgA (Dako A0430) was diluted 1:1000 in Dako antibody diluent (s3022) and applied for 30 minutes at room temperature. MaxPoly-One polymer HRP rabbit (Max Vision Biosciences) was applied for 10 minutes at room temperature and again washed in TBST. The chromogen Nova Red (Vector Laboratories, SK-4800) was applied for 5 minutes and washed in dH2O followed by Dako hematoxylin (s3302) diluted 1:3 in dH2O for 5 minutes, rinsed in dH2O, rinsed in TBST to blue, run down to xylene and coverslipped.
RNA extraction and cDNA synthesis

Total RNA was extracted from one intestinal mucosal biopsy sample of each dog (IBD group and CG2) by use of a commercial RNA extraction kit in accordance with the manufacturer's protocol for extraction of RNA from animal tissue. The concentration of RNA was determined by measuring the absorbance at 260 nm ($A_{260}$) in a spectrophotometer. One microliter of RNA-containing solution was applied directly to obtain first-strand cDNA by use of specially optimized mix of oligo-dT and random primers in accordance with the manufacturer's instructions.

QRT-PCR Assays

5HT$_{2B}$ receptor (5-HT$_{2B}$), THP-1, and SERT were assayed with asymmetric cyanine dye–based 2-step real-time QRT-PCR assay. For this assay, cDNA prepared from each total RNA sample was used. Gene-specific primer sets previously published for 5-HT$_{2B}$ and THP-1 were used.$^{52}$ An individual primer assay was used to analyze SERT. Target sequences in cDNA samples were detected with QRT-PCR assay by use of a dye mixture. Expression of the canine cYWHAZq gene (Appendix) was used as the reference house gene. Each cDNA sample was diluted to a concentration of 1.0 ng/µL prior to QRT-PCR assays. Addition of 2.5 µL of cDNA to each 10-µL assay mixture resulted in a final cDNA concentration of 0.25 ng/µL. The reaction mixture also contained forward and reverse primers 5µM each (Appendix), Power SYBR mix,$^{5}$ and nuclease-free water. The cDNA samples and negative control samples without template sequences were placed in triplicate in wells of 96-well QRT-PCR assay plates. Plates were placed in a sequence detection system. 5HT$_{2B}$ and THP-1 reaction mixtures were
exposed to the following thermoprotocol: 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C, then melt curve analysis at 60°C. Based on the primer assay recommendations, SERT reaction mixtures were exposed to the following thermoprotocol: 15 minutes at 95°C, 40 cycles of 15 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C, then melt curve analysis at 60°C. Cycle values were determined for each target using of software, and relative quantitative analysis was performed utilizing custom spreadsheet files. Based on assessment of melt curves, signals were excluded if thought to be created from primer-dimer formation. The relative target sequence expression values were normalized relative to cYWHAZq gene expression.
RESULTS

Study population

Two hundred thirty-seven dogs diagnosed with IBD were identified from the database review. Two hundred twenty-four dogs were excluded due to incomplete records, unavailability of records, and/or inadequate sample size of biopsy specimens available for review. Eleven GI biopsy specimens from nine dogs were included in the retrospective aspect of the study. All of the dogs had evidence of inflammation within the intestinal mucosa and a histopathologic diagnosis of IBD. A summary of histopathologic scores of the IBD dogs is shown in Table 1. There were 3 spayed females and 6 neutered males. The mean age was 6.2 years (range, 1 to 14 years). Breeds included one of the following: Australian shepherd, Basset hound, Bernese mountain dog, Bichon frise, Border collie, Boxer, Cockapoo, Labrador retriever, Shih tzu. There was a mean CIBDAI score of 4.6. The mean serum albumin concentration was 2.9 g/dL (range, 2.2 to 3.2 g/dL).

GI biopsies of eleven dogs were identified for the prospective aspect of the study. Based on normal histopathology examinations, 4/11 dogs were excluded. Biopsy specimens of 7/11 dogs were included in the prospective aspect of the study. There were 3 spayed females and 4 neutered males. The mean age was 9.3 years (range, 4 to 14 years). Breeds included one of the following: American Staffordshire Terrier, Border collie, German Shepherd Dog, Golden Retriever, Lhaso Apso, Schnauzer, Shetland Sheepdog. There was a mean CIBDAI score of 4.4. The mean serum albumin concentration was 2.8 g/dL (range, 2.2 to 3.2 g/dL).
CG1 and CG2 included nine and eight healthy control dogs, respectively, from an unrelated project. Both groups included mixed breed, adult dogs in which the age and neuter status were unknown. Results of physical exams were unremarkable and results of the histologic examination of intestinal biopsy specimens obtained from these control dogs were unremarkable.

**Immunohistochemistry Assays**

Both the IBD group and CG1 showed strong IHC staining for 5HT (Fig 1A, 1B). The number of 5HT immunopositive cells was overall significantly increased ($p<0.001$) in the IBD group compared to CG1 (Fig 2). The mean 5HT immunopositive expression for the IBD group and CG1 was 2.1 cells/hpf (1.1-3.1 cells/hpf) and 1.0 cells/hpf (0.40-2.15 cells/hpf), respectively. Both the IBD group and CG1 showed strong IHC staining for CgA (Fig 1C, 1D). The number of CgA immunopositive cells was overall significantly increased ($p<0.05$) in the IBD group compared to CG1 (Fig 3). CgA immunopositive expression for the IBD group and CG1 was 2.6 cells/hpf (1.5-4.4 cells/hpf) and 1.7 cells/hpf (0.90-3.50 cells/hpf), respectively. There was a significant correlation between 5HT and CgA positive cells/hpf across all dogs (Fig 4; Pearson $r^2=0.2433; p=0.016$).

**QRT-PCR Assays**

The $5HT_{2B}$ receptor expression was significantly lower in the dogs with IBD ($p<0.05$). The RQ values in the IBD group overall were approximately 0.5 times those of CG2. The $5HT_{2B}$ mean relative target sequence expression value was 0.5 for the IBD
group and was 1.1 for CG2. There was no significant difference in the amount of THP-1 expressed in the IBD dogs versus CG2 (p=0.7413). The mean relative target sequence expression value was 2.9 for the IBD group and was 1.0 for CG2. There was no significant difference in the expression of SERT in the IBD group versus CG2. The mean relative target sequence expression value of SERT was 0.78 for the IBD group and was 1.33 for CG2.
Figure 1. Photomicrographs of IHC staining for 5HT in an IBD dog (A), CG1 dog (B), and IHC staining for CgA in an IBD dog (C), CG2 dog (D).
Figure 2. Vertical scatter plot of average 5HT-positive cells/HPF in the group and CG1.
Figure 3. Vertical scatter plot of average CgA-positive cells/hpf in the group and CG1.
Figure 4. Correlation plot demonstrating that average 5HT-positive and average CgA-positive cells/hpf showed a significant correlation ($r^2=0.243$, $p=0.016$).
Table 1. Summary of histopathologic scores based on the WSAVA standards in eleven duodenal mucosal biopsy samples of the nine IBD dogs from the retrospective study. IBD, inflammatory bowel disease; IELs, intraepithelial lymphocytes; LPLs, lamina propria lymphocytes; LPNs, lamina propria neutrophils; LPEs, lamina propria eosinophils. WSAVA, World Small Animal Veterinary Association. The numbers in the cells are representative of the number of dogs.

<table>
<thead>
<tr>
<th>Score</th>
<th>Villous Stunting</th>
<th>Epithelial Injury</th>
<th>Crypt Distention</th>
<th>Lacteal Dilation</th>
<th>Mucosal Fibrosis</th>
<th>IELs</th>
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<tr>
<td>0 (normal)</td>
<td>8</td>
<td>10</td>
<td>11</td>
<td>7</td>
<td>11</td>
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</tr>
<tr>
<td>1 (mild)</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>2 (moderate)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 (marked)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
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<td>11</td>
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<td>11</td>
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<table>
<thead>
<tr>
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<th>LPNs</th>
<th>LPEs</th>
<th>Total WSAVA Score</th>
</tr>
</thead>
<tbody>
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<td>8</td>
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<td>≤4 (insignificant)</td>
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<tr>
<td>1 (mild)</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>5-9 (mild)</td>
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<tr>
<td>2 (moderate)</td>
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<tr>
<td>3 (marked)</td>
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<td>0</td>
<td>0</td>
<td>15-19 (severe)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>≥20 (very severe)</td>
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<td>11</td>
<td>11</td>
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</tbody>
</table>
DISCUSSION

This study demonstrated that there was increased expression of 5HT and CgA in the ECCs of the small intestine of the IBD group versus that of the control group. Also, the relative expression of 5HT$_{2B}$ was decreased in the IBD versus the control group. There was no significant difference in the relative expression of THP-1 or SERT between the groups.

From the IHC aspect of this study, we were able to demonstrate that the expression of both 5HT and CgA is significantly increased in the intestine of IBD dogs versus the control population. 5HT is thought to activate the submucosal sensory branch of the enteric nervous system, and control and accelerate GI motility via interneurons and motor neurons. CgA is a protein that is co-stored and co-released with monoamines and peptide hormones of various endocrine cells, including those of the GI tract. CgA thus serves as a marker for intestinal endocrine cells. In a human study, the density of CgA-immunoreactive cells in patients diagnosed with lymphocytic colitis was significantly higher than that in control patients. Although the study did not identify the specific cell type to which this increase may be credited, the cells that contain 5HT are the major endocrine cell type in the colon and constitute approximately 88% of the total endocrine cell population as revealed by CgA. It is plausible that the increase in colonic CgA in the lymphocytic colitis patients accounts for an increase in 5HT cells. Thus, it is possible that colonic hormones, and almost certainly 5HT, are involved in the pathophysiology of this disease in humans. If this set of circumstances stands, clinical signs of diarrhea, abdominal cramping and nausea could be explained on this basis.
in the colon, 5HT containing ECCs comprise the single largest endocrine cell population in the small intestine. In the case of IBD involving the small intestine of dogs, 5HT involvement could explain the clinical signs in these patients.

In human medicine, IBD includes two chronic GI diseases, ulcerative colitis and Crohn’s disease. These diseases are chronic inflammatory conditions of unknown etiology. Inflammation of the intestinal mucosa has been shown to affect 5HT signaling in animal models and humans. Changes in ECC numbers and in 5HT content have been reported in association with Crohn’s disease and ulcerative colitis. Approximately 50% of human patients with IBD in long-standing remission have irritable bowel syndrome-like symptoms (diarrhea, constipation, discomfort), which may be related to these inflammation-induced alterations in ECCs and 5HT signaling. It has also been shown that consumption of selective 5HT reuptake inhibitors is associated with microscopic colitis. The juncture between 5HT-expressing ECCs and the immune responses that drive IBD are principal to the pathogenesis of this disease.

We hypothesized that the expression of THP-1 in the IBD group would be altered compared to the control group. However, in this study, there was no significant difference in the mean relative target sequence expression of THP-1 between the groups. This finding is different than what has been documented in a human study, in which levels of mRNA encoding THP-1 was decreased in ulcerative colitis and IBS specimens relative to those from healthy controls patients. Irritable bowel syndrome (IBS) is a condition in humans usually diagnosed based on symptoms, including diarrhea, constipation, bloating, and discomfort. While IBS and IBD have distinct pathophysiologic properties, shared
defects in 5HT signaling may underlie the altered motility, secretion, and sensation in the GI tract. 5HT has been shown to be a major player in this alteration. Diarrhea-predominant IBS is associated with elevated 5HT whereas constipation-predominant IBS is associated with decreased levels of 5HT in the colon mucosa. Recent clinical trials using a small molecule inhibitor of THP-1 have shown the ability to alleviate the symptoms associated with IBS, especially diarrhea predominate IBS. Treatment with THP-1 inhibitor decreased blood 5HT level, relieved discomfort, and increased stool consistency. Modulation of tryptophan metabolism, especially 5HT synthesis may be a novel target for developing therapies for GI disorders. In this study, the lack of a significant difference in THP-1 expression between the two groups could be due to the small population size. Also, although based on histopathologic findings the IBD group had evidence of mucosal inflammation, the CIBDAI guidelines (Table 1) identified that most of the dogs had clinically insignificant or mild disease. It could be possible that more severely affected dogs may demonstrate altered THP-1 expression. Although THP-1 was not altered in the IBD group, inhibiting this enzyme in dogs diagnosed with IBD, could potentially decrease the expression of 5HT and therefore relieve clinical signs associated with the disease including anorexia considering 5HT is anorexigenic. This may prevent or delay the use of glucocorticoids in some IBD dogs, and therefore potentially avoid serious side effects including, secondary infections, muscle wasting, and thromboembolic disease.

The relative target sequence expression of 5HT2B in the IBD dogs was decreased compared to CG2. In the gut, activation of 5HT2B leads to contraction of smooth muscle
of the gastric fundus and the small intestine.\textsuperscript{73} Also, epithelial cells in intestinal crypts have been found to express 5HT\textsubscript{2B}. Crypt epithelial cells represent a site where 5HT\textsubscript{2B} is likely to affect growth and/or differentiation. The crypts of the gut contain a self-renewing stem cell population that continually differentiates throughout life to replace the cells that line the luminal surface of the small and large intestines.\textsuperscript{74} It is somewhat surprising that this receptor expression is decreased in the IBD population compared to normal dogs. Taking into account the effects of chronic inflammation on the enterocytes, one would consider that perhaps there would be a greater need for self-renewing of enterocytes during this diseased state. With this thought in mind, an increased expression of 5HT\textsubscript{2B} would suit this theory. However, another concept is that the decreased expression of 5HT\textsubscript{2B} could potentially manifest as a decreased epithelial cell self-renewing and therefore a decreased ability for patients with chronic inflammation in the intestine to replace damaged enterocytes and be more susceptible to pathogen invasion and diarrhea. The receptors could also be damaged from the inflammation that’s present and thereafter clinical signs worsen.

It was hypothesized that SERT expression would be altered in the IBD group. Although there was a trend towards a lower relative target sequence expression value for SERT in the dogs with IBD, there is no significant difference found. In this case, there is a lack of a difference between the groups when one would expect there to be. Considering the effects of 5HT on the gut, there should conceivably be an upregulation of SERT, in order to decrease the amount of mucosal 5HT available. However, it has been reported that mRNA encoding SERT and SERT protein are diminished in an animal
model of colitis. SERT expression has also been shown to be decreased in human patients with IBD (ulcerative colitis). In the current study, IHC showed increased 5HT expression in ECC of the dogs with IBD. Perhaps this change could be due to decreased expression of SERT, which could increase availability of 5HT, and exacerbate clinical signs of IBD including hypermotility and diarrhea. One control dog had an unexplained increase in SERT expression that was an apparent outlier. With removal of this outlier, the trend towards increased expression of SERT in the IBD group does not exist.

There were inherent limitations to the study. The retrospective nature prevented the availability of complete records of some patients that would have been included otherwise. Records from a large number of samples submitted from outside sources were not available. Also, there was a small population size, and a larger population size may allow a difference to identified between groups, when evaluating THP-1 expression. This study also lacked dogs with more severe disease. This would allow for comparison of severity of disease between groups. There could potentially be more of a difference in 5HT2B expression or a difference in THP-1 expression in more severely affected dogs.

One main limitation for inclusion into the retrospective aspect of the study specifically, was poor, undersized endoscopic biopsy mucosal tissue samples retrieved via endoscopy. During the study period, this issue was identified, and Boston Scientific biopsy forceps replaced the previous forceps, in an attempt to obtain larger biopsy samples and reduce crush artifact. If these biopsy forceps were used during the entire study period, perhaps the number of dogs included would have been larger.
CONCLUSION

In conclusion, in this study we were able to demonstrate that there was increased expression of 5HT and CgA in the small intestine of IBD dogs versus that of a control group that correlated with previous studies in humans. There was a decrease in the relative target sequence of 5HT2B in the IBD dogs. However, there was no significant difference in the relative target sequence of THP-1. Although there was no significant difference in THP-1 expression, inhibition of this enzyme could provide a specific mechanism by which to decrease 5HT levels in the GI tract and therefore control clinical signs in dogs with IBD. The limitation of a small study population could have prevented the discovery of alterations of THP-1 between the groups. Further investigation with a larger, more severely affected population could potentially allow demonstration of significant alterations in THP-1.
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APPENDIX
Primer sequences used for QRT-PCR amplification of mRNA of 5HT_2B, THP-1, and SERT genes in intestinal mucosal biopsy samples.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Probe Sequence (5' to 3')</th>
<th>Description</th>
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<td>5HT_2B receptor</td>
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<td>Forward</td>
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<tr>
<td>THP-1</td>
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<td></td>
<td></td>
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<tr>
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<tr>
<td>SERT</td>
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<td></td>
<td></td>
</tr>
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</tr>
<tr>
<td>cYWHAZq</td>
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<td>Reverse</td>
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