Neuroimmune Crosstalk: A Role for Neuropeptide Y in Inflammatory Bowel Disease

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NEUROIMMUNE CROSSTALK: A ROLE FOR NEUROPEPTIDE Y IN INFLAMMATORY BOWEL DISEASE

by

Henry H. Ruiz

A dissertation submitted to the Graduate Faculty in Neuropsychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

NEUROIMMUNE CROSSTALK: A ROLE FOR NEUROPEPTIDE Y IN INFLAMMATORY BOWEL DISEASE

by

Henry H. Ruiz

Adviser: Professor Susan D. Croll

Neuropeptide Y (NPY) is a 36-amino acid peptide widely expressed in the central and peripheral nervous systems. In addition to other cells, NPY is also synthesized and co-released from sympathetic nerve fibers functioning as a potent sympathetic neuromodulator. NPY has been implicated in playing important roles in the regulation of energy balance, appetite, anxiety, vascular tone, and immune cell functioning. In addition, immune cells of both the innate and adaptive immune systems express functional NPY receptors. Some immune cells can produce and secrete NPY, and genetic alteration of these receptors results in altered immune cell functioning. Its direct association with the immune system, its presence in sympathetic neurons innervating primary and secondary immune organs and its close association with vasculature, make NPY a candidate for mediating, at least in part, the neuroimmune crosstalk. The gene expression results presented here suggest that DSS is a valid model of human IBD and that pain-related behavior in the open field is closely associated with DSS-induced gene changes. Furthermore, the data suggest that NPY signaling via its Y₁ receptor plays some regulatory role in the immune process induced by DSS. Y₂ receptor antagonism resulted in a mild attenuation of immune activity but also slightly attenuated pain-related behavior.
in the open field. In sum, it appears that NPY signaling via its Y_1 and Y_2 receptors plays a role in various features of DSS induced disease.
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DEDICATION

After a long journey, I have achieved what I set out to achieve. The road to my success however was not always straight, sunny and clear. On the other hand, it was wavy and bumpy. Being an immigrant and with an obvious accent, I was once told by my introduction to biology professor that I “could never succeed in science because English is an integral part” and that I should “look for a profession in another field.” Today I stand before all people who at one point in their lives have been told that they cannot do something as what I hope is an example that if you want, you can!!! Never let words, regardless of their origin, stop you from trying.

With this in mind, I dedicate this work to the past, current and future immigrants (and those who have been told that they cannot succeed) as proof that it can be done. In particular, I dedicate this dissertation to the future generations in my family:

Joan Gomez
Yennyfer Ruiz
Jonathan Gomez
Natalia Muñoz
Santiago Muñoz
Anthony Muñoz
Brianna Ruiz

May you all believe and be as blessed as I have been!!!
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Chapter 1

INTRODUCTION

The Involvement of the Nervous System in Inflammatory Disease

The nervous and immune systems are two distinct body systems that have traditionally been thought to underlie very distinct functions and diseases. Recent research has revealed more crosstalk between the two systems than was previously appreciated. The role of neuroimmune interactions in both normal physiology and pathophysiology is becoming increasingly apparent, but research more fully elucidating the mechanisms and breadth of these functions is in its infancy. One system that is gaining increased attention in this regard is the autonomic nervous system. Because the autonomic nervous system innervates the vasculature and most peripheral organs, it has the potential to be involved in a wide range of physiological conditions. It is for this reason that many laboratories have launched into research programs designed to better understand the role of the autonomic nervous system in immune diseases. The current paper will introduce work being done to understand some of the potential mechanisms of autonomic nervous system involvement in immune disease.

The Autonomic Nervous System

The autonomic nervous system represents just one component of the nervous system. The nervous system encompasses two major systems; the central nervous system (CNS), which is made up of the brain and spinal cord, and the peripheral nervous system (PNS), which is made up of all nerves and ganglia outside the CNS. The peripheral nervous system is further subdivided into two subsystems: the somatic nervous system, which includes sensory and motor fibers, and the autonomic nervous system (ANS). The
ANS is further subdivided into three components. The two major components that often work in harmonic opposition to one another: the sympathetic nervous system (SNS) and the parasympathetic nervous system (PaNS) with the third being the enteric nervous system (ENS) and compromising neurons controlling the functions of the gastrointestinal system. Through mainly opposing mechanisms, the SNS and the PaNS play major roles in maintaining an organism’s internal environment at homeostatic balance. This maintenance occurs automatically, and is usually outside the voluntary control of the organism.

Some of the functions mediated by the SNS include pupil dilation, stimulation of hormonal release from glands, inhibition of digestion and constriction of blood vessels all characteristic of the “fight or flight” response. The PaNS on the other hand mediates the opposing effects, such as pupil constriction, stimulation of digestion and blood vessel dilation. Signaling for both systems originates in the CNS and is carried out by clusters of neurons known as preganglionic cells. For both the SNS and PaNS, the neurotransmitter secreted by preganglionic cells is acetylcholine (Ach). The preganglionic cells then project to postganglionic cells which project throughout the body to signal autonomic regulation. The neurotransmitter released by postganglionic cells is different for the two branches of the autonomic nervous system, with PaNS cells releasing Ach onto target organs whereas SNS signaling is mediated by the catecholamine, norepinephrine (NE). These two systems must work as negative feedback mechanisms in order to maintain homeostatic balance and healthy functioning.

There is growing evidence that ANS disruption is often associated with pathological states. Several cardiovascular (Abboud et al, 2012), murine inflammatory
human inflammatory diseases such as inflammatory bowel disease (Belai et al., 1997; Furlan et al., 2005), primary Sjögren syndrome (pSS), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) have been linked to alterations in ANS functioning (Stojanovich et al., 2007). The observation that ANS dysfunction is associated with several immune disease states has contributed to the perspective that the immune and nervous system interact, and may be capable of modulating each other's activity.

Though there has been evidence of nerve fibers innervating immune organs such as the lymph nodes for more than a century, only in the last forty years have there been attempts to elucidate the connection between the brain and the immune system or the neuroimmune connection (Elenkov et al., 2000). This relatively recent integration of fields of study has provided a great amount of insight into the mechanisms by which the CNS and immune system communicate, with a large body of literature pointing to the crucial involvement of the ANS as the key mediator for this cross system communication (Nance & Sanders, 2007).

**The Immune System**

The immune system is an important line of defense for an organism’s body to be protected against foreign bodies. In addition to this internal line of defense, the body also has other defense mechanisms. In the CNS, protection is largely mediated by bone encompassing the brain and spinal cord and a protective vascular barrier known as the blood brain barrier (BBB), which forms a tight selective vascular barrier. The PNS is largely unshielded from the general circulation and is vulnerable to infiltration by external factors. The skin is the body’s largest organ in charge of keeping foreign
substances out of the body. In addition, the epithelial lining of organs exposed to outside agents, such as gastrointestinal organs or lung alveoli are designed to provide a barrier to unrestricted passage of foreign substances. At times, however, these barriers can be broken as is the case during lesions or antigen infiltration in compromised barriers such as in the nasal passages or the gastrointestinal (GI) track. When this occurs, cells that make up the immune system, which are constantly surveying the internal state of the body, begin an immune response.

There are two main components of the immune system that work in concert to identify and destroy foreign threats to the body: the innate and adaptive immune systems. The innate immune response is mediated by cells that rapidly respond to any foreign threat detected in the body. This response does not lead to long lasting immunity and is not specific to any individual antigen. The innate immune response is mainly mediated by innate leukocytes (neutrophils, eosinophils, basophils, monocytes and macrophages) (Murphy, 2012). Upon recognition of a body or substance as foreign, immune cells become activated and begin a phagocytic process resulting in the degradation and removal of the substance. This response can and often does result in inflammation, an important component of most immune responses.

Inflammation is a phenomenon first identified by Cornelius Celsius nearly 2000 years ago when it was clinically described by its four cardinal signs of redness and swelling with heat and pain (rubor et tumor cum calore et dolore) (Scott, 2004). Inflammation is characterized by the presence of immune cells in the affected region, and its symptoms are thought to be mediated by the immune cells themselves. In addition to
the innate immune system, inflammation is also associated with responses of the adaptive immune system.

An adaptive immune response is initiated when antigens are able to evade the innate immune system or be incompletely cleared by the innate response. Dendritic cells are thought to be primary players in the transition between innate and adaptive responses. The adaptive immune response is primarily carried out by T and B-lymphocytes. T-Cells can be further subdivided into effector or helper T-Cells. Effector T-Cells can perform three main functions: 1) destroying cells expressing a protein antigen through cytotoxic responses, 2) activating macrophages and other immune cells for antigen removal or 3) regulatory where they help control immune responses by suppressing other immune cells. Helper T-Cells (Th) enhance the phagocytic function of macrophages and activate B-Cells by presenting the antigen on their cell surface to be recognized by B-Cells (Murphy, 2012). B-cells express large numbers of receptors on their cell surface and each B-cell has a different combination of receptors. In this manner, each B-Cell is programmed to respond to only a very select set of antigens, but there can be millions of potential antigen receptors circulating in the body at any given time to encounter antigens that have bypassed innate immunity. Upon activation of a B-Cell receptor, that cell begins a cloning process to divide and create more cells that express the same antigen receptor. When activated, B-Cells can differentiate into plasma cells that release antibody (Abs) proteins, which bind to foreign antigens, targeting them for immune attack.

It is clear that both the innate and acquired immune systems are crucial for the protection and maintenance of an organism’s health. However, when these systems fail to maintain homeostatic harmony or become dysregulated in concert, immune diseases
such as autoimmunity can occur. Autoimmunity refers to the immune system’s inability to distinguish foreign antigens from self-antigens (antigens associated with the body’s own tissue), leading to autoimmune diseases characterized by tissue damage (Murphy, 2012). Currently, there are more than 80 chronic autoimmune illnesses affecting an estimated 5-8% of the American population, rapidly increasing in prevalence and affecting women and minorities disproportionally (NIH, 2005). In light of the debilitating nature of these diseases, it is of importance to elucidate the mechanisms responsible for the development and progression of these diseases. A solid line of support for the involvement of the neuroimmune pathway in inflammatory diseases comes from clinical and experimental studies showing altered SNS function during the active phase of the disease (Boisse et al., 2009) and no difference from controls during stable disease phases (Flachenecker et al., 2001). Irregular SNS activity in humans is highly associated with autoimmune diseases such as systemic sclerosis and Raynaud’s phenomenon (Pancera et al., 1999) and these observations have also been corroborated in experimental models (Kasselman et al., 2006).

The relevance for neuroimmune crosstalk in autoimmune and inflammatory diseases has received much attention over the past four decades. Kasselman et al. (2006) and Pancera et al. (1999) both presented results suggesting that sympathetic dysfunction plays a crucial role in the development of perivascular inflammation and in autoimmunity respectively. Though the link between the CNS and SNS in these diseases has been established (Nance & Sanders, 2007), the question remains as to what underlying mechanisms are responsible for the induction of these pathological states. Emphasis is currently being placed on the neurotransmitters and neuromodulators of the SNS as
potential therapeutic targets. These include the catecholamines (epinephrine, norepinephrine), regulatory neuropeptides particularly from the Y family and their respective receptor subtypes.

**Neuropeptide Y**

Neuropeptide Y (NPY) is a neuropeptide widely expressed in both the CNS and PNS, which may play a role in neuroimmune cross talk (Bedoui et al., 2003). NPY is a 36 amino acid peptide sequenced within the last three decades and part of the larger Y peptide family consisting of NPY, peptide YY (PYY) and pancreatic polypeptide (PP) (Tatemoto, 1982). NPY is synthesized and released by neurons that, in the PNS, are predominantly sympathetic neurons. Upon NPY release, its signals are mediated via G-protein coupled receptors (GPCRs). GPCRs are seven transmembrane domain metabotropic receptors commonly found throughout the nervous system but also found in other tissues. There are five types of GPCRs for NPY (Y$_{1,2,4,5,6}$), each with a distinct pattern of distribution in the brain and periphery (Dumont et al., 1993; Jönsson-Rylander et al., 2003). These activated GPCRs initiate a downstream cascade via the G$_i$ subunit resulting in the inhibition of cyclic adenosine monophosphate (cAMP) production from adenosine triphosphate (ATP) thereby altering second messenger cascade signaling (Lobaugh & Blackshear, 1990).

In the CNS, NPY signaling has been associated with food intake, energy balance and anxiolytic effects and in the periphery it modulates metabolic (Li et al., 2012), gastrointestinal and renal functioning as well as being a potent vascular constrictor and sympathetic modulator (Michel et al., 1998). Recently, the role of NPY as a mediator of
the sympathetic link between the neuroimmune crosstalk has been explored and has generally been well supported.

Evidence for the idea of neuroimmune crosstalk being mediated by the SNS comes from observations that noradrenergic sympathetic fibers innervate the major peripheral immune organs including the thymus, bone marrow, spleen, and primary lymph nodes (Felten et al., 1985; Ericsson et al., 1987; Madden et al., 1997). In addition to the direct innervation of lymphatic tissue by the SNS, the presence and potential synthesis (Ericsson et al., 1987) of several neuropeptides including NPY at these immune organs has been reported (Felten et al., 1985). Furthermore, it has been observed that NPY is co-transmitted in large vesicles with catecholamines (CA) such as norepinephrine (NE) and epinephrine (E) in sympathetic fibers but not sensory fibers innervating the lymph nodes (Fink & Weihe, 1988). It is thought that these fibers contribute to the regulation of immune cell functioning (Straub et al., 2000). Taken together, the fact that sympathetic fibers carrying signals from the central nervous system via neurotransmitters such as NE and E and neuromodulators such as NPY innervate and have synaptic-like connections on immune organs suggests that this mechanism serves as a control and feedback mechanism between the brain and the immune system.

**Neuropeptide Y and Immunity**

NPY has also been demonstrated to play a crucial role in the direct modulation of the neuroimmune crosstalk at the cellular level. Sympathetic fibers releasing NPY have synaptic-like connections with immune cells (Phillips et al., 2012), and NPY receptors have been observed on those cells (Bedoui et al., 2002; Straub et al., 2000). It is widely accepted that the release of catecholamines and NPY from sympathetic terminals, is
dependent on the stimulation pattern. That is, NE is preferentially released during stimulation representative of baseline sympathetic functioning whereas NPY release dominates sympathetic signaling during sympathetic hyperactivity (Bedoui et al., 2002). In addition, activated monocytes, macrophages and B-lymphocytes secrete NPY, further implicating this neuropeptide in the modulation of immune responses (Schwarz et al., 1994).

The nature of NPY’s involvement during immune responses is dependent on the cell type and Y receptors expressed (Wheway et al., 2005). Several studies point to the expression of different Y receptors on different immune cell populations with $Y_1$, $Y_2$ and $Y_5$ expression being most often reported (Bedoui et al., 2002; Dimitrijevic et al., 2005; Mitic et al., 2011). Peripheral blood granulocytes such as neutrophils, eosinophils and basophils affect immune responses via $Y_1$, $Y_2$ and $Y_5$ receptors. In addition, $Y_2$ and $Y_5$ receptors appear to influence the adherence properties of granulocytes whereas $Y_2$ and $Y_1$ are reported to be important in regulating phagocytic activity (Mitic et al., 2011). Though there is clear evidence from multiple laboratories on the involvement of NPY in immune functioning, these observations have been shown to vary between in vitro and in vivo studies. Mitic et al. reported that in vivo, NPY decreases inflammatory cell adherence and phagocytosis via $Y_{2/5}$ and $Y_{1/2}$ receptors respectively as determined by pharmacological manipulations. In vitro however, the same authors report that NPY mediated enhancement of both adhesiveness and phagocytic properties of immune cells that are mediated by the $Y_1$ receptor (Mitic et al., 2011). The authors attribute these differential effects between the in vivo and in vitro assays to the possibility that in the in vivo experiment, Dipeptidyl peptidase-4 (DPP4) or CD26 activity may be cleaving NPY into
an isoform not recognized by the $Y_1$ receptor but with high affinity to the $Y_2$ receptor. This explanation is supported by reports that $Y_2$ receptor activation suppresses phagocytic activity by immune cells (Dimitrijevic et al., 2005). An alternative explanation comes from observations that during insult with the endotoxin lipopolysaccharide (LPS) and in the presence of NPY, monocyte migration is reduced at early time points (4 hrs.) but then NPY enhances migration 72 hrs. after insult (Nave et al., 2004). Mitic et al. observations took place on the same day of NPY treatment but the specific time points were not provided. Additionally, Nave et al. demonstrated that *in vivo*, NPYs enhances monocyte, macrophage and T-lymphocyte adhesion via its $Y_2$ receptor. $Y_2$ receptor selective antagonism resulted in a decreased macrophage mobilization whereas selective agonism of the same receptor enhanced macrophage mobilization. Furthermore, these authors demonstrated that $Y_2$ receptor expression on PBMCs is only apparent following activation by LPS. These observations are in conflict with Mitric et al. who report the $Y_2$ receptor to suppress cell adhesion in vivo. This discrepancy in findings may be partially explained by Nave at al. observation that $Y_2$ receptor expression does not begin until some time after PBMCs become activated. However, there is still a need for more in vivo studies to replicate and assessed these findings.

NPY has also been implicated in the mobilization of leukocytes as the application of exogenous NPY increases the adherence of macrophages and T-Cells (Nave et al., 2004) and other immune cells. Mobilization of natural killer (NK) cells, monocytes and B-lymphocytes is modulated by NPY via its $Y_1$ and $Y_5$ receptors as demonstrated by pharmacological agonism and antagonism of each of these receptors (Bedoui et al., 2002)
as well as Y₂ receptors for monocytes, macrophages and T-Cells (Nave et al., 2004). Because these leukocytes were only reported to express the Y₁ receptor, it was suggested that the Y₂ and Y₅ receptor mediated modulation of leukocyte mobilization occurs via an indirect pathway (Bedoui et al., 2002). However, Nave at al., demonstrated that Y₂ receptors though not expressed at baseline, upon immune cell activation, their transcription is initiated and the same could be the case for the Y₅ receptor, however, this question has yet to be addressed. In vitro, Y₁ activation was shown to inhibit leukocyte mobilization while Y₅ facilitates leukocyte mobilization to affected areas (Bedoui et al., 2002). In a different in vivo model of immunological responses (septic shock), it was demonstrated that monocyte mobilization initially decreases after insult (4 hrs.) but three days later monocyte count is significantly increased only in the NPY treated groups. In addition, on the fourth day, there is a 60% monocyte increase compare to the third day and 600% increase when compared to the 4 hr. time point suggesting a continuously increasing monocyte perfusion in the presence of NPY (Nave et al., 2004).

In macrophage-dependent models of degradation and phagocytic activity, NPY has been demonstrated to be necessary for activation and proper functioning of macrophages and NPY knock out mice show impaired APC functioning (Wheway et al., 2005), a phenomenon shown to be dependent on the receptor subtypes activated (Dimitrijevic et al., 2005). Dimitrijevic et al. showed that in the phorbol myristate acetate (PMA) model of macrophage degradation, the observed increases in oxidative stress by NPY are mediated by Y₁ and Y₂ receptors. However, in the zymosan-stimulated macrophage model, Y₂ and Y₅ receptors mediated the suppression of the NPY-induced oxidative stress. Y₅ also mediated oxidative stress suppression in the PMA model.
(Dimitrijevic et al., 2005). It is therefore apparent that the regulatory role of NPY in neuroimmune signaling is not homogenous but rather varies with cell type and the Y receptors expressed and activated.

Similar to the heterogeneous response of NPY depending on cell type and receptors expressed, NPY signaling in neuroimmune crosstalk is differentially modulated depending on sympathetic tone. NPY levels have often been reported to be altered in SNS-dependent inflammatory and autoimmune diseases such as RA, SLE, pSS, fibromyalgia and inflammatory bowel diseases but decreased in other diseases (Bedoui et al., 2003). Sympathetic innervation of lymphatic organs is necessary to regulate cytokine release as demonstrated by increases in pro- and anti-inflammatory cytokine release from animals exposed to chemical sympathectomy and reversal of this effect by pharmacologically preventing chemical sympathectomy (Kruszewska et al., 1995).

Sympathetic tone and NPY significantly interact to alter leukocyte mobilization. *In vivo* and *in vitro* studies looking at different doses of epinephrine in the presence of NPY have provided insight into the role of NPY under different levels of sympathetic tone. Low and moderate sympathetic tone in the presence of NPY enhances the mobilization of leukocytes such as NK, monocytes and B-lymphocytes while high sympathetic tone in the presence of NPY inhibits these effects (Bedoui et al., 2002). In addition, and as is expected from high sympathetic tone resulting in increased NPY levels, administration of high levels of NPY results in enhanced mobility of NK cells, monocytes and T-cells while low levels of NPY result in inhibition of these effects (Bedoui et al., 2001). Both of these studies suggest a direct modulation of immune cell mobilization by the sympathetic system. Though these findings appear contradictory in
that Bedoui et al. (2000) found high sympathetic tone in the presence of NPY to inhibit leukocyte mobilization and Bedoui et al. (2001) found that high levels of NPY (as would be expected in high sympathetic tone) showed increased leukocyte mobilization, the difference may lay in the baseline levels of NPY. That is, when NPY is already present, it may modulate a protective role and inhibit leukocyte migration whereas during high sympathetic tone, elevations in NPY drive leukocyte mobilization. Hence, it could be that at baseline sympathetic conditions, NPY plays a regulatory role whereas high levels of NPY during sympathetic hyperactivity may initiate proinflammatory cascades.

Sympathetic tone also has modulatory effects on macrophage-dependent cytokine release. Under low sympathetic tone, catecholamine signaling is largely mediated by the α-adrenoreceptors while high sympathetic tone preferentially activates β-adrenoreceptors. Under low sympathetic tone, NPY enhances the inhibitory effects of NE on cytokine release as measured by the macrophage production of the proinflammatory cytokine interleukin 6 (IL-6). However, under high sympathetic tone or direct pharmacological stimulation of β-adrenoreceptors, NPY enhances the proinflammatory macrophage-dependent cytokine IL-6 release (Straub et al., 2000). These results indicate that in part, sympathetic activity determines the role of NPY on neuroimmune crosstalk particularly via macrophage-dependent proinflammatory cytokine release.

In line with the data supporting a role for NPY in immune disease, preliminary data from our laboratory suggests that peripheral NPY Y₁ receptor antagonism in an experimental autoimmune model results in decreased inflammation of some peripheral organs often affected in human autoimmune diseases (Ruiz et al., 2011). NPY has also been implicated in mediating neurogenic dermal inflammation in other immune models.
Naveilhan et al. (2001) looked at capsaicin-induced inflammation (neurogenic), mustard oil induced inflammation (mixed: neurogenic and non-neurogenic component) and carrageenan induced inflammation (non-neurogenic). These authors observed a complete amelioration of dermal neurogenic inflammation, a partial suppression of dermal inflammation in the mixed model and no difference in the non-neurogenic model for mice lacking the Y₁ receptor. These findings suggest that dermal inflammation with neurogenic components is largely mediated by the Y₁ receptor. Another major inflammatory area where the Y₁ receptor has been largely implicated is in inflammatory bowel disease.

**Inflammatory Bowel Disease**

Inflammatory bowel disease (IBD) is a category of diseases characterized by chronic or recurring immune responses leading to inflammation of the gastrointestinal tract. IBD symptoms include diarrhea, cramping abdominal pain, fever, bloody stool and often, significant weight loss. Due to its detrimental effects, IBDs impose a burden on patients affecting interpersonal and intrapersonal relationships as well as posing a problem for other family members and the family’s financial status. Ulcerative colitis (UC) and Crohn’s disease are the two primary and most common types of IBD. Crohn’s disease was first described by the German surgeon Wilhelm Fabry (also known as Guilhelmus Fabricus Hildanus) in 1623 and later on described and named in the United States by the physician Burril B Crohn. The British physician Sir Samuel Wilks first described UC nearly two centuries later in 1859 (Baumgart and Carding, 2007a).

Though many centuries has passed since the first description of the primary IBDs, there is currently a need for a global clinical diagnostic criterion to allow for consistent
and accurate disease classifications. Stemming from this issue, current epidemiology IBD studies though informative may not be providing the most precise global incidence and prevalence of IBD. With this in mind, there is a large body of literature providing informative patterns about IBDs that may aid in unveiling the etiology for these diseases, which has yet to be uncovered.

**Epidemiology of IBD**

Crohn’s disease and UC symptoms and clinical diagnosis is most commonly observed during late adolescence and early adulthood with a mean age range from 33.4 to 45 and a median of 29.5 years of age. IBD diagnosis in children under the age of 18 is estimated to be about 10% and has not risen in recent years. Thought this is the general pattern, some reports suggest a bimodal age distribution with some cases appearing in the later years of life (Loftus, 2004). According to the center for disease control (CDC), There are marginal sex differences in the incidence of IBD. Women have a tendency to experience Crohn’s disease more often whereas UC tends to be more frequently observed in men (Sonnenberg, 1989, Loftus, 2004). A more pronounce difference in IBD incidence is observed with ethnic and geographical differences. Historically, IBD has been more often observed in Caucasians than minorities, however, these differences have been narrowing with time with recent reports showing the prevalence of Crohn’s disease among African Americans being two thirds that of whites (Baumgart and Carding, 2007a). Geographically, there are also differences in the epidemiology of IBD. Historically, IBD has most often been observed in developed, more industrialized nations than in underdeveloped countries pointing to a potential contribution to disease development by urbanization (Lakatos, 2006). However, it is not fully clear whether this
observed pattern might be driven by improvement of diagnosis or increased development in the less urbanized countries. Regional changes in the epidemiology of IBD traces back to the 1930’s when IBD incidence rates began to climb in the United States, a pattern mimicked by Northern and western European countries in the 1950’s with UC increases preceding Crohn’s disease by an average of 17.5 years (Lakatos, 2006). One of the latest available meta-analysis on IBD incidence indicates 19.2 and 24.3 cases per 100,000 people with UC and 20.2 and 12.7 cases per 100,000 people with Crohn’s disease in North America and Europe respectively. The same report shows prevalence rates of 249 and 319 per 100,000 people for UC and Crohn’s disease respectively in North America. These same statistics for other regions including Asia, Middle East and South America are several orders of magnitude lower. Furthermore, incidence of UC and Crohn’s disease has been significantly increasing over time (Molodecky et al., 2012). Extrapolating these estimates suggests that IBD currently affects as many as 1.4 million people in the United States. While the regional differences in IBD epidemiology point to an important environmental contribution, twin studies have provided strong evidence for a substantial role of genetics.

Studies looking at twin concordance rates are scarce in the literature but the reports available show a high degree of consistency. In general and as would be expected from a strong genetic component, monozygotic twins show significantly greater rates of concordance than dizygotic twins. Monozygotic twins showed 44.4-50% concordance rates for Crohn’s disease and 6.3-18% for UC as well as an increased probability of IBD development by first-degree relatives, the rates for dizygotic twins were not different from the general population rates (Orholm et al., 2000; Halfvarson et al., 2003). These
findings suggest a strong genetic component to IBD, which is stronger in Crohn’s disease. Taken together, the life-long debilitating nature of IBD along with its severe socioeconomic impact on the worldwide younger population, it becomes apparent that an effective therapeutic treatment for IBD should be sought out in light that the current treatments have failed to retain effectiveness across time.

**Clinical Presentation and Treatments for IBD**

Although both UC and Crohn’s disease share clinical manifestation overlap, there are major presentation differences between these diseases. UC and Crohn’s disease both present with similar clinical symptoms including bloody diarrhea, abdominal pain, passage of pus and mucus, weight loss and fever as well as bowel obstruction which is most often reported in Crohn’s disease patients. To distinguish these diseases, objective endoscopic, radiologic and histological analyses are required (Baumgart and Sandborn, 2007b). Objective studies of Crohn’s and UC patients have revealed a differential inflammatory pattern for these diseases in the gastrointestinal tract. UC is characterized by an inflammatory patterns that is limited to the colon whereas Crohn’s disease patients suffer from inflammatory patches that can localize at any place along the tract with the most distal areas (away from the mouth) more commonly affected.

Current treatments for IBD fall under four major categories: aminosalicylates (mesalizine, sulfasalazine, olsalazine), corticosteroids (hydrocortisone, budesonide), immune modifiers (infliximab), and antibiotics (metronidazole, ampicillin, ciprofloxin). Aminosalicylates (mesalizine and sulfasalazine for UC and Crohn’s respectively) appear to be the most effective and first-line therapies for mild to moderate IBD (Baumgart and Sandborn, 2007b). With more complicated cases, the specific treatment has to be tailored
to the individual and appears to be largely based on trial and error. The current rates of induction of disease remission attest to the lack of efficacy of the current treatments with about 60% of IBD patients showing a chronic intermittent course of disease progression (with rate being slightly higher for Crohn’s disease patients). Eventually, a large proportion of IBD patients require surgical removal of the affected areas such as colectomy (removal of the colon). However, this surgical procedure appears to only be efficacious in UC patients. Though the quality of life for IBD patients is not optimal, life expectancy for UC is normal and only slightly reduced in Crohn’s disease (Baumgart and Sandborn, 2007b). Nevertheless, with increasing insight to the etiology of IBD, there is a need for more efficacious treatments to become available.

**Potential Risk Factors for IBD**

One risk factor that has been associated with IBD is appendectomy or the removal of the appendix. Several meta-analysis and large cohort studies have reported that patients who undergo an appendectomy had a 69% reduce rate of developing UC. For patients at risk for Crohn’s disease, appendectomy was not beneficial and rather increased the chances of disease development. Oral contraceptive intake by women has been linked to increased risk for developing IBD. This is particularly true for women who have used oral contraceptives for long periods of time. Some reports estimate between 30-40% greater likelihood of developing IBD when using oral contraceptives. Diet also appears to play a key role in the development and prevention of IBD. Patients who engage in high sugar, chocolate and fat dietary patterns have an increased risk for developing IBD with fiber, fruits ad vegetables showing a protective role in IBD. Perinatal and childhood factors also have been linked to an increased risk for IBD.
Maternal contraction of the measles virus as well as attenuated live measles vaccine has been linked to a three-fold likelihood of developing IBD. In addition, breastfeeding appears to have a protective effect against the development of IBD (Loftus, 2004). This protective effect of breastfeeding may be linked to the immunoregulatory actions of lactoferrin, a protein found in high concentrations in breast milk, which has been found to attenuate experimental colitis by increasing production of anti-inflammatory cytokines and reducing levels and synthesis of proinflammatory cytokines (Togawa et al., 2002).

Another major risk factor for IBD development is smoking history. The earliest report linking cigarette smoking to IBD was reported in 1982 by Harries et al. who found that patients with Crohn’s disease were more likely to smoke than patients suffering from UC. This original report established the relationship between smoking and IBD but left the question unanswered as to what the nature of this relationship is, since then, several reports have shed light on this relationship. Current smokers have a significantly reduced risk of developing UC, former smokers on the other hand showed an increased risk of developing UC even if cessation happened for a long time. Furthermore, in one study, 45% of patients who resumed smoking showed clinical improvement, similar findings have been reported from UC patients treated with transdermal nicotine patches. However, this pattern does not hold for Crohn’s disease patients. On the other hand, smoking has been associated with a two-fold increase risk and worsening of disease for this patient population (Loftus, 2004). Though the reports on the effectiveness of nicotine in treating IBD have been mixed, there is an emerging theory that can explain this phenomenon. Wang et al., in 2004 showed that a specific nicotinic receptor subtype has anti-inflammatory actions, however, the specific pathways were not known at the time.
Recently, it has been reported that a specific nicotinic receptor subtype (α7 nAChR) involved in autonomic nervous system regulation of immunity plays a major role in inhibiting the production of proinflammatory cytokines known to be involved in IBD (Anderson and Tracey, 2013). Hence, it can be conceived that the nicotine in cigarette smoke may directly regulate the immune system.

**IBD and Immunity**

The homeostatic balance of the immune systems is perturbed in patients suffering from IBD. Though no known cause has been elucidated, for some time it has been known that both types of IBD manifest with increased cellular infiltration in inflamed regions, a phenomenon that is more accentuated in Crohn’s disease (Furgeson et al., 1975). Cellular infiltrates, the first line of immune defense, tends to be compromised of granulocytes and other immune cells that play important roles in the initiation and execution of phagocytosis (Beeken et al., 1987). However, this observation may depend on the stage of disease. Marks et al. (2006) reported an overall decrease in neutrophil accumulation following trauma to the intestines in patients suffering from Crohn’s disease. Furthermore, these researchers noted that this hyporesponsiveness of neutrophils is generalized as the same pattern is observed following skin trauma and it is not limited to neutrophils since cultured macrophages from these patients demonstrated reduced responsiveness to stimulatory agents. Hence, it is yet to be determined whether IBD causes a decrease in granulocyte availability or if the disease is a result of this observed deficiency. The large mobilization of immune cells during inflammatory phases of IBD and the alteration in responsiveness of these cells have led to the theory that IBD may be driven by penetration of intestinal flora into the intestines. Under normal conditions,
bacteria are recognized when immune cells identify the pathogen associated molecular pattern (PAMP) via pathogen recognition receptors (PRR). Upon recognition, PPRs activate membrane bound Toll-like receptors (TLR) and cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors (NLR). Activation of TLRs and NLRs results in the activation of the transcription factor nuclear factor (NF)-κB thereby initiating the production of inflammatory cytokines for bacteria removal. In support of this hypothesis are observations that IBD patients have defective intestinal epithelium (Geremia et al., 2013) and that NOD2 mutations are common in patients with Crohn’s disease leading to inadequate NF-κB activation and inability to fight off intruding bacteria (Ogura et al., 2001). An alternative role for NOD2 comes from reports that NOD2 regulates TLR2, which in turn regulate Th1 inflammatory responses (Watanabe et al., 2004). Based on these observations, the reported alterations in NOD2 observed in Crohn’s disease patients may results in disinhibition of the Th1 inflammatory response characteristic of this disease (Fichtner-Feigl et al., 2005) and would explain the hyperreactive inflammatory profile observed during acute IBD.

Autoantibodies have been observed in patients with IBD suggesting the involvement of the adaptive immune system in these diseases. Adaptive immunity is largely driven by T-cells that can differentiate into Th1, Th2 or the more recently recognized Th17 profiles. UC has often been characterized as demonstrating a Th2 profile with overproduction of cytokines such as Interleukin 4, 5, 10, which are associated with the induction of eosinophil accumulation. Crohn’s on the other hand has been associated with a Th1 profile characterized by overproduction of tumor necrosis factor (TNF), interferon gamma (IFN-γ) and Interleukin 2 and 12. Though autoantibodies
have been observed in samples from IBD patients including from colon samples and serum, neither disease meets the criteria to be considered typical autoimmune diseases (Wen and Fiocci, 2004). These autoantibodies have been reported to be against epithelial cells and to cross-react with *Escherichia coli*. This observed cross-reaction between bacteria and self-antigens suggests that IBD is associated with a faulty adaptive immune system. In addition to wrong targeting of epithelial cell in IBD, several reports have observed the presence of autoantibodies against epithelial cell-associated components (ECAC), more specifically against goblet cell glycoproteins (Aronson et al., 1983). This observation is validated by findings that mucin-producing cells (goblet cells) are depleted in humans suffering from IBD (McCormik et al., 1990) and in experimental models of IBD (Elson et al., 1995). Two mucin layers made from MUC2 glycoproteins provide protection from intestinal flora, one loose outer layer in direct contact with the lumen and where bacteria colonize and a more rigid inner layer completely devoid of bacteria (Johansson et al., 2008). In an experimental model of IBD, Johansson et al. (2010) found that disruption of the outer mucus layer results in colonic permeability that allows for bacteria to infiltrate the colon mucosa after 12 hours of treatment administration, a time period that well precedes inflammation. These results suggest that IBD may be in part due to gastrointestinal epithelial barrier disruption leading to bacteria infiltration initiating an uncontrollable immune response, a phenomenon postulated by other as being driven by high salt diets (Kleinewietfeld et al., 2013).

*Experimental Models of IBD*

Over the past decades, there has been a surge of experimental models for inflammatory bowel disease based on genetic or environmental manipulations. Though
no one animal model exactly reproduces human IBD, they each have pathological aspects that mimic human IBD and can help to inquire about the etiology and potential therapeutics for these diseases (Elson et al., 1995). One of the most widely used models for IBD is the dextran sulfate sodium (DSS) experimental model. The DSS model is commonly used due to its simplicity and similar histological (monocyte infiltration, mucosa ulcerations and erosion) and clinical (weight loss, diarrhea, lethargy etc.) pathology to human IBD, though extreme precaution must be paid to several parameters including, compound molecular weight, concentration, exposure time (Yan et al., 2009), intestinal flora and animal species (Karlsson et al., 2007) and strain (Mahler et al., 1998), all parameters that can alter disease presentation (Perse & Cerar, 2012). The DSS model was first postulated by Okayasu et al. (1990) and consists of oral administration of this salt for short consecutive time period (5-7 days) resulting in acute inflammation or in cycles consisting of an acute administration followed by a recovery period (10-21 days) resulting in a more chronic inflammatory profile. DSS exposure reliably results in diarrhea, bloody stool, weight loss, colonic crypt damage, colon hyperplasia, immune cell infiltration to the colon mucosa including leukocytes and lymphocytes (Yan et al., 2009), mucin depletion (Qualls et al., 2006), shortening of the colon (Okayasu et al., 1990; Dieleman et al., 1998; Bento et al., 2012) and muscle wall thickening (Larsson et al., 2006). Though DSS-induced colitis has been reported to affect multiple areas of the GI tract, most studies observed greater damage to the distal colon (the most posterior part of the colon) with little damage to the proximal colon (Dieleman et al., 1998; Mahler et al., 1998; Yan et al., 2009).
In line with Kleinewietfeld et al. data pointing to high salt content as a risk factor for the development of immune diseases due to increased permeability, Yan et al. (2009) observed that DSS, a salt, increases colon epithelium permeability allowing for labeled DSS molecules and bacteria to infiltrate the colonic mucosa. This DSS-induced colonic epithelium permeability allows for immune cell infiltration and damage to colonic crypts. Cell infiltrates in this model consist of -activated lymphocytes, macrophages and neutrophils (Dieleman et al., 1998; Yan et al., 2009; Bento et al., 2012). The high neutrophil activity translates into elevated levels of the enzyme myeloperoxidase (MPO), which is commonly used as a marker for neutrophil activity and a hallmark of DSS-induced colitis. Though it is possible that these immune cells may drive DSS-induced colitis, it has also been demonstrated that the complete absence of these phagocytic cells results in worse pathology (Qualls et al., 2006). Therefore it appears that DSS administration results in permeable colonic epithelium allowing for bacteria and potentially other pathogen infiltration into the colon mucosa. This in turns initiates an immune response mainly driven by phagocytes, which then exacerbates and results in the recruitment of cytokine producing lymphocytes. DSS-induced colitis is characterized by both a Th1 (IL-1β, IFN-γ, IL-12) and Th2 (IL-4, IL-10) cytokine profile (Dieleman et al., 1998). This magnified immune response then translates into colonic microstructure damage including aberration of mucus producing cells and colonic crypt structures. The importance of the colonic epithelium permeability in the DSS model becomes apparent when considering the fact that during recovery periods, a time point when DSS molecules are not present in the animals, epithelium permeability is resolved (Yan et al., 2009).
In addition to being simplistic and mimicking human disease, the DSS model of induced colitis is advantageous in that it has been validated with drugs currently being used for the treatment of human IBD. It has been reported that treatment of DSS-induced colitis with currently conventional drugs (and in equivalent human dose ranges) for the treatment of human IBD such as sulfasalazine, olsalazine (Axeksson et al., 1998), cyclosporine A, methotrexate, anti-IL-12p40 and CD3 antibodies (Melgar et al., 2008) ameliorates DSS-induced colitis albeit to different degrees. Furthermore, it was reported that validation treatments were more often efficacious after the disease had been established from some time (chronic colitis) than during the most acute phases (Axeksson et al., 1998). Taken together, these data suggest that though several experimental models for human IBD exist, DSS is a good model for the assessment of potential therapeutic agents and it is often amongst the top model of choice due to its simplicity and similarity to human IBD.

**Pain-Related Measures and the DSS Model**

Though DSS induced colitis has been reliably shown to very closely mimic human IBD parameters including colonic inflammation, weight loss and bloody stool, studies looking at visceral pain, which is a symptom often reported by human patients (Baumgart and Carding, 2007a) are scarce in this model. Most of the available literature looking at DSS-induced (and other experimental models of colitis) visceral pain has focused on measuring visceral hypersensitivity (VHS) in response to colorectal distention (CRD). However, the results from CRD in the DSS model are not in accord with data generated from other colon inflammation models that suggest increased nociception during colonic inflammation (Kamp et al., 2003; Bercik et al., 2004; Larsson et al.,
Larsson et al. (2006) showed that DSS-induced colitis does not affect VHS in response to CRD at either acute or chronic inflammatory phases. These same authors then sought to determine if psychological stress (either before or after DSS administration) can trigger VHS in DSS-induced colitis and observed that only a transient hyperalgesia during acute colitis (Larsson et al., 2009). In contrast to these findings, Chen et al. (2013) reported that although DSS does not alter VHS in response to CRD, the paring of DSS-induced inflammation with psychological stress does result in visceral hypersensitivity. Hence, these data suggest a potential exacerbation of the nociceptive component of IBD by psychological stress. Though other behavioral paradigms have been used in the DSS model, those usually focus on assessing anxiety and depression (Painsipp et al., 2011) and not pain-related behavior. It is therefore clear that more emphasis should be directed at assessing visceral nociception in experimental models of colitis and particularly in the DSS model.

**Neuropeptide Y and the DSS Model**

With increasing evidence that NPY and its receptors are involved in the neuroimmune crosstalk (Prod’homme et al., 2006; Wheway et al., 2007b), there is a growing line of research interested in assessing the involvement of NPY in human inflammatory diseases using animal models, in particular, the role of NPY in DSS-induced colitis. Over the last decade, there have been a handful of reports presenting data that suggest a key role for NPY and its Y1 receptor in experimental colitis.

The role of NPY in colonic motility and secretion is well established in both human (Cox et al., 2001; Cox et al., 2007) and mice (Tough et al., 2011; Chandrasekharan et al., 2008) during non-inflamed states. Klompus et al. (2010) sought
to determine if the antisecretory effects of NPY are altered with DSS administration. These authors provided further support that NPY application to murine distal but not proximal colon results in inhibition of ion transport across the colonic epithelium (antisecretory effect), an effect that is not likely under direct neurogenic control as application of tetrodotoxin (TTX) does not alter these results. Using pharmacological agents, it was shown that NPY’s inhibitory effects on ion transport are mediated largely via the Y₁ with a small contribution from the Y₂ receptor. In DSS-induced inflamed colons, both the proximal and distal colons failed to respond to NPY stimulation suggesting a loss of NPY-mediated inhibitory control during experimental colitis. This observation was accompanied with decreased levels of Y₁ mRNA in colon tissue and decreased Y₁ protein expression in the colon epithelium. In addition and in line with the lack of proximal colon responsiveness to NPY application, proximal colon segments express less Y₁ mRNA (10-fold less) than the distal colon. These data suggest that the NPY system plays an important role in regulating colonic ion transport, which is dysregulated in DSS-induced colitis and may account for the observed dehydration and weight loss observed in these animals.

In addition to a role for NPY in colon secretion, there is also a growing body of literature providing evidence for an interaction between NPY signaling and the immune system in DSS-induced colitis. In vitro, NPY induces the production of neuronal nitric oxide synthase (nNOS), a marker for oxidative stress, and the inflammatory cytokine TNF-α. In vivo, DSS administration induces an increase in the expression of NPY that is as high as a 20-fold as well as a significant increase in nNOS in enteric ganglia from the murine distal colon (Chandrasekharan et al., 2008; Pang et al., 2010). Making use of
mice genetically altered to not express the NPY gene (NPY−/−), it has been shown that these mice do not demonstrate the DSS-induced nNOS increases suggesting that NPY regulates oxidative stress in the DSS model. In addition, NPY−/− mice were shown to be resistant to DSS-induced colitis as evident from decreased clinical and histological scores along with decreased MPO activity in these mice. In line with the decreased MPO activity, NPY−/− mice had less neutrophil infiltration suggesting that neutrophil mobilization in DSS-induced colitis is regulated by NPY (Chandrasekharan et al., 2008). The importance of NPY signaling in the progression of DSS-induced colitis has also been determined by preventing NPY signaling after disease development by preventing its transcription using antisense oligodeoxynucleotides (ODN). In line with the findings from Chandrasekharan et al., NPY ODN treatment after acute colitis development results in attenuation of DSS-induced colitis. NPY ODN reversed clinical and histological scores as well as decreasing MPO activity and TNF-α expression (Pang et al., 2010). The fact that ODN treatment was initiated after acute inflammation was in place provides strong evidence for the idea that NPY is one of the major drivers of DSS-induced pathology.

With these observations in mind, it is apparent that NPY signaling plays a key role in experimental colitis; the question then is, via which of its five known receptors does NPY exacerbate DSS-induced colitis. The limited literature currently available using receptor specific small molecule antagonists and mice genetically altered to not express the Y1 receptor (Y1−/−) suggests a strong role for this receptor in experimental colitis. Hassani et al. (2004) observed that DSS-induced colitis was attenuated in Y1−/− mice as determined by the clinical and histological scores. In addition, in Y1−/− mice,
DSS-induced weight loss, though not eliminated, followed a slower course and these mice recovered faster than wild type mice. A similar pattern was observed using a Y1 receptor antagonist for the duration of the disease. The involvement of the Y1 receptor was corroborated by Wheway et al. (2005) who also observed attenuation of DSS-induced colitis in Y1-/- mice, likely driven by decreased IFN-γ production by Y1-/- mice. Furthermore, these authors elucidated a previously unknown bimodal role of NPY signaling via Y1 in immunity. Y1-/- mice had smaller spleens with decreased numbers of mature B-cells, CD8+ T–cells and other effector T-cell populations. In line with altered immune cell expression in mice lacking Y1 were observations that APC cells in these mice and T-cells are functionally defective. Antigen presentation abilities are impaired in macrophages and dendritic cells from Y1-/- mice as evident from their inability to activate T-cells and to induce the production IL-12 expression upon stimulation. Similarly, T-cell function from Y1-/- mice is altered. T cells from these mice were hyperresponsive to stimulation. Transplantation of Y1-/- T-cells to a RAG-/- mice who lack mature B and T-cells induces a more rapid and aggressive colitis. This role of Y1 in immunity is further supported by observations that dendritic, natural killer, T, B cells and macrophages all express the functional Y1 receptor (Wheway et al., 2005). This series of findings complement those of Chandrasekharan et al. (2008) and Pang et al. (2010) in providing evidence for the importance of NPY signaling in the development and progression of experimental colitis and further implicate the Y1 receptor as playing a crucial role in neuroimmune inflammation.

Though it is clear that NPY signaling via Y1 plays an important role in neuroimmune inflammation, there is very limited information about the role of its other
receptors, in particular, the role of Y2 and Y5 receptors signaling, which has been implicated to play key roles in immune cell regulation (Dimitrijevic et al., 2005; Bedoui et al., 2008; Mitic et al., 2011). With increasingly available pharmacological agents and genetic manipulations specifically targeting these receptors, it is possible to determine their potential role in mediating NPY’s effects on the neuroimmune crosstalk.

To date, there are no available reports assessing the role of the Y2 receptor in DSS-induced colitis even though several reports indicate that these receptors are expressed in colon tissue. This paper seeks to replicate the findings that Y1 receptor antagonism has beneficial effects on DSS-induced colitis and looks to extend these findings to the assessment of any potential role of the Y2 receptor in this model. The Y2 receptor was selected over the Y5 receptor, which has also been implicated in immune processes due to the fact that Y2 but not the Y5 receptors have been demonstrated to be expressed in the bowel.
Chapter 2

SPECIFIC AIMS AND RATIONALE

Specific Aim 1: To assess disease progression and the regulation of NPY in the DSS-induced model of colitis across time.

The disease severity and colonic inflammation in the DSS-induced model of colitis is contingent upon [among other things] the concentration of DSS, time of exposure and recovery period. The progression of colonic inflammation across time (4, 7 and 10 days) will be assessed using body weight, distal colon histological assessment, MPO and cytokine protein expression. In addition, NPY protein expression levels will be determined at each time point to determine the potential involvement of NPY in this model. Lastly, general open field activity will be assessed as an indicator of disease-related discomfort.

Specific Aim 2: To determine the role of peripheral NPY via its Y\textsubscript{1} receptor in the mouse model of DSS-induced colitis.

NPY fibers comprise a significant component of the sympathetic nervous system innervation of the distal colon and NPY receptors are expressed on immune cells. Based on this potential neuroimmune interaction and the reports suggesting inflammation mediated via Y\textsubscript{1} receptors, the role of Y\textsubscript{1} in 7-day DSS-induced colitis will be assessed. Once daily subcutaneous injections of the selective Y\textsubscript{1} receptor antagonist BIBP-3226 at 1mg/kg or 3mg/kg will be administered for peripheral Y\textsubscript{1} receptor inhibition. Colon histology, cytokine and NPY protein expression and general open field behavior will be assessed.
The potential involvement of NPY via its Y₁ receptor may be differential between a chronic inflammatory state and during disease recovery periods. To address this question, colon histology, cytokine and NPY protein levels and general open field behavior will be assessed at a chronic inflammatory stage (7-day DSS) and during a recovery period (7-day DSS with a 3-day recovery). To assess the potential involvement of NPY, BIBP 3226 (Sigma-Aldrich) will be administered subcutaneously once daily at 1mg/kg or 3mg/kg.

**Specific Aim 3:** To determine the role of peripheral NPY via its Y₂ receptor in the mouse model of DSS-induced colitis.

NPY Y₂ receptors are autoreceptors reportedly expressed on immune cells and playing substantial roles during immune responses. To assess the potential involvement of the Y₂ receptor in the DSS-induced model of colitis, BIIE-0246 (Tocris), a selective Y₂ receptor antagonist will be administered subcutaneously once daily at 10mg/kg. Colon histology, cytokine and NPY protein levels and general open field behavior will be assessed at a chronic inflammatory stage (7-day DSS) and during a recovery period (7-day DSS with a 3-day recovery).

**Specific Aim 4:** To evaluate the possibility that NPY plays a role in the pathophysiology of DSS-induced colitis via its Y₁ and Y₂ receptors in a pathology-stage dependent manner.

Pilot data from our laboratory suggests a potential differential effect of the Y₁ and Y₂ receptors at the different disease stages. It appears that the Y₁ receptors plays a major role during the acute inflammatory state and the Y₂ receptor appears to be most important during the recovery phase of the disease. To further study these effects, a factorial design
will be conducted with a experimental group receiving daily subcutaneous injections of BIBP-3226 at 3mg/kg for the first 7 days while the animals are exposed to DSS followed by BIIE-0246 subcutaneous injections at 10mg/kg for the remaining 3 days of recovery.

**Specific Aim 5: To assess DSS-induced gene expression changes, identify a DSS-induced genetic meta-signature from multiple independent studies and compare this to genetic profiles generated from human IBD patient samples.**

The DSS-model of inflammatory bowel disease is one of the most common used experimental animals models to study human IBD due to its relative simplicity and robust similarity in pathological presentation to human disease. First, it will be necessary to determine if the potential DSS-induced gene alterations are reliable enough so that a DSS genetic meta-signature can be computed. Should this be the case, it would be useful, for translational purposes, to determine if these similarities between the DSS model and human IBD are also observed at the genetic level. This will be addressed by comparing the potential DSS-induced genetic meta-signature to genetic profiles from human Crohn’s and Ulcerative Colitis patients. To address these questions, colon samples from four independent DSS studies will be genetically profiled and compared to develop a DSS genetic meta-signature. This meta-signature will then be compared to genetic profile samples using publically available databases.
Chapter 3

GENERAL METHODS

Animals

C57Bl/6 adult male mice (Jackson Labs) between 16-20 weeks of age and weighing on average 26 grams were used for these experiments. Animals were randomly assigned to control and experimental conditions. Animals were housed in groups of 3-5 per cage in a 12:12 hour light:dark cycle (lights on at 7:00) and were provided with food and water ad libitum. All animals were acclimated to the housing colony and water bottles for at least one week before treatment initiation. All procedures were conducted in full compliance with the Regeneron Pharmaceuticals, Inc. Institutional Animal Care and Use Committee regulations.

DSS Preparation and Colitis Induction

Dextran Sodium Sulfate (Average molecular weight 9,000-20,000, Sigma-Aldrich) was mixed for at least two hours in distilled water to yield a 4% DSS solution (e.g. 40g of DSS in every 1 liter of distilled water). This solution was placed in 250mL glass bottles with metal spouts and was given to experimental animals ad libitum as a replacement for drinking water. Control mice received tap water in similar glass containers. Fluids were replenished as needed for the duration of the experiments. The acute inflammatory model consisted administration of 4% DSS for 7 consecutive days. The recovery model consisted of the acute inflammatory model treatment plus a 3-day recovery period where treated animals were placed back on tap water until euthanized.

Body Weight
All animals were weighed once daily for the duration of the experiments. A plastic container was placed on a calibrated Ohaus CS200 scale (Ohaus Corporation, NJ), the scale was then equilibrated at 0 grams and the animals were placed in the container. Body weight was recorded to the nearest tenth of a gram. Animal health was carefully monitored, and if weight loss was greater than 25% of starting body weight, or if animals appeared to be in poor health, they were immediately euthanized.

**Pharmacological Treatment**

*Y*₁ Receptor Antagonism —

Systemic peripheral NPY Y₁ receptor antagonism was achieved by once daily subcutaneous injection of the selective Y₁ inhibitor BIBP 3226 (Sigma Aldrich) reconstituted in distilled water to a final injectable dose of 1mg/kg or 3mg/kg. Control animals received a vehicle (dH2O) injection instead.

*Y*₂ Receptor Antagonism —

Systemic peripheral NPY Y₂ receptor antagonism was achieved by one daily subcutaneous injection of the selective Y₂ inhibitor BIIE 0246 (Tocris). For one experiment, BIIE 0246 was reconstituted in 100% DMSO and diluted with distilled water to a 50% DMSO solution. For all subsequent experiments, BIIE 0246 was reconstituted in a 1mL of 100% dimethyl sulfoxide (DMSO) and then diluted in distilled water to a 12.5% DMSO solution with a final injectable dose of 10mg/kg. Control animals received a vehicle (50% or 12.5% DMSO in dH2O accordingly) injection instead.

**Open Field**

On the last day of each experiment, animals were placed in a *smart frame* open field system configured with the automated tracking software for Windows (Microsoft)
called Motor Monitor (Kinder Scientific). This 16” (length) x 16” (width) x 15” (height) inch Plexiglas open field chamber was encompassed by two horizontal laser beam detection frames. The lower frame rested 3/4” and the upper frame was 2½” inches from the chamber floor. Each frame contained 16 beams per side (times 4 sides) that were 1” inch apart. Eight animals counterbalanced across experimental conditions were tested for 60 minutes each in the field. The automated software recorded responses in five minute intervals and a total count for each measure for the 60 minutes was calculated by summing the twelve five minute intervals for each measure. The following measures were collected: basic movements (operationally defined as any horizontal beam cross), immobility time (operationally defined as a lack of horizontal and vertical beam crosses), fine movements (operationally defined as changes in body position not meeting criteria for ambulation, includes grooming and head movements), X+Y axis ambulation (operationally defined as a complete relocation of the animal’s body), rears (operationally defined as vertical beam crosses), rearing time (the time spent crossing vertical beams), rest time (operationally defined as a lack of beam crosses lasting longer than 15 seconds), total distance traveled (calculated from known distances between beams and total beam crosses), normalized rears (defined as the ratio of total rears to X+Y ambulation) and normalized rear duration (defined as the ratio of time spent rearing to total rears).

**Euthanasia and Tissue Collection**

Animals were euthanized by carbon dioxide (CO₂) asphyxiation. Blood samples were collected using the cardiac puncture method. Collected blood samples were immediately placed in serum separator tubes (Becton, Dickinson and Company), kept on wet ice for the length of the sacrifice, spun in an centrifuge (Eppendorf, model 5415R) at
13,200 revolutions per minute and 4° C for 20 minutes. The resulting serum (supernatant) was collected in 1.5mL Eppendorf tubes and stored at -80° C. The large intestine and cecum were identified, surgically removed, cleaned in a neutral PBS solution and dried on an absorbent tissue. The large colon was then divided in half and labeled as proximal (closest to the cecum) or distal (closest to the anus). For the time course characterization experiment, the proximal and distal colons were then subdivided into two approximately equal parts. The most proximal and most distal segments were placed into tissue embedding cassettes (VWR) and sunk in a 10% formalin neutral buffered solution for fixation. The inner segments were flash frozen in 200mL of 2-methylbutane (Fisher Scientific) for immunoassay analyses and stored at -80° C. For all subsequent experiments, only the distal colon was collected always keeping the most distal 1-1.5cm of tissue for histology and the most proximal 1-1.5cm of tissue for immunoassays. For experiments where gene profiling was conducted, the next proximal 1-1.5cm of tissue was collected and placed in 5ml RNASlater tubes (Qiagen) stored overnight at 4° C and then transferred to -20° C until processed.

**Gross Anatomical Measures**

Colon and cecum were collected, cleaned, dried and positioned flat while avoiding stretching on a dissecting board with an engraved ruler (Fisher Scientific) to measure the full length of the colon and cecum in centimeters. Next, the colon was placed on a calibrated scale that recorded weights to the nearest hundredth of a gram. The same procedure was conducted for the cecum.
**Tissue Processing**

**Histology**

Proximal and distal colon samples in embedding cassettes were immersed in 10% formalin neutral buffered for at least 24 hours. The samples were washed at least three times with phosphate buffered saline (PBS) and immersed in 70% alcohol until processed for paraffin embedding. Samples were placed in a metal rack cassette holder and then in the chamber of an automated tissue processor (TBS, model ATP1) and allowed to process overnight. The processing procedure consisted of repeated and increasing gradients of alcohol concentrations (70, 95 and 100%), two xylenes and two wax immersions at 58° C. Samples were then placed in the liquid paraffin chamber of a tissue-embedding center (TBS, TEC-120) and completely embedded in paraffin. Once embedded, all samples were placed on a frozen stage for at least an hour for paraffin solidification.

Paraffin embedded tissue blocks were then individually placed and secured to a microtome cassette holder (Leica, Model RM 2165). Colon sample were sectioned at thickness of 7µm. Recently cut tissue ribbons were placed in a warm water bath and mounted from there onto Histobond coated slides (VWR). At least three slides, each with four or more sections, were collected per animal. Once completely dry, slides were stored in slide boxes until stained.

**Immunoassays**

Colon samples used for immunoassays were first pulverized and then homogenized in a buffer containing 150mM NaCl, 20mM Tris, pH 7.5, and 1% TritonX and protease inhibitor tablets (1 tablet for every 25mL of buffer). For pulverization, samples were removed from the -80° C freezer and placed on dry iced. The Eppendorf
tubes were then opened one at a time and the tissue was placed in the tissue compartment of an ice-cold stainless steel Bessman tissue pulverizer mortar (Spectrum Labs). Using a lead hammer, the tissue was completely pulverized (hammering about 8 times per sample). Following pulverization, the mortar tissue compartment was placed above a 10mL falcon round bottom tube sitting on dry ice and inverted for tissue transference. Any tissue that did not transfer from the mortar was scooped with a dry ice-cold spatula and placed in the tube. Once the pulverized tissue was transferred into the 10mL tube, it was left on dry ice to prevent protein degradation and awaiting homogenization.

Pulverized samples in 10mL falcon tubes were removed from dry ice for buffer addition. 1mL of the homogenization buffer was added to the pulverized tissue and placed on wet ice. All tissue samples were kept in wet ice awaiting homogenization. Each 10mL falcon tube containing the sample and buffer was then placed into a 50mL beaker filled with wet ice to keep the sample from degrading during the homogenization process. Using a dispensing ULTRA-TURRAX T-8 (IKA) instrument, the tissue was homogenized in three 10-second intervals with 10 second waiting periods in between when the probe was placed in wet ice to prevent excessive heating. When the third homogenization period was concluded, the homogenate was placed back on wet ice and allowed to sit until the solution settled (when bubbles disappeared). The homogenate was then transferred into 1.5mL Eppendorf tubes and centrifuged at 4°C and 1400 revolutions per minute (RPM) for a 20 minute period. Homogenized samples were separated in aliquots to prevent repeated freeze-thaw cycles and protein degradation. A small volume (20µL) aliquot was placed on wet ice for protein analysis using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific).
The BCA protein assay kit was used following the manufacturer’s protocol. Briefly, 25µL of standards (ranging from 25-2000µg/mL) and unknown samples were added to a non-binding 96-well round-bottom clear plate in duplicates. 200µL of working reagent (50 parts reagent A: 1 part reagent B) were added to each well and mixed thoroughly for 30 seconds on a plate shaker. The plate was then placed in an oven at 37°C and allowed to incubate for 30 minutes. Following incubation, the plate was allowed to reach room temperature and then read in a spectrophotometer at a wavelength of 562nm providing a total protein concentration in milligrams for each sample. The remaining aliquot samples were stored at -80°C for future immunoassay analyses.

The homogenized colon aliquots were then used to quantify different proteins of interest including neuropeptide Y, MPO, and inflammatory cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 [p40/p70] and TNF-α).

Genetic Profile

Barcoded distal colon samples submerged in 5ml RNALater tubes were removed from the -20°C storage and trimmed to a total weight of 33mg. After weighing, tissue samples were placed under the hood and transferred to 4ml round bottom tubes containing 1ml of TRIzol solution. A plastic tissue cutter was placed into each sample tube for tissue homogenization using an Omni AH96 homogenizing workstation (Omni International). Samples were homogenized at 20,000 rpm for a period of two minutes with the first minute consisting of an oscillating pattern for a more complete homogenization. After homogenization, the tubes were capped and stored at -20°C until RNA extraction. RNA extraction was done using the MagMax Total RNA Isolation kit.
(Life Technologies) and following the suppliers provided protocol. Next, the resulting RNA was quantified using a standard protocol. Briefly, 2µl of RNase free water was added to each sample and placed in a Nanodrop 8000 spectrophotometer (Thermo Scientific) for quantification. Lastly, RNA quality was determined using a QIAxcel RNA quality control kit (Qiagen) following the manufacturer’s protocol.

**Tissue Analysis**

**Histology**

All colon samples were first stained with hematoxylin and eosin (H&E) for pathological assessment (see protocol in supplement), cover-slipped and allowed to dry over night. All excess adhesive was removed from dried slides using a glass cleaning solution (50:50 Windex-dH2O solution). Cleaned slides were then placed in the slide rack compartments (hotels) of an automated imager (Leica, Model SL801). The imager was set to scan all slides up to the maximum magnification of 40X. The resulting scanned images were uploaded to an Internet-based network provider (http://slidepath/dih/login.php) from where digital images were capture of each colon sample. At least 4 images for each colon were captured and digitally stored. Using the publically available software Image J (NIH), five length measurements of each of the different anatomical areas of interest (colon muscle layers and colon crypts) of the colon were recorded. With 4 images per sample, a total of 20 length measurements were collected for each measure for each animal.

**Histopathological Analysis**

H&E stained distal colon sections were scored by an experimenter blind to treatment conditions using a modified scale from that used by T. ten Hove et al., 2002. At
least four different images from each animal along different levels of the distal colon were scored to obtain an average score per animal. Tissue was scored for the following criteria: a) percent of area involved on a scale of 0-4 where 0=0%, 1=0-10%, 2=10-20%, 3=20-50% and 4=>50%, b) erosion and ulceration, c) crypt loss, d) number of follicle aggregates, e) monocyte infiltration, f) edema and g) goblet cell loss with b-g being scored on a 0-3 scale where 0=none, 1=weak, 2=moderate and 3=severe.

Immunoassay

All protein expression assays were conducted using commercially available assays and in accordance with the provided manufacturer protocols. NPY (Phoenix Pharmaceuticals) and MPO (Hycult Biotech) colon expression levels were quantified using an enzyme-linked immunosorbent assay (ELISA). Multiple inflammatory cytokine expression levels were quantified in single samples by making use of the multiplexing capabilities of a 10-Plex Mouse magnetic capture bead cytokine panel Luminex (Invitrogen).

RNAseq Read Mapping

Sequenced reads in Illumina Hiseq2000 image files (BCL files) were converted to FASTQ format via Illumina Casava 1.8.2. Reads were decoded based on their barcodes and merged for each individual samples. The overall read quality per sample was evaluated with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to retain only samples with sufficient quality. Subsequently, a two-step hierarchical mapping strategy was employed to retrieve the raw read counts mapped to each gene. Reads in each sample were first mapped against mouse transcriptome (http://data/ncbi/GBK/mouse) using Bowtie (http://bowtie-
bio.sourceforge.net/index.shtml) with two allowed mismatches. For each gene, reads mapped to the sense-strand exons of the gene were identified and counted. The resulting unmapped reads were then mapped against the genomic sequence of each gene (gDNA) by Bowtie using the same mapping parameters. The reads mapped to the sense-strand introns were also added to the counts for each gene.

Statistical Analysis

All quantitative measures and graphical representations were conducted using either the Statistical Package for the Social Sciences (SPSS, Version 20 for Mac) or Graphpad Prism (Prism 6b for Mac OS X) unless otherwise specified. Any data value that fell 2 or more standard deviations from the mean in either direction was considered an outlier and removed from statistical analysis. Two or three-way Factorial analyses of variance (ANOVAs) were conducted for each measure using either a fully independent or mixed Factorial ANOVAs as appropriate. All statistical analyses were conducted at an alpha level of .05 for statistical significance. Post-hoc analyses were conducted to probe significant main effects or interactions using either Tukey tests, Sidak tests, or a Bonferroni tests, as appropriate. All graphs are expressed as group means ± the standard error of the mean (SEM).

Statistical Analysis of Differentially Expressed RNA

Read counts summarized at gene level represented the raw gene expression measures. An empirical minimum read count of 10 was applied to flag the “absence” and “presence” of genes in each sample, assuming the quantitation of a gene with less than 10 mapped reads is not reliable. We normalized the raw gene expression in each sample by global scaling to match the median library size (i.e. the total number of mapped reads) as
well as the 75% quartile of the gene-level read counts across all samples, as described in previous studies [BMC Bioinformatics 2010, 11:94]. For each comparison between two groups of samples, we first eliminated genes that were not flagged as “presence” in all samples of the higher expressing group, resulting in an average of about 13,000 out of total 35,161 genes for subsequent analysis. Next, fold changes associated with the comparison were calculated as the ratio between the arithmetic mean expressions in the two groups. The statistical significance (p-value) of the differential expressions was assessed under negative binomial distribution models using DESeq package (version 1.6, Genome Biology 2010, 11:R106). At the end, we selected genes with fold changes no less than 1.5 in either up or down directions with p-values of at most 0.01 as the significantly perturbed gene signatures. The final number of signatures in the present work range from a few hundreds to a couple thousand.
Chapter 4

SPECIFIC AIM METHODS AND RESULTS

Specific Aim I: To assess disease progression and the regulation of NPY in the DSS-induced model of colitis across time.

The DSS model of colitis is an animal model often used and well characterized in the literature. However, from experiment to experiment, there are variations in DSS used, the concentration of DSS and the time of exposure. In general, the model is characterized by distal colon shrinkage and microscopic damage including immune cell infiltration, crypt structure disorganization, muscle wall thickening and increased expression of inflammatory mediators. All these parameters however, can potentially be altered by differences in protocols, yielding differential results across experiments. The current experiment was designed to assess the progression of DSS-induced disease at three commonly used DSS exposure time points, including recovery periods. In addition, based on some reports indicating sympathetic involvement in immune diseases such as SLE and RA as indicated by elevated serum levels of NPY (Harle et al., 2006) as well as elevated colon expression of NPY in patients suffering from Crohn’s disease (Belai et al., 1997), colon NPY levels were assessed for all animals in this experiment to determine any potential regulation of NPY in DSS-induced disease. It was hypothesized that colon NPY protein levels would be increased in DSS treated animals indicating the involvement of the SNS in this model. Additionally, all open field behavioral measures specified in the methods section were collected at each time point to determine any potential DSS-induced behavioral signature including the animal’s discomfort level. It was hypothesized that mice treated with DSS would show behavioral alterations suggestive of general
discomfort such as decreased locomotion and decreased general movement. In addition, rearing behavior was measured as a potential indicator of visceral pain with the reasoning that rearing requires stretching of the viscera, a behavior that is expected to be decreased under painful conditions (Neubert et al., 2007).
Experiment 1

Aim and Design

The aim of this experiment was to characterize the DSS-induced disease progression using 3 different time courses. Model characterization included the assessment of colon tissue damage, the quantification of inflammatory cytokine, MPO and NPY at each time point studied. In addition, open field behavior was studied to determine the potential DSS signature on behavior. The time points studied were: 4, 7 and 10 days. Animals in the 4-day condition were exposed to DSS the entire time. The 7-day condition consisted of two group, one receiving DSS for 4 days and allowed to recover on water for 3 extra days and a group receiving 7-days of consecutive DSS exposure. Animals in the 10-day condition received 7-days of DSS exposure followed by a 3 day recovery period. All the characteristic landmarks of DSS-induced disease including weight loss, colon and cecum length and width, histological score and cytokine profile were assessed at each time point for every group. There was a control group administered regular drinking water for each time point with the exception of the day 7 groups. The 7-day control group served as control for both the 7-day DSS only and the 4-day DSS plus recovery groups. Data points from the 7-day control group were duplicated to have an even number of groups in order to compute the appropriate statistical analyses.

A design table summarizing the different treatment conditions is depicted below.

<table>
<thead>
<tr>
<th></th>
<th>4 Day</th>
<th>4 Day + 3 Day Recovery (7 Day)</th>
<th>7 Day</th>
<th>7 Day + 3 Day Recovery (10 Day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (H₂O)</td>
<td>6</td>
<td>8*</td>
<td>8*</td>
<td>8</td>
</tr>
<tr>
<td>4% DSS</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

* Indicates that the same control group was used.
Results

DSS Treatment Induces a Progressive Pattern of Weigh Loss

DSS treatment induces a rapid and steady decrease in body weight with leveling off only observed in the 10-recovery group after replacement of DSS with regular drinking water (see Figure 1). DSS treated mice had significantly greater weight loss at terminal time points than control mice in all disease time courses assessed: day 4 (F(1,14)=30.236; p < .001), day 7 recovery group (F(1,16)=198.713; p < .001), day 7 DSS only (F(1,16)=89.522; p < .001) and at day 10 (F(1,14)=103.945; p < .001). These findings are in line with the available literature providing support for the observation that DSS induces weight loss. In addition however, these findings add to the available literature the observation that adding a recovery period after 4 days of DSS treatment does not prevent further weight loss and that adding a recovery period following 7 days of exposure to DSS does prevent further weight loss.
Figure 1. **DSS induces rapid and progressive weight loss.** Animals treated with DSS showed significant weight loss compared to control mice. Recovery from DSS does not prevent weight loss after a 4-day exposure to DSS but does halt weight loss following 7 days of exposure. Data presented as mean ± SEM.

**DSS Induces Colon and Cecum Shrinkage and Cecum Weight Loss**

Treatment with DSS results in colon and cecum tissue shrinkage at all disease time points studied (see Figure 2). Colons from animals treated with DSS were significantly shorter than colons from control mice ($F(1,59)=40.768; p <.001$) across all times studied ($F(1,59)=0.490; p = .690$). Colon weight however was not significantly changed by DSS treatment ($F(1,57)=2.510; p = .119$). Ceca from DSS treated mice were
significantly shorter (F(1,58)=185.106; \( p < .001 \)) and weighed significantly less (F(1,59)=4.526; \( p =.038 \)) than ceca from control mice at all times studied. Taken together, these results demonstrate that the DSS-induced colon and cecum shrinkage is present by the fourth day from DSS treatment initiation and does not resolve with the instillation of a 3-day recovery period.

**Figure 2.** DSS treatment results in colon (A) and cecum shrinkage (C) and an overall decrease in cecum (D) but not colon weight. DSS-induced tissue shrinkage and weight loss was observed at all times studied. Data presented as mean ± SEM. **(p<.01)** indicates a statistically significant main effect of DSS.
DSS Induces Colon Muscle Wall Thickening and Mucosal Crypt Shortening

DSS treatment results in distal colon tissue damage including colon muscle wall thickening. Animals treated with DSS had a significantly thicker colon muscle wall in comparison to control mice (F(1,54)=47.577; \( p < .001 \)). Since the distal colon muscle wall is made up of two smooth muscle layers (muscularis interna or circular and muscularis externa or longitudinal), a refined analysis was conducted and revealed significant thickening of both the circular (F(1,54)=53.735; \( p < .001 \)) and the longitudinal (F(1,54)=24.963; \( p < .001 \)) muscle layers in DSS treated mice, an effect that was consistent at all time points assayed (F(3,54)=0.952; \( p = .422 \)). These findings corroborate at the microscopic level similar findings reported in the literature but using magnetic resonance imaging (MRI) as the assessment tool. It is therefore apparent that DSS induces colon muscle wall thickening (see Figure 3 and 4) potentially as a result of edema.

DSS treatment significantly alters the microstructure of the colon mucosa by shortening distal colon crypts. Animals treated with DSS had significantly shorter colon crypts than control mice (F(1,58)=7.281; \( p = .009 \)), with this effect consistently observed at all times studied (F(3,58)=1.143; \( p = .340 \)). Anecdotal observations of these tissues stained with H&E suggested that this DSS-induced crypt shortening could be the result of immune cell infiltration to the mucosa inducing non-specific immune cell phagocytosis of mucosal enteric cells. This cascade could be the result of bacterial penetration into the mucosa following DSS-induced permeability or damage to the surface epithelium membrane. In addition, DSS damaged colon tissue can vary in its histological appearance between groups, which may be the result of different degrees of edema. This variability in appearance was also observed in subsequent experiments.
Figure 3. **DSS exposure results in significant colon tissue damage.** DSS-induced colon damage includes muscle wall thickening, colon mucosa shortening and crypt structure damage and immune cell infiltration. Representative micrographs (10X) of distal colon samples are shown for each time point studied. Representative sections from animals receiving water (A) or 4% DSS (B) for 4 days. Representative sections from animals receiving water (C), 4% DSS for 4 days and 3 days of water (d) and 4% DSS for 7 days (E). Representative sections from animals receiving water (F) and 4% DSS for 7 days followed by 3 days on water (G). Scale bars = 100µm.
Figure 4. DSS induces significant mucosal crypt shortening (B) and colon muscle wall thickening (A) affecting both the circular (C) and longitudinal (D) smooth muscle layers. These DSS-induced microstructural changes did not vary across the different disease times studied. Data presented as mean ± SEM. 

** (p<.01) indicates a statistically significant main effect of DSS.

**DSS Induces a Significant Distal Colon Upregulation of MPO Protein**

MPO, an enzyme abundantly expressed by activated neutrophils, is highly expressed in the distal colon in response to DSS (see Figure 5). Animals treated with DSS had significantly greater colon MPO protein expression than control mice (F(1, 58)=7.647; p = .008) and this effect was similarly observed across all disease time points.
(F(3,58)=1.025; \( p = .388 \)). These results are in accord with a large body of literature and indicative of a strong neutrophil involvement in this inflammatory disease model.

**Distal Colon MPO Protein Expression**

![Distal Colon MPO Protein Expression](image)

**Figure 5.** DSS induces significant mean increases in distal colon MPO expression. These increases in MPO are observed at each disease time point studied. Data presented as mean ± SEM. **(p<.01)** indicates a statistically significant main effect of DSS.

**DSS Induces Distal Colon Upregulation of Both Pro and Anti-Inflammatory Cytokines**

DSS induces an inflammatory profile composed of both pro and anti-inflammatory cytokine with IL-1β, IL-6, IL-12, TNF-α, IL-2 and IL-4 all being reliably expressed at detectable levels in the distal colon. DSS treated mice expressed significantly more protein for the cytokines mentioned above than control mice except for
IL-1β and IL-6 which did not reach statistical significance but showed a slight statistical trend to be elevated with DSS treatment (see Table 1). These elevated cytokines in the colon were similarly observed at all disease time points studied. Table 1 shows the calculated concentrations for each cytokine assayed and the associated $p$ value only for those cytokines whose protein expression was reliably detected. Values for the other cytokines are reported using detectable protein expression in only some animals and hence should be interpreted with caution. For this reason, the statistical $p$ values for these cytokines are not reported.
### Table 1. DSS induces a cytokine inflammatory response resulting in elevated distal colon pro and anti-inflammatory cytokine expression.

All cytokine concentrations are expressed as group mean in pg/ng of total sample protein. *p* values refer to the main effect of DSS independent of treatment. No statistically significant interactions were observed for treatment by time.

**NPY Protein Expression in the Distal Colon is Significantly Increased in Response to DSS Treatment**

Treatment with DSS results in significant increases in distal colon NPY protein expression (see Figure 6). DSS treated mice had significantly greater concentrations of NPY protein in the distal colon than control groups (*F*(1,61)=12.723; *p* < .001) and this
effect was observed at all disease time points studied (F(3,61)=0.355; p =.785). This observed DSS-induced upregulation of the sympathetic neuromodulator NPY provides support for the idea of increased sympathetic involvement during inflammatory disease.

**Distal Colon NPY Protein Expression**

![Graph](image)

**Figure 6.** DSS induces a significant upregulation of NPY protein in the distal colon. This significant upregulation NPY was observed at all disease time points studied. Data presented as mean ± SEM. ** (p<.01) indicates a statistically significant main effect of DSS.

**DSS Induces Significantly Alterations in General Movement, Locomotion and Pain-Related Behaviors in the Open Field**

DSS treatment significantly altered all open field behavioral measures assessed except for total rest time (F(1,57)=0.613; p =.437). DSS treated mice showed significant
decreases in total locomotion and general movement behaviors including basic movements (F(1, 57)=31.653; p <.001), fine movements (F(1,58)=74.785; p <.001), X-Y axis ambulation (F(1,58)=21.911; p <.001), total distance traveled (F(1,58)=20.047; p <.001) and significantly increased the total time animals spent immobile (F(1,54)=8.597; p =.005). All these DSS-induced behavioral changes showed statistically significant interactions with disease time point except for immobility. All interactions followed the same pattern such that DSS treatment did not significantly alter open field behavior in the 4-day group but significantly altered behavior for the 7 day, and in some cases for the 10-day, DSS groups (see Figures 7-11.)

Visceral pain-related behaviors were observed in DSS treated compared to control mice. DSS treatment significantly decreased the total number of rears (F(1,58)=55.548; p <.001) and the total time animal spent rearing (F(1,56)=93.601; p <.001). Similar to what was observed with general movements and locomotion, DSS treatment resulted in decreased rearing (F(3,58)=4.838; p =.005) and total time spent rearing (F(3,56)=5.242; p =.003) in all time disease time points except on day 4 post treatment initiation. In order to rule out the possibility that the observed decreases in rearing and rearing time in DSS treated mice were due to general sickness-related discomfort, these two measures were normalized to total ambulation and total rears respectively. Normalization of rears was done by dividing the total number of rears by total X-Y axis ambulation, similarly, the average duration per rear was calculated from the ratio of total rearing time by total number of rears. Statistical analysis of the ratios revealed that DSS treated mice reared significantly less (F(3,57)=53.452; p < .001) and the average duration per rear was significantly shorter than that of control mice (F(3,57)=34.337; p < .001), see Figure 9. In
line with the observation before normalizing rearing, decreases in rearing were observed at all disease time points except for day 4 (F(3,57)=4.126; \( p = .010 \)). Mean rearing duration however, showed that DSS-induced decreases in rearing duration were observed at all disease time points including at day 4. Taken together, this data suggest that even though inflammation is present after 4 days of DSS treatment, a DSS-induced behavioral profile has not developed by this time point but is present in all subsequent disease time points studied. Furthermore, the normalized rearing ratios appear to be a more sensitive measure than total counts to identify visceral pain.

Figure 7. DSS induces significant decreases in general movement behaviors in the open field. Mean total (A, C) and per 5-minute block counts (B, D) for general...
movements measured. DSS decreased the total number of basic and fine movements at all time points studied except at day 4. Data presented as mean ± SEM.

** (p<.01), * (p<.01) indicates a statistically significant main effect of DSS.

Figure 8. DSS significantly decreases total ambulation and distance traveled in the open field. Mean total counts (A, C) and counts per 5-minute blocks (B, D) for locomotion measures. DSS treatment significantly decreases ambulation distance traveled in the 7-day groups but for the 4 and 10-day groups. Data presented as mean ± SEM. ** (p<.01), * (p<.01) indicates a statistically significant main effect of DSS.
Figure 9. DSS does not alter the total time animals spent resting for 15 seconds or more but significantly increases the total time spent immobile. Mean total (A, C) and per 5-minute blocks (B, D) time spent resting and immobile. DSS treatment does not affect resting time but significantly increases immobile time in the 7-day DSS-only group. Data presented as mean ± SEM. ** (p<.01), * (p<.01) indicates a statistically significant main effect of DSS.
Figure 10. DSS significantly decreases total rears and time spent rearing. Mean total (A, C) and per 5-minute blocks (B, D) rearing counts and time. Animals treated with DSS reared significantly less and spent significantly less time rearing than control mice at all disease time points assessed except at day 4. Data presented as mean ± SEM. ** (p<.01), * (p<.01) indicates a statistically significant main effect of DSS.
Figure 11. Normalized rears and rearing duration revealed significant decreases induced by DSS treatment. Mean total (A,C) and per 5 minute blocks (B,D) rearing counts normalized to ambulation and rearing time normalized to total. DSS treated mice reared less with respect to overall movement at all time points except day 4. The average duration per rear was significantly reduced by DSS treatment at all time points assessed. Data presented as mean ± SEM. ** (p<.01), * (p<.01) indicates a statistically significant main effect of DSS.
Specific Aim II: To determine the role of peripheral NPY signaling via its Y$_1$ receptor in the mouse model of DSS-induced colitis.

The role of NPY in immune and inflammatory disease is well established (Wheway et al., 2007b), particularly in inflammation with neurogenic components (Naveilhan et al., 2001). Though 3 receptors for NPY have been consistently observed in the immune system, namely, Y$_{1,2}$ and Y$_5$ (Bedoui et al. 2008; Dimitrievic et al., 2008), at this point it is not clear how each of these receptors contributes to the involvement of NPY in inflammation. Naveilhan et al. (2001) conducted a series of experiments to ascertain the involvement of Y$_1$ in different types of inflammation (neurogenic, non-neurogenic and mixed). These authors concluded that NPY Y$_1$ signaling is both required and sufficient to induce neurogenic inflammation. Similarly and specific to experimental IBD, Y$_1$ has been implicated to play a key role in experimental colitis potentially by activating antigen presenting cells (Wheway et al., 2005) as well as reports that Y$_1$ antagonism or NPY gene knock-out attenuates DSS-induced colitis (Hassani et al., 2004). Hence, the elevated levels of distal colon NPY observed in the previous experiment and elsewhere in the literature along with reports supporting the involvement of Y$_1$ in inflammation led to the assessment of the effect of systemic peripheral Y$_1$ antagonism in the DSS model of colitis using both an acute inflammatory model where DSS is continuously given for 7 days and a recovery model where 3 DSS is withdrawn and replaced with regular drinking water for 3 days after the acute inflammatory phase. It was hypothesized that Y$_1$ receptor antagonism would decrease disease severity in DSS-induced colitis.
Experiment 2

Aim and Design

To assess if systemic peripheral Y₁ receptor antagonism attenuates DSS-induced disease parameters including tissue damage, cytokine, MPO and NPY expression and open field behavior during the acute inflammatory phase. Due to the limited literature available on daily systemic administration of the selective Y₁ antagonist BIBP-3226, for this experiment, a safe dose of 1 mg/kg was chosen based on unpublished dose response studies conducted by the author. Animals were randomly assigned to one of four groups: water-vehicle, water-BIBP 3226, DSS-Vehicle or DSS-BIBP 3226 where the vehicle was distilled water (dH₂O). All animals received a once daily subcutaneous injection around the same time of day for the length of the experiment (7 days). This first exploratory experiment with BIBP 3226 did not include a recovery phase group. All statistical analyses for this experiment were computed as described in the general methods. The specific experimental design is depicted in the table below.

<table>
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<th>Vehicle (dH₂O)</th>
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Results

Y₁ Receptor Antagonism at 1 mg/kg Slows Down and Significantly Improves DSS-induced Body Weight Loss at the Terminal Time Point

Similar to the observations from Experiment 1 and in the literature, DSS induced significant weigh loss. DSS treated mice showed significantly more weight loss than control mice (F(1,36)=202.745; p <.001). The observed DSS-induced body weight loss is first observed on day 4 post DSS administration when all animal in the DSS groups loss...
significantly more weight than control mice (F(1,21)=25.208; \( p < .001 \)). Treatment with the Y\(_1\) receptor antagonism significantly attenuated DSS-induced body weight loss after 5 days of DSS exposure and this protective effect persevered until day 7, the terminal time point for this experiment (F(1,36)=4.672; \( p = .037 \)), see Figure 12.

**Figure 12.** DSS induces severe and progressive weight loss, an effect that is attenuated by Y\(_1\) receptor antagonism. Animals exposed to DSS loss significantly more weight than control mice starting at day 4 post DSS initiation. BIBP 3226 treatment significantly slows down DSS-induced weight loss and significantly attenuates this effect at the terminal time point. Data presented as mean ± SEM.


**DSS-induced Colon and Cecum Shrinkage is not Significantly Affected by Y₁ Receptor Antagonism at 1 mg/kg**

In line with the findings from *Experiment 1*, DSS exposure results in colon and cecum shrinkage and this effect is not attenuated by Y₁ receptor antagonism (see Figure 13). Animals exposed to DSS had colons that were significantly shorter (F(1,35)=39.811; \( p < .001 \)) and weighed significantly more (F(1,35)=18.968; \( p < .001 \)) than colons from control mice. Similar to the effects observed in the colon, cecum animals exposed to DSS were significantly shorter (F(1,36)=63.540; \( p < .001 \)) and weighing significantly less (F(1,36)=10.410; \( p < .003 \)) than cecum from control mice. These findings are in agreement with those from *Experiment 1* and provide further evidence that DSS induces both colon and cecum shrinkage. Treatment with the Y1 receptor antagonist did not prevent DSS-induced colon (F(1,35)=0.117; \( p = .735 \)) or cecum (F(1,36)=0.277; \( p = .602 \)) shrinkage, see Figure 12. The observation that animals exposed to DSS demonstrated signs of colon tissue atrophy yet had colons that weighed significantly more than those of control mice potentially indicates greater fluid retention and may be indicative of edema. However, this speculation was not directly assessed.
Figure 13. Exposure to DSS results in colon (A) and cecum shrinkage (C), increased colon weight (B) and decreased cecum weight (D). Treatment with a $Y_1$ receptor antagonist did not prevent these effects. Data presented as mean ± SEM. ** (p<.01), indicates a statistically significant main effect of DSS.

$Y_1$ Receptor Antagonism at 1mg/kg Does Not Significantly Alter DSS-induced Colon Muscle Wall Thickening and Mucosa Crypt Shortening

As observed in Experiment 1, DSS exposure results in significant colon wall thickening and mucosa crypt shortening (see Figures 14 and 15). Animals treated with DSS had colon muscle walls that were significantly thicker than those of control mice (F(1,30)=10.275; $p = .003$). Upon closer examination, it was observed that both the circular (F(1,29)=28.292; $p < .001$) and longitudinal (F(1,28)=13.113; $p < .001$) muscle
layers making up the distal colon wall were significantly thickened. BIBP 3226 treatment did not attenuate these effects of DSS. In addition, DSS-induced shortening of distal colon mucosa crypts (F(1,35)=47.590; p <.001) and this effect was not significantly altered by the Y1 receptor antagonist (F(1,35)=3.511; p=.069), see Figure 13. The effects of DSS on colon muscle wall and mucosa crypt observed in Experiment 1 were replicated in this experiment providing further evidence for this effect. In light that these effects may be due to edema, it is not surprising that Y1 receptor antagonism, which is expected to act on immune cell functioning, does not alter these observations.

Figure 14. Exposure to DSS results in significant colon tissue damage that is not attenuated by Y1 receptor antagonism. Representative micrographs (10X) of a non-treated control animal (A), a control animal treated with the Y1 antagonist (B), a non-
treated DSS animal (C) and a DSS animal treated with the Y₁ receptor antagonist (D). Scale bar = 100µm.

Figure 15. Y₁ receptor antagonism does not attenuate DSS-induced mucosal crypt shortening (B), full colon muscle wall (A), circular (C) or longitudinal (D) smooth muscle layers thickening. Data presented as mean ± SEM. ** (p<.01), indicates a statistically significant main effect of DSS.

Y₁ Antagonism at 1 mg/kg Attenuates DSS-Induced Distal Colon MPO Protein Overexpression

Distal colon MPO protein quantification revealed that DSS induces neutrophil activity as was also observed in Experiment 1. DSS exposure significantly increased distal colon MPO protein expression (F(1,30)=95.612; p < .001), an effect that was
significantly decreased with Y₁ receptor antagonist Treatment (see Figure 16). Animals treated with the Y₁ receptor antagonist had significantly less MPO expression than those treated with the vehicle (F(1,30)=6.252; p = .018) and this reduction in MPO expression was mainly driven by the effect of the antagonist in DSS treated mice (F(1,30)=6.524; p = .016). These observations suggest that Y₁ signaling inhibition significantly reduces the expression of MPO, thereby potentially tampering with the neutrophil-mediated inflammatory response to DSS and hence implicating NPY signaling as playing an important role in this experimental model of IBD.

**Distal Colon MPO Protein Expression**

![Graph showing MPO protein expression](image)

**Figure 16.** Y₁ receptor antagonism reduces DSS-induced MPO expression in the mouse distal colon. DSS treated animals expressed significantly more MPO than control mice. BIBP 3226 treatment significantly decreased MPO expression in DSS treated mice. Data presented as mean ± SEM.** *(p<.01), * (p<.01) indicates a statistically significant
main effect of DSS. $ (p<.05)$, indicates a significant interaction of BIBP 3226 treatment with DSS

**Exposure to DSS Results in Elevated Levels of Inflammatory Cytokines in the Distal Colon, an Effect Not Altered by Y$_1$ Receptor Antagonism at 1 mg/kg**

Animals exposed to DSS showed increased levels of all cytokines assessed except for IL-10, which was not in the detectable range for any sample (see Table 2). This DSS-induced cytokine profile is in line with the findings from *Experiment 1* and extends those findings to include the upregulation of GM-CSF, IL-5 and IFN-γ. Y$_1$ receptor antagonism did not significantly regulate DSS-induced cytokine increases. On the other hand, animals treated with this antagonist showed significantly higher levels of TNF-α (F(1,35)=7.060; $p = .012$) and IL-5(F(1,35)=5.362; $p = .027$). However, this was a general effect and not specific to DSS treated mice. Taken together, this data corroborate the DSS-induced cytokine profile and suggest that NPY signaling has the potential to modulate cytokine expression.
DSS Induced Cytokine Profile and the Effect of Y₁ Receptor Antagonism

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Table 2. DSS exposure results in increased expression of pro and anti-inflammatory cytokines in the distal colon, an effect not regulated by Y₁ receptor antagonism. Mean distal colon cytokine protein expression is presented in pg/mg of total sample protein. ** (p<.01), * (p<.01) and p values indicate a significant main effect of DSS. $ indicates a statistically significant main effect of BIBP 3226 treatment.

DSS-Induced Distal Colon NPY Protein Upregulation is not Significantly Changed by Y₁ Receptor Antagonism at 1 mg/kg

Similar to the observations from Experiment 1, exposure to DSS resulted in significant increases in distal colon NPY expression in mice (see Figure 17). DSS treated mice showed significantly greater levels of colon NPY protein than control mice.
(F(1,34)=33.214; p <.001). Treatment with the Y₁ receptor antagonist BIBP 3226 did not significantly altered this observation (F(1,34)=1.882; p =.179) These observations provide further support for the direct involvement of the sympathetic neuromodulator NPY in this model of inflammatory disease. Y₁ receptor antagonism did not significantly regulate colon NPY expression, a potential indication that other Y receptors are available for NPY signaling in the colon.

**Distal Colon NPY Protein Expression**

![Distal Colon NPY Protein Expression](image)

**Figure 17.** Exposure to DSS results in increased colon expression of NPY. Y₁ Receptor antagonism does not alter this observation. Data presented as mean ± SEM.

** (p<.01), indicates a statistically significant main effect of DSS.
**Y_1 Receptor Antagonism at 1 mg/kg Does Not Significantly Alter DSS-induced Behavioral Changes in the Open Field**

Consistent with the findings from *Experiment 1*, DSS administration significantly altered all open field behavior measures assessed (see Figures 18-22). DSS treated mice demonstrated significant decreases in general movements including basic ($F(1,36)=20.63; p <.001$) and fine movements ($F(1,36)=25.89; p <.001$) when compared to control animals. These animals also demonstrated significant decreases in general locomotion including less movement along the X-Y axis ($F(1,34)=46.640; p <.001$) and total distance traveled ($F(1,33)=55.43; p <.001$) as well as increases in total rest time ($F(1,36)=5.073; p =.031$) and time spent immobile ($F(1,35)=11.05; p =.002$). Visceral pain-related measures including rearing ($F(1,35)=91.12; p <.001$), rearing time ($F(1,34)=104.50; p <.001$), normalized number of rears to overall ambulation, ($F(1,35)=42.400; p <.001$) and normalized rearing time to total rears ($F(1,36)=27.970; p <.001$) were all significantly decreased in DSS treated mice when compared to control mice. Peripheral Y_1 receptor antagonism did not significantly alter the behavioral profile induced by DSS.
Figure 18. DSS exposure significantly decreased general movement in the open field. Y₁ receptor antagonism does not alter these observations. Mean total counts (A, C) and counts per 5-minute blocks (B, D) for basic and fine movements. DSS treatment significantly decreases the total count of basic and fine movements. Data presented as mean ± SEM. ** (p<.01), indicates a significant main effect of DSS.
Figure 19. DSS exposure significantly decreased overall locomotion in the open field. Y₁ receptor antagonism does not alter these observations. Mean total counts (A, C) and counts per 5-minute blocks (B, D) for locomotion. DSS treatment significantly decreases both X-Y ambulation and total traveled distance. Data presented as mean ± SEM. ** (p<0.01), indicates a significant main effect of DSS.
Figure 20. DSS exposure significantly increases the time animals spent immobile and resting in the open field. Y₁ receptor antagonism does not alter these behaviors. Mean total (A, C) and per 5-minute blocks (B, D) time spent resting and immobile. DSS treated mice spent significantly more time resting and immobile than control mice. Data presented as mean ± SEM. ** (p<.01), * (p<.01) indicate a significant main effect of DSS.
Figure 21. DSS exposure significantly decreases the occurrences and duration of rearing behavior in the open field. $Y_1$ antagonism did not alter these observations. Mean total (A, C) and per 5-minute blocks (B, D) rearing counts and time in the open field. DSS treated mice showed significantly less occurrences and less time spent rearing compared to control mice. Data presented as mean ± SEM. ** ($p<.01$), indicate a significant main effect of DSS.
Figure 22. After normalizing rearing behavior with overall activity, DSS exposure significantly reduces these behaviors. Y₁ antagonism does not alter these observations. Mean total (A, C) and per 5 minute blocks (B, D) rears normalized to ambulation and rearing time normalized to total rears in the open field. DSS treatment significantly decreases rearing counts and time normalized to overall X-Y ambulation. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.
Experiment 3

Aim and Design

The observation from Experiment 2 that systemic Y<sub>1</sub> receptor antagonism with BIBP 3226 at 1mg/kg significantly decreases DSS-induced MPO increases in the distal colon suggests that NPY signaling via Y<sub>1</sub> plays an important role in regulating neutrophil activity in DSS-induced inflammation. In addition, Y<sub>1</sub> receptor antagonism at 1mg/kg of BIBP 3226 attenuates one clinical manifestation of DSS induced colitis, namely, weight loss. Together, these observations provide support for a potential involvement of the sympathetic neuromodulator NPY in DSS-induced colitis, specifically its signaling via the Y<sub>1</sub> receptor. In order to further assess the role of NPY signaling via its Y<sub>1</sub> receptor in this experimental model of IBD, the current experiment was designed to replicate Experiment 2 at the higher concentration of the antagonist BIBP 3226 of 3mg/kg and further to determine the role of Y<sub>1</sub> antagonism at this higher concentration not only in the acute inflammatory model but also after a 3-day recovery period when animals are placed back on regular drinking water while still receiving the antagonist treatment. Data from Experiment 1 provides sufficient evidence that at this time point, DSS-induced bowel inflammation, general and pain-related behavioral abnormalities are still observed. To address these questions, two experiments were designed and executed concurrently with the specific designs described in the tables below. By the end of both experiments, all animals from the acute model survived until the terminal time point whereas 5 animals from the DSS vehicle recovery group had died by their terminal time point. Hence, statistical analysis for this group was carried out with a maximal sample size of 5. All statistical analyses for these experiments were computed as a 2X2 factorial ANOVA or
as a 3 way mixed Factorial ANOVAs (when adding a repeated measures variable) as appropriate.

### 7-Day Acute Inflammation Model

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<th>Vehicle (dH2O)</th>
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<td>Control (H2O)</td>
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<td>4% DSS</td>
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### 10-Day Recovery Model

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<td>Control (H2O)</td>
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<td>4% DSS</td>
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### Results

Y$_1$ Receptor Antagonism at 3 mg/kg Slows Down and Significantly Improves DSS-induced Body Weight Loss at the Terminal Time Point for Both the Acute Inflammatory and Recovery Models

Similar to the observations from *Experiment 1* and *Experiment 2*, DSS induces a progressive weight loss pattern that alleviates upon withdrawal (see Figure 23). Animals exposed to DSS showed significant weight loss in both the acute inflammatory (F(1,36)=183.935; $p < .001$) and recovery models (F(1,30)=286.178; $p < .001$). In the acute inflammation model, BIBP 3226 treatment at 3mg/kg significantly reduced DSS induced weight loss at the terminal time point (F(1,36)=4.248; $p = .047$). Similarly, in the recovery model, animals exposed to DSS and treated with BIBP 3226 at 3mg/kg showed significantly less weight loss than those receiving the vehicle injection (F(1,30)=7.842; $p = .009$). These observations replicate the findings from *Experiment 2* indicating a
protective effect of Y₁ receptor antagonism in the pathological weight loss induced by DSS.

![Diagram](image)

**Figure 23.** DSS induces a progressive pattern of weight loss in both models with weight leveling off when DSS is withdrawn in the recovery model. Y₁ receptor antagonism significantly attenuates this effect. Mean percent body weight change across time in the acute inflammation model (A) and in the recovery model (B). Data presented as mean ± SEM.
DSS-induced Colon and Cecum Shrinkage, Tissue Weight and Colon Histopathological Damage Are Not Significantly Altered by Y₁ Receptor Antagonism at 3mg/kg

Treatment with the Y₁ Receptor Antagonist at 3mg/kg does not attenuate DSS-induced histopathological damage, tissue shrinkage or weight changes (see Figures 24-26). Animals exposed to DSS had significantly more histopathological damage in both the acute F(1,35)=506.67; \( p < .001 \) and recovery periods F(1,34)=310.57; \( p < .001 \) than water treated mice and colons that were significantly shorter than those of control mice in both the acute inflammatory (F(1,36)=28.36; \( p < .001 \)) and recovery models (F(1,31)=21.85; \( p < .001 \)). Y₁ receptor antagonism did not significantly altered this observation at the acute nor recovery time points. Colon weight was not significantly different between animals exposed to DSS and control mice in the acute inflammatory model (F(1,34)=1.672; \( p = .205 \)). In the recovery model however, colons from animals exposed to DSS were significantly heavier than those from control mice (F(1,30)=33.65; \( p < .001 \)). Y₁ receptor antagonism did not affect colon weight in either the acute (F(1,34)=0.902; \( p = .349 \)) or recovery models (F(1,30)=2.743; \( p = .108 \)).

Cecum length was significantly decreased in animals exposed to DSS in both the acute (F(1,36)=18.99; \( p < .001 \)) and recovery models (F(1,29)=8.302; \( p < .007 \)) while no main effect of DSS was observed for cecum weight in either the acute (F(1,35)=1.313; \( p = .260 \)) or recovery models (F(1,31)=0.043; \( p = .837 \)). Y₁ receptor antagonism did not significantly change any of these DSS-induced effects for either model but significantly decreased cecum weight in control mice (F(1,35)=6.720; \( p = .014 \)).
Figure 24. DSS exposure induces colon and cecum shrinkage, an effect not attenuated by Y₁ receptor antagonism. Mean dry colon and cecum length following acute (A,C) and recovery (E,G) phases and mean weight following acute (B,D) and
recovery (F,H) phases. DSS induces significant colon and cecum shrinkage at both time points and increases colon weight following the recovery period. Y₁ receptor antagonism significantly decreases cecum weight following the recovery period in control but not DSS mice. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.

**Y₁ receptor Antagonism at 3 mg/kg Does Not Significantly Alter DSS-Induced Colon Wall Thickening or Crypt Shortening in in Either Model**

Similar to the observation from previous experiments, DSS exposure results in significant muscle wall thickening and mucosal crypt shortening (see Figure 27). Animals exposed to DSS demonstrated colon muscle wall thickening in both the acute (F(1,33)=15.07; p <.001) and recovery (F(1,29)=9.01; p <.001) models. Closer examination of the individual muscle layers making up the colon muscle wall revealed that both the circular (F(1,33)=31.12; p <.001) and longitudinal (F(1,34)=13.44; p <.001) muscles were significantly thickened by DSS in both models; (F(1,28)=31.82; p <.001) and (F(1,29)=12.28; p <.001) for circular and longitudinal muscle layers respectively. In the acute model, Y₁ receptor antagonism did not significantly affect overall colon wall thickness (F(1,33)=1.895; p =.178) nor the individual circular (F(1,33)=3.176; p =.084) or longitudinal (F(1,34)=4.054; p =.052) muscle layers. In the recovery model, animals treated with BIBP 3226 had significantly longer colon muscle wall (F(1,29)=6.165; p =.019) than control mice and this effect was mainly driven by a significant elongation of the longitudinal (F(1,29)=5.683; p =.024) but not the circular (F(1,28)=2.358; p =.136) muscle layer and was not specific to DSS treated mice.
In line with previous observations, DSS exposure resulted in significantly shorter mucosal crypt in the acute model (F(1,33)=39.05; \( p < .001 \)). In the recovery model however, crypt lengths were not significantly different between animals exposed to DSS and control mice (F(1,31)=3.886; \( p < .058 \)) but instead showed a statistical trend towards longer crypts than control mice potentially indicating a rebound effect during recovery. \( Y_1 \) receptor antagonism did not have an effect on DSS-induced crypt shortening in either the acute (F(1,33)=0.156; \( p = .695 \)) or recovery (F(1,31)=1.453; \( p < .237 \)) models.

![Figure 25](image)

**Figure 25.** DSS exposure in the acute inflammatory model results in significant colon tissue damage and \( Y_1 \) receptor antagonism does not attenuate this effect. Representative micrographs (10X) of a non-treated control animal (A), a control animal treated with the \( Y_1 \) antagonist (B), a non-treated DSS animal (C) and a DSS animal treated with the \( Y_1 \) receptor antagonist (D). Scale bar = 100µm.
Figure 26. DSS exposure in the recovery model results in significant colon tissue damage and $Y_1$ receptor antagonism does not attenuate this effect. Representative micrographs (10X) of a non-treated control animal (A), a control animal treated with the $Y_1$ antagonist (B), a non-treated DSS animal (C) and a DSS animal treated with the $Y_1$ receptor antagonist (D). Scale bar = 100µm.
Figure 27. DSS exposure results in colon wall thickening and mucosal crypt shortening, an effect not significantly altered by Y$_1$ receptor antagonism. Mean distal colon muscle wall thickness for the acute inflammatory (A, C, D) and recovery models.
(E, G, H), and crypt length during acute (B) and recovery periods (F). DSS treatment results in significant muscle wall thickening at both time points and BIBP 3226 does not alter this effect. Crypt lengths are shortened in the acute but not the recovery phase while BIBP 3226 does not alter these DSS-induced effects. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.

A Statistical Trend for Y₁ Receptor Antagonism at 3mg/kg to Attenuated DSS-Induced Colon MPO Expression is Observed in the Acute inflammatory but Not in the Recovery Model

In line with previous observations, DSS induces a significant increase of MPO in the distal colon (see Figure 28). Animals exposed to DSS expressed significantly more MPO protein than control mice in both the acute inflammatory (F(1,34)=20.53; p <.001) and recovery (F(1,30)=15.82; p <.001) models. This DSS-induced increase in distal colon MPO was not significantly altered by Y₁ receptor antagonism during acute inflammation (F(1,34)=1.706; p =.200) or after recovery (F(1,30)=2.115; p =.156). However, in the acute inflammatory phase, treatment with the antagonist resulted in a slight statistical tendency to decrease MPO protein expression in DSS treated animals (F(1,34)=2.925; p =.096).
Figure 28. DSS exposure results in significant elevations of MPO in the colon. Y1 receptor antagonism shows a statistical trend to attenuate this effect in the acute inflammatory model. Mean distal colon MPO protein concentrations are expressed as ng/mg of total sample protein for animals in the acute inflammatory (A) and recovery (B)
models. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.

**Y<sub>1</sub> Receptor Antagonism at 3mg/kg Results in a Tendency to Decrease DSS-Induced Distal Colon IL-1β Overexpression in the Acute Inflammatory Model and Significantly Decreases this Cytokine in the Recovery Model**

In line with the previous results presented here, DSS exposure significantly increased distal colon for IL-1β, IL-6 and IL-12 in the acute inflammatory model and IL-1β in the recovery model (see Table 3). Y<sub>1</sub> receptor antagonism showed a strong statistical tendency to decrease IL-1β expression in DSS treated mice F(1,33)=3.913; p = .056) during DSS-induced acute inflammation while no significant changes were observed in response to the antagonist for any of the other cytokines assessed in this model. Similarly, in the recovery model, Y<sub>1</sub> receptor antagonism significantly decreased DSS-induced IL-1β expression F(1,30)=6.521; p<.016), see Table 4. Together, these findings suggest that Y<sub>1</sub> signaling plays a key role in DSS-induced IL-1β overexpression in the distal colon.
### Acute Phase Cytokine Profile

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>H2O-Vehicle</th>
<th>H2O-BIBP 3226</th>
<th>DSS-Vehicle</th>
<th>DSS-BIBP 3226</th>
<th>p Value</th>
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**Table 3.** Mean distal colon cytokine protein expression measured using a Multiplex assay and standardized to total sample protein (pg/mg). DSS significantly upregulates the proinflammatory cytokines IL-1β, IL-6, and IL-12 during the acute inflammatory phase. BIBP 3226 does did not have a significant effect on cytokine expression but showed a tendency to decrease IL-1β expression in DSS treated mice. ** (p<.01), * (p<.01) and p values indicate a statistically significant main effect of DSS.
Recovery Phase Cytokine Profile

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<th>Cytokine</th>
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<th>DSS-Vehicle</th>
<th>DSS-BIBP 3226</th>
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<tr>
<td>IL-1β</td>
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<tr>
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Table 4. Mean distal colon cytokine protein expression measured using a Multiplex assay and standardized to total sample protein (pg/mg). DSS significantly upregulates IL-1β and shows strong statistical tendencies to increase other cytokines. BIBP 3226 significantly decreases IL-1β expression in DSS treated mice while decreasing GM-CSF, IL2 and IL-4 for animals in the control water group.

** (p<.01) and p values indicate a statistically significant main effect of DSS. $ indicates main effect of BIBP 3226. & (p<.05) indicates a significant interaction between treatments.

Y₁ Receptor Antagonism at 3mg/kg Does Not Significantly Alter DSS-Induced Distal Colon NPY Protein Overexpression in either Model Assessed
In line with the observations from previous studies discussed, animals exposed to DSS had significantly greater NPY protein expression in the distal colon in both the acute inflammatory (F(1,35)=25.45; \( p < .001 \)) and recovery (F(1,30)=10.95; \( p < .001 \)) models (see Figure 29). These DSS-induced distal colon NPY protein elevations are not significantly altered by BIBP 3226 in the acute (F(1,35)=0.346; \( p = .560 \)) or recovery (F(1,30)=2.313; \( p = .139 \)) models, see Figure 25. These findings provide further support for the involvement of NPY in DSS-induced inflammation and suggest the potential involvement of the sympathetic nervous system in peripheral inflammation.
Figure 29. Mean distal colon NPY protein concentration measured by ELISA and standardized to total sample protein (ng/mg). DSS treatment significantly increased distal colon NPY protein expression at both acute and recovery phases. Y₁ receptor
antagonism does not alter these observations. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.

**Y1 Receptor Antagonism at 3mg/kg Does Not Significantly Alter the DSS-Induced Behavioral Profile in the Open Field in Either Model**

In line with the findings from Experiments 1 and 2, animals exposed to DSS showed significant behavioral alterations in both the acute inflammatory and recovery models for all behavioral measures assessed in the open field except for total time spent resting in the acute (F(1,36)=3.190; p =.083) and recovery (F(1,30)=1.285; p =.266) models (see Figures 30-34). General movements are significantly decreased in DSS treated mice in comparison to control mice. In the acute phase, both basic (F(1,36)=16.23; p <.001) and fine movements (F(1,36)=53.13; p <.001) are significantly decreased in DSS treated mice. A similar observation was made during the recovery phase for both basic (F(1,30)=26.65; p <.001) and fine movements (F(1,31)=41.58; p <.001).

Locomotion was also significantly reduced by DSS administration. During the acute phase, DSS exposure induced significant decreases in X-Y ambulation (F(1,35)=18.00; p <.001), total distance traveled (F(1,36)=20.66; p <.001) and immobility (F(1,35)=11.56; p <.001). Similar observations were made for X-Y ambulation (F(1,29)=27.85; p <.001), distance traveled (F(1,30)=30.59; p <.001) and immobility (F(1,30)=38.45; p <.001) for animals in the recovery model.

DSS induced visceral pain-related behavioral changes at both time points. Rearing behavior was significantly reduced in DSS treated mice in both the acute inflammatory (F(1,36)=83.18; p <.001) and recovery (F(1,30)=56.19; p <.001) models. In line with
these observations, the total time spent rearing was also significantly decreased in animals exposed to DSS in both the acute (F(1,36)=121.7; p < .001) and recovery (F(1,30)=61.41; p < .001) models. These same behaviors assessed while normalizing rearing behavior to total ambulation and rearing time to total rears support the observation that DSS induces pain-like states. With the normalized data, DSS treated animals in the acute inflammatory (F(1,34)=59.60; p < .001) and recovery (F(1,31)=17.70; p < .001) models reared significantly less than control mice and spent significantly less time per rear, (F(1,36)=42.790; p < .001) and (F(1,31)=62.68; p < .001) for acute and recovery respectively. Y₁ receptor antagonism did not significantly alter any of the DSS-induced behavioral changes. These results corroborate the findings from Experiment 2, which suggests that Y₁ signaling may not play a significant role in DSS-induced visceral pain.
Figure 30. DSS exposure results in significant decreased in general movements in the open field. Y₁ receptor antagonism does not alter these observations. Mean basic and fine movement total (A,C,E,G) and per 5-minute block (B,D,F,H) counts in the acute and recovery models. DSS treatment significantly decreases the total count of basic and
fine movements. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.

**Figure 31.** DSS exposure results in significant decreases in locomotor activity in the open field. Y₁ receptor antagonism does not alter these observations. Mean X-Y ambulation and total distance traveled (A,C,E,G) and per 5-minute block (B,D,F,H)
counts in the acute and recovery models. DSS treatment significantly decreases both X-Y ambulation and distance traveled at both time points. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.

Figure 32. DSS exposure results in significant increases in immobility time in the open field. Y₁ receptor antagonism does not alter these observations. Mean rest and
immobility (A,C,E,G) time and per 5-minute block (B,D,F,H) counts in the acute and recovery phases in the open field. DSS does not significantly alter rest time but does increase total immobility time. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.
Figure 33. DSS exposure significantly decreases total number of rears and rearing time. Y1 receptor antagonism does not alter these observations. Mean number of rears and rearing time (A,C,E,G) and per 5-minute block (B,D,F,H) counts in the acute and recovery phases in the open field. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.
Figure 34. DSS-Induced significantly decreases reduced normalized rearing and rearing time. Y₁ receptor antagonism does not alter these observations. Mean number of rears and rearing time (A,C,E,G) and per 5-minute block (B,D,F,H) counts normalized to overall ambulation in the acute and recovery phases in the open field. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.
Specific Aim 3: To determine the role of peripheral NPY via its Y2 receptor in the mouse model of DSS-induced colitis.

Up until this point, Experiment 1 provided an in depth characterization of the DSS model of colitis at 3 different disease time points with the inclusion of recovery periods. Experiment 1 corroborated the current experimental disease landmarks reported in the literature including elevated levels of MPO, cytokines, microscopic damage to the colon epithelium and body weight loss. In addition, Experiment 1 provided a microscopic pathological characterization of DSS-induced damage not readily available in the literature including colon crypt length, colon muscle wall thickness including changes in the specific smooth muscle layers making up the colon wall as well as DSS-induced behavioral profile in the open field. Experiments 2 and 3 sought to determine the effect of NPY Y1 receptor antagonism on DSS induced colitis along all the previously mentioned variables. Findings from Experiments 2 and 3 suggested that NPY signaling via its Y1 receptor plays an important role in this experimental model of human inflammatory disease. Specifically, it was observed that Y1 receptor signaling inhibition results in significantly less body weight loss as well as decreased inflammatory cytokine and MPO expression in the distal colon. These observations were most often observed in the acute inflammatory model than following the recovery period suggesting what appears to be a specific involvement of Y1 signaling in the development of DSS-induced acute colon inflammation. These experiments provide strong evidence for the involvement of NPY in neuroimmune mediated inflammation but it is also apparent that Y1 signaling is not the sole mechanism of NPY’s involvement. In light of these observations and the well publicized role of Y2 receptors in immunity, the studies in Experiment 4 are aimed at
unveiling the potential involvement of NPY signaling via its Y2 receptor in the DSS experimental model of colitis.

**Experiment 4**

**Aim and Design**

To study the role of the Y2 receptor in DSS-induced inflammation and pain-related behavior, the small molecule selective Y2 receptor antagonist BIIE 0246 given once daily via subcutaneous injections at a dose of 10mg/kg during the length of the experiment. Because BIIE 0246 is not soluble in water but rather ethanol or DMSO, both of which have the capacity to alter behavior on their own, the first study was designed with the highest concentration of diluent (DMSO) available in the literature as a gauge for subsequent studies within this experiment.

For this first study, BIIE 0246 was reconstituted in 100% DMSO for the stock solution and then diluted in a 50:50 ratio of dH2O to DMSO to obtain an injectable solution of 50% DMSO and a dose of 10mg of BIIE 0246 per kg of mouse weight. The recovery model was used to assess any potential detrimental effects of this compound across the longer model (10-day administration period). Briefly, the control group received drinking water for 10 days whereas the experimental group received 4% DSS for 7 days then animals were changed to drinking water for the following 3 days. Due to higher than expected mortality rates in the DSS groups for this study, only body weight and behavior was assessed. The specific experimental designed is depicted in the table below. All statistical analyses for this experiment were computed as independent samples t-test or as a 3 way mixed Factorial ANOVAs (when adding a repeated measures variable) as appropriate.
Results

**BIIE 0246 Reconstituted in 50% DMSO as well as 50% DMSO Alone Exacerbate DSS-Induced Weight Loss and Increase Death Rate in DSS Treated Mice**

In line with previous observation, DSS exposure results in significant body weight loss compared to mice provided with regular drinking water (see Figure 35). Similar to previous experiment, significant DSS-induced body weight loss is observed as early as day 4 post DSS administration ($F(1,35)=21.621; p <.001$) and continued to decrease throughout the length of the experiment. However, unlike the previous recovery experiment, mice exposed to DSS did not show body weight leveling following initiation of the recovery phase. In fact, by day 7 post DSS exposure (DSS withdrawal day), only 8 out of 20 DSS treated animals survived (5 from DSS-BIIE 0246 group) while 17 animals from the water control groups were still alive. By day 8 post DSS administration (day 1 of recovery), the remaining DSS mice were humanely sacrificed as they continued to loose weight below the critical point of 25% body weight loss and that experimental leg was concluded. Both water control groups were maintained and weight daily to assess the role any potential detrimental effects of DMSO in body weight. As seen in Figure 31, though there were weight variations in these groups, no obvious detrimental effect of DMSO was observed in these mice. These findings suggest that a 50% DMSO solution is lethal for DSS treated mice and call for a lower DMSO concentration for future reconstitution of the $Y_2$ receptor antagonist.
Figure 35. 50% DMSO is lethal for animals exposed to DSS. Mean percent body weight change across time in the recovery model of DSS-induced colitis. DSS treated mice experience significant body weight loss as early as day 4 post DSS exposure and continue to loose weight even when tap water is reinstalled in the presence of 50% DMSO. Water control groups maintain body weight throughout the length of the experiment with minimal fluctuation. Data presented as mean ± SEM.

**BIIE 0246 Reconstituted in 50% DMSO Does Not Significantly Alter Open Field Behaviors Compared to 50% DMSO-Alone Control Groups**

Due to high mortality rates in the DSS treated groups, only the two water control (water-50% DMSO and water-BIIE 0246 in 50% DMSO) groups were assessed in the open field. General movements in the open field were not affected by
BIIE 0246 reconstituted in 50% DMSO in comparison to the vehicle (50% DMSO-alone) 
group (see Figures 36-40), this was true for basic movements ($t(14)=0.527; p=0.606$) and 
fine movements ($t(14)=0.421; p=0.680$). Similarly, measures of locomotion did not differ 
significantly between these two groups. Animals from both groups showed similar X-Y 
ambulation patterns ($t(14)=0.747; p=0.468$), traveled similar total distances ($t(14)=0.551; 
p=0.591$), had similar total resting time ($t(13)=0.626; p=0.542$) as well as total time spent 
immobile ($t(14)=0.103; p=0.919$). In addition, these two groups did not differ 
significantly in the visceral pain-related measures assessed including total rearing counts 
($t(14)=1.431; p=0.175$), rearing time ($t(14)=1.308; p=0.212$), and no difference was 
observed when these two measures normalized to overall ambulation ($t(13)=0.605; 
p=0.556$) and total rears ($t(14)=0.770; p=0.454$) respectively. Though there is no significant 
difference between the 50% DMSO and BIIE 0246 in 50% DMSO groups, it is worth 
noting that all values for each measure assessed appear to be at very low levels in 
comparison to the values from control animals in previous experiments which is in line 
with the reported numbing properties of DMSO.
Figure 36. BIIE 0216 in 50% DMSO does not significantly alter general movement behaviors in the open field. Mean total counts (A, C) and counts per 5-minute blocks (B, D) for general movements measures. BIIE 0246 treated animals did not differ significantly from control groups for either measure of general movement. Data presented as mean ± SEM.
Figure 37. BIIE 0216 in 50% DMSO does not significantly alter general locomotion behaviors in the open field. Mean total counts (A, C) and counts per 5-minute blocks (B, D) for locomotion. BIIE 0246 treated animals did not differ significantly from control groups for either measure of locomotion. Data presented as mean ± SEM.
Figure 38. BIIE 0216 in 50% DMSO does not significantly total rest and immobility time in the open field. Mean total (A, C) and per 5-minute blocks (B, D) time spent resting and immobile. BIIE 0246 does not alter resting time or time spent immobile when compared to animals in the vehicle group. Data presented as mean ± SEM.
Figure 39. BIIE 0216 in 50% DMSO does not significantly alter general rearing behaviors in the open field. Mean total (A, C) and per 5-minute blocks (B, D) rearing counts and time. BIIE 0246 treated mice showed similar number of rears and time rearing as animals from the vehicle group. Data presented as mean ± SEM.
Figure 40. BIIE 0216 in 50% DMSO does not significantly alter normalized rearing behaviors in the open field. Mean total (A, C) and per 5 minute blocks (B, D) rears normalized to ambulation and rearing time normalized to total rears. Normalizing for overall ambulation, BIIE -0246 treated mice did not differ significantly from animals in the vehicle group. Data presented as mean ± SEM.
Experiment 5

The study from Experiment 4 demonstrated that administration of BIIE 0246 diluted in 50% DMSO or 50% DMSO alone results in high mortality rates for animals exposed to DSS. In addition, though no significant differences were observed in behavioral patterns between animals injected with 50% DMSO alone or the Y₂ blocker diluted in this solution, post hoc observations suggest that generally, animals treated with 50% DMSO showed decreased activity in the open field (half values for most measures) in comparison to values from control animals in all previous experiments. These observations suggest that treatment with a 50% DMSO solution is detrimental for animals exposed to DSS and dampers overall activity in the open field, findings which are suggestive of a “numbing effect” of DMSO at a concentration of 50%. In order to address this issue, different dilution of BIIE 0246 were made at varying degrees of DMSO concentrations to assess the lowest DMSO concentration that would maintain the Y₂ antagonist in solution. The DMSO concentrations assessed were 50%, 25%, 12.5% and 6.25% after letting the solutions sit for over 72 hours. BIIE 0246 was completely in solution at all concentration except at the lowest where some crystals were still visible. Based on these observations the 12.5% concentration was selected. A pilot study was designed to determine if a12.5% DMSO solution injected daily subcutaneously would also induced the higher mortality rates in mice exposed to DSS mice as was observed with the 50% DMSO solution. Since this was a pilot study, only percent weight change and open field behaviors were assessed. This pilot study design is depicted in the table below. A t-test or repeated measures ANOVA were used for statistical analysis where appropriate.
Results

A 12.5% DMSO Solution Administered to Mice Exposed to DSS Results in Nearly Indistinguishable Body Weight Loss Patterns Compared to Control Mice

Daily subcutaneous injection of 12.5% DMSO to DSS treated mice does not induced the high mortality rate observed when a 50% DMSO solution is administered nor does it exacerbate body weight loss (see Figure 41). In fact, both groups showed percent body weight changes that were in line with the previously observed DSS-induced weight loss patterns but no significant difference was observed between the groups across the 7 day period (F(1,8)=0.586; p=.466).

Percent Body Weight Change
Figure 41. Mean percent body weight change across time in the acute model of DSS-induced colitis. Treatment with a 12.5% DMSO solution does not alter DSS-induced body weight loss. Data presented as mean ± SEM.

A 12.5% DMSO solution Administered to Mice Exposed to DSS Does Not Alter DSS-Induced Behavioral Changes in the Open Field

Administration of a 12.5% DMSO solution to mice exposed to DSS does not significantly alter general movements, locomotion or pain-related behaviors in the open field (see Figures 42-46). In DSS treated mice, total basic (t(8)=0.620; p=.552) and fine movements (t(8)=1.366; p=.210) are not significantly different between vehicle (dH2O) or 12.5% DMSO treated animals. Similarly, measures of locomotion including X-Y ambulation (t(8)=0.466; p=.654), total distance traveled (t(8)=0.711; p=.497), total time spent resting (t(8)=0.377; p=.716) and total immobility time (t(8)=1.337; p=.218) were not significantly different between these groups. In addition, these groups also did not differ in pain-related measures including total rears (t(8)=0.626; p=.549), total rearing time (t(8)=1.073; p=.315) even after normalizing rearing to overall ambulation (t(8)=0.198; p=.848) and rearing time to total rears (t(8)=0.879; p=.405).
Figure 42. 12.5% does not significantly alter DSS-induced changes in general movements in the open field. Mean total counts (A, C) and counts per 5-minute blocks (B, D) for general movements measures. DSS treated mice injected with 12.5% DMSO do not differ significantly in general movements from control mice receiving the vehicle injection. Data presented as mean ± SEM.
Figure 43. 12.5% does not significantly alter DSS-induced changes in general locomotion in the open field. Mean total counts (A, C) and counts per 5-minute blocks (B, D) for locomotion. DSS treated mice injected with 12.5% DMSO do not show significant differences from control mice in overall locomotion. Data presented as mean ± SEM.
Figure 44. 12.5% does not significantly alter DSS-induced changes in rest and immobility times in the open field. Mean total (A, C) and per 5-minute blocks (B, D) time spent resting and immobile. Injections of 12.5% DMSO to DSS treated mice does not significantly alter total rest time or immobility compare to control mice. Data presented as mean ± SEM.
Figure 45. 12.5% does not significantly alter DSS-induced changes in rearing behaviors in the open field. Mean total (A, C) and per 5-minute blocks (B, D) rearing counts and time. DSS treated mice injected with 12.5% DMSO do not show differential rearing patterns compared to control mice. Data presented as mean ± SEM.
Figure 46. 12.5% does not significantly alter DSS-induced changes in normalized rearing behavior in the open field. Mean total (A, C) and per 5 minute blocks (B, D) rearing normalized to ambulation and rearing time normalized to total rears. 12.5% DMSO injections do not alter rearing behavior even when normalizing to overall ambulation in DSS treated mice. Data presented as mean ± SEM.
Experiment 6

Both previous studies sought to determine the optimal concentration of DMSO to reconstitute the Y$_2$ receptor antagonism BIIE 0246 while not affecting mortality in DSS treated mice or behavior when administered systemically for long periods of time. The data from these studies suggests that DMSO at high concentrations (50%) increases mortality rates in DSS treated mice and affects behavior in both water and DSS treated mice via what appears to be a “numbing-like effect”. At the lower concentration of DMSO (12.5%) where BIIE 0246 is still maintained in solution for long periods of time, mortality in DSS treated mice is unaffected as well as behaviors in the open field. Based on this data, the role of NPY signaling via its Y$_2$ receptor in the DSS model of colitis in both the acute inflammatory and recovery models was assessed in a similar design to Y$_1$ in Experiment 3. Briefly, for the acute inflammatory model, experimental animals received 4% DSS in their drinking water for 7 days at which point they were tested in the open field and sacrificed for tissue collection. For the recovery model, animals were allowed a 3-day recovery period after the acute inflammatory phase. All animals in the experimental group received the Y$_2$ antagonist for the duration of the experiment. The specific designs are depicted in the table below. All statistical analyses for these experiments were computed as a 2X2 factorial ANOVA or as 3 way mixed Factorial ANOVAs as appropriate. One animal from each DSS group in the acute model died in the course of the experiment while none did in the recovery model. Data from dead animals was only used for statistical analysis when available.
Results

Y\textsubscript{2} Receptor Antagonism Slows Down DSS induced Weight Loss and Significantly Improves Terminal Weight in DSS Treated Mice in Both the Acute Inflammatory and Recovery Models

As observed in all previous experiments, administration of DSS to mice caused significant weight loss in both the acute (F(1,34)=638.65; p < .001) and recovery models (F(1,36)=281.39; p < .001) compared to their respective control groups (see Figure 47). Y\textsubscript{2} receptor antagonism results in a slower weight loss pattern in animals exposed to DSS and significantly improves terminal body weight for these mice in both the acute (F(1,34)=8.88; p = .005) and recovery (F(1,36)=11.25; p = .002) models while showing no significant effect on body weight for any of the control groups, see Figure 43.
Figure 47. DSS induces a progressive pattern of weight loss in both models with weight leveling off after DSS withdrawal in the recovery model. Y2 receptor antagonism significantly attenuates this effect. Mean percent body weight change across time in the acute inflammation model (A) and in the recovery model (B). Y2 receptor antagonism slows down DSS-induced weight loss and significantly improves
body weight at both terminal points. Data presented as mean ± SEM. ** (p<.01), indicate a significant difference between DSS groups.

**Y2 Receptor Antagonism at 10mg/kg Does not Alter DSS-induced Histopathological damage, Colon and Cecum Shrinkage or Tissue Weight Changes in the Acute Inflammatory Model But Does Alter Tissue Weight in the Recovery Phase**

Similar to the observations from previous studies, DSS administration results in significant colon and cecum shrinkage and weight loss at both stages of disease (see Figure 48). Animals exposed to DSS had greater histopathological damage than control mice at the acute F(1,36)=491.723; p <.001) and recovery periods F(1,36)=149.25; p <.001) and colons that were significantly shorter in both the acute (F(1,33)=58.720; p <.001) and recovery phases (F(1,36)=6.635; p =.014) compared to colons from control mice. Y2 antagonism did not significantly change these observations. Colon weight was not significantly altered in the acute phase (F(1,33)=0.2919; p =.593) but was significantly increased in mice exposed to DSS in the recovery model (F(1,35)=5.334; p =.027).

Cecum from mice exposed to DSS were significantly shorter than those of control mice in both the acute (F(1,34)=42.820; p <.001) and recovery (F(1,36)=53.480; p <.001) models. Y2 antagonism did not alter these observations at either time point. Cecum weight was significantly decreased by DSS administration in both the acute (F(1,34)=12.75; p <.001) and recovery (F(1,36)=8.631; p =.005) models. Y2 antagonism did not alter these observations in either model.
Figure 48. DSS induces colon and cecum shrinkage and tissue weight change in both models. Y$_2$ receptor antagonism does not alter these observations. Mean dry colon and cecum length following acute (A,C) and recovery (E,G) phases and mean weight following acute (B,D) and recovery (F,H) phases. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.
Y$_2$ Receptor Antagonism Does Not Significantly Alter DSS-Induced Colon Wall Thickening or Mucosa Crypt Shortening in either Model Assessed

In line with previous observations, DSS induces significant colon muscle wall thickening and mucosa crypt shortening (see Figures 49-51). Animals exposed to DSS had significantly thicker colon muscle walls than control mice in both the acute (F(1,31)=15.400; $p <.001$) and recovery (F(1,31)=8.887; $p =.006$) models. Examination of the individual smooth muscle layers making up the colon wall revealed significant thickening of both the circular (F(1,30)=34.49; $p <.001$) and longitudinal (F(1,31)=4.668; $p =.039$) muscle layers in the acute inflammatory model. For the recovery model, colon muscle wall thickness was mainly driven by thickening of the circular (F(1,32)=6.083; $p =.019$) but not the longitudinal (F(1,31)=0.349; $p =.559$) muscle layer. Y$_2$ receptor antagonism did not have an effect on DSS-induced colon wall thickening in either model.

Animals exposed to DSS had significantly shorter mucosal crypts in the acute (F(1,32)=4.736; $p =.037$) model but significantly longer crypts in the recovery (F(1,34)=6.407; $p =.016$) model. Y$_2$ receptor antagonism did not have an effect in DSS-induced crypt shortening during the acute inflammatory (F(1,32)=0.012; $p =.915$) or recovery (F(1,34)=1.708; $p =.200$) models.
Figure 49. DSS exposure in the acute inflammatory model results in significant colon tissue damage and Y₂ receptor antagonism does not attenuate this effect. Representative micrographs (10X) of a non-treated control animal (A), a control animal treated with the Y₂ antagonist (B), a non-treated DSS animal (C) and a DSS animal treated with the Y₂ receptor antagonist (D). Scale bar = 100µm.
Figure 50. DSS exposure in the recovery model results in significant colon tissue damage and $Y_2$ receptor antagonism does not attenuate this effect. Representative micrographs (10X) of a non-treated control animal (A), a control animal treated with the $Y_2$ antagonist (B), a non-treated DSS animal (C) and a DSS animal treated with the $Y_2$ receptor antagonist (D). Scale bar = 100µm.
Figure 51. DSS induces significant colon wall thickening and mucosa crypt shortening. Y2 receptor antagonism does not alter these observations. Mean full distal colon muscle length and specific muscle layers for the acute (A, C, D) and recovery phases (E, G, H), and crypt length during acute (B) and recovery periods (F). Data
presented as mean ± SEM. ** (p<.01), * (p<.05) indicate a significant main effect of DSS.

**Y₂ Receptor Antagonism Does Not Significantly Alter DSS-Induced Distal Colon MPO Protein Expression in the Acute Inflammatory Model but Shows A Statistical Tendency To Attenuate This Effect in the Recovery Model**

In line with observations from all previous experiments presented here, exposure to DSS significantly increases distal colon MPO protein expression (see Figure 52). MPO protein was significantly overexpressed in DSS treated mice in both the acute (F(1,31)=34.370; p <.001) and recovery (F(1,33)=19.140; p <.001) phases. Y₂ receptor antagonism does not alter DSS-induced distal colon MPO increases in the acute inflammatory model (F(1,31)=0.148; p =.703) but shows a statistical tendency to decrease MPO expression in the recovery model (F(1,33)=3.519; p =.077).
Figure 52. DSS induces significant upregulation of distal colon MPO in both models. 

**Y$_2$ receptor antagonism does not significantly alter these observations.** Mean distal colon MPO protein concentration expressed in ng/mg of total sample protein for animals in the acute inflammatory (A) and recovery (B) models. Y$_2$ receptor antagonism does not alter DSS-induced increases in distal colon MPO expression in the acute inflammatory model but shows a strong statistical tendency to decrease MPO expression in the recovery model. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.
Y₂ Receptor Antagonism Does Not Alter DSS-Induced Distal Colon Cytokine Increases in Either Model

In line with previous findings, DSS exposure induced significant increases in distal colon cytokine expression in the acute inflammatory model (see Tables 5 and 6). In the recovery model however, DSS did not increase distal colon cytokine expression with only a slight trend to increase IL-1β observed. In fact, from all the cytokines assessed, only IL-1β, IL-6 and IL-12 demonstrated consistent detectable protein expression in both models with only very few animals showing protein expression for the other cytokines. When expression was below the detectable range, animals were assigned the lowest detectable limit value for those cytokines. Y₂ receptor antagonism did not have an effect on distal colon cytokine expression in either model.
## Acute Phase Cytokine Profile

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<th>DSS-BIIE0246</th>
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Table 5. DSS induces significant increases in distal colon cytokine expression. Y2 receptor antagonism does not significantly alter these observations. Mean distal colon cytokine protein expressed as pg/mg of total sample protein. Animals exposed to DSS showed significant increases in IL-1β, IL-6 and IL-12.

* (p<.01) indicate a statistically significant main effect of DSS.
### Recovery Phase Cytokine Profile

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Table 6. DSS did not significantly increase distal colon cytokine expression in the recovery model. Mean distal colon cytokine protein expressed in pg/mg of total sample protein

**Y2 Receptor Antagonism Does Not Significantly Alter DSS-Induced Distal Colon Increases in NPY Protein Expression at either Disease Time Point**

As previously observed, exposure to DSS results in a significant upregulation of distal colon NPY protein expression (see Figure 53), an effect that was observed at both the acute inflammatory (F(1,33)=19.440; p < .001) and recovery (F(1,34)=11.160; p < .002) phases. Treatment with BIIE 0246 does not alter distal colon NPY protein...
expression in neither the acute (F(1,33)=2.421; \( p = .129 \)) nor recovery (F(1,34)=2.547; \( p = .120 \)) models.

**Figure 53.** DSS induces significant increases of NPY in the distal colon in both models. Mean distal colon NPY protein concentration expressed in ng/mg of total sample protein. DSS treatment induces distal colon NPY protein upregulation in both the acute and recovery models. \( Y_2 \) receptor antagonism does not significantly alter these observations but shows a statistical tendency to decrease NPY expression in DSS treated
mice. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.

**Y2 Receptor Antagonism Does Not Significantly Alter DSS-Induced Behavioral Changes in the Open Field**

Similar to the observations from previous experiments, DSS exposure significantly alters all behavioral measures assessed in the open field in both models except for total rest time at both time points and total distance traveled and X-Y ambulation in the recovery model (see Figures 54-58). DSS significantly alters general movement at both time points in the open field. Basic movements were significantly decreased in mice exposed to DSS in the acute inflammatory (F(1,34)=22.160; p <.001) and recovery (F(1,36)=4.127; p =.500) models. Similarly, fine movements were significantly decreased in mice exposed to DSS in comparison to control mice in both the acute (F(1,34)=29.860; p <.001) and recovery (F(1,36)=16.910; p <.001) models. Y2 receptor antagonism did not significantly changes these DSS-induced behavioral changes in the acute inflammatory model but showed a slight statistical tendency to increase fine movements in these mice (F(1,36)=2.860; p =.100).

Locomotion in the open field was also significantly decreased in animals exposed to DSS when compared to control mice. DSS treated mice showed significantly less X-Y ambulation in the acute inflammatory model (F(1,32)=69.720; p <.001) and a statistical tendency in the same direction was observed for recovery model (F(1,36)=3.308; p =.077). Similarly, the total distance traveled was significantly decreased in animals exposed to DSS in the acute inflammatory model (F(1,32)=70.410; p <.001) but for animals in the recovery model (F(1,35)=1.939; p =.173). Though no significant effect of
DSS was observed in total time spent resting, there was however an effect of this treatment in total time spent immobile. DSS treated mice spent significantly more time immobile than control mice in both the acute inflammatory (F(1,33)=25.250; \( p < .001 \)) and recovery (F(1,34)=5.185; \( p = .029 \)) models. \( \gamma \text{}_2 \) receptor antagonism did not significantly alter these DSS-induced behavioral changes in either model.

Visceral pain-related behaviors were significantly induced by DSS as seen in all previous experiments in both the acute inflammatory and recovery models. Total rearing counts were significantly decreased in mice exposed to DSS compared to control mice both during acute inflammation (F(1,31)=114.700; \( p < .001 \)) and recovery (F(1,35)=39.030; \( p < .001 \)). Similarly, the total amount of time animals spent rearing was significantly decreased in DSS treated mice during acute inflammation (F(1,31)=114.700; \( p < .001 \)) and recovery (F(1,31)=114.700; \( p < .001 \)). Analyzing the effects of DSS on rearing behavior normalized to the animal’s total X-Y ambulation resulted in similar findings. Rearing counts normalized to total X-Y ambulation revealed significant decreases in rearing in the acute inflammatory (F(1,32)=65.990; \( p < .001 \)) and recovery (F(1,35)=35.090; \( p < .001 \)) models. Rearing time normalized to total rears was also decreased in mice exposed to DSS in both the acute inflammatory (F(1,34)=30.070; \( p < .001 \)) and recovery (F(1,36)=80.490; \( p < .001 \)) models.

\( \gamma \text{}_2 \) receptor antagonism did not significantly affect DSS induced rearing or rearing time but it showed strong statistical trends to increase overall total rearing (F(1,31)=4.082; \( p = .052 \)) and total rearing time (F(1,32)=3.637; \( p = .066 \)) in the acute inflammatory model. Normalizing total rears to total ambulation revealed a significant effect of BIIE 0246 on rearing. Overall, animals treated with the antagonist showed
significantly more rearing than animals receiving the vehicle solution (F(1,32)=7.673; \(p < .009\)). Though not statistically significant, the statistical tendency for BIIE 0246 to increase rearing duration was also observed after normalizing for total rears (F(1,34)=3.439; \(p = .072\)). In the recovery model, BIIE 0246 did not have a significant effect on total rears (F(1,35)=0.207; \(p = .652\)) or total rearing time (F(1,36)=0.038; \(p = .847\)). This observation was also seen after normalizing total rears by ambulation (F(1,35)=1.648; \(p = .207\)). However, when total rearing time was normalized to total rears, an effect of BIIE 0246 was revealed. Overall, treatment with the \(Y_2\) receptor antagonist resulted in significantly longer rears (F(1,36)=8.857; \(p < .005\)).
Figure 54. DSS induces significant decreases in general movements in the open field. \( \text{Y}_2 \) receptor antagonism does not alter these observations. Mean basic and fine movement total (A,C,E,G) and per 5-minute block (B,D,F,H) counts in the acute and recovery models. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.
Figure 55. DSS induces significant decreases in general locomotion in the open field. Y$_2$ receptor antagonism does not alter these observations. Mean X-Y ambulation and total distance traveled (A,C,E,G) and per 5-minute block (B,D,F,H) counts in the acute phase.
and recovery models. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.

Figure 56. DSS does not alter rest time but significantly increases immobility in the open field. Y₂ receptor antagonism does not alter these observations. Mean rest and
immobility (A,C,E,G) time and per 5-minute block (B,D,F,H) counts in the acute and recovery models. Data presented as mean ± SEM.

**Figure 57.** DSS induces significant decreases in rearing behavior in the open field. 

_Y_2 receptor antagonism does not alter these observations._ Mean number of rears and rearing time (A,C,E,G) and per 5-minute block (B,D,F,H) counts in the acute and recovery phases.
recovery models. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.

Figure 58. DSS induces significant decreases in normalized rearing behavior in the open field. Y2 receptor antagonism does not alter these observations. Mean
normalized rears and rearing time (A,C,E,G) and per 5-minute block (B,D,F,H) counts in the acute and recovery models. Data presented as mean ± SEM. ** (p<.01), indicate a significant main effect of DSS.
Specific Aim 4: To evaluate the possibility that NPY plays a role in the pathophysiology of DSS-induced colitis via its Y₁ and Y₂ receptors in a pathology-stage dependent manner.

Experiments 3 and 6 sought to determine any potential involvement of NPY signaling via its Y₁ and Y₂ receptors in modulating several parameters associated with DSS-induced colitis. When looking at the data collectively, it is apparent that blocking either receptor does not result in any robust disease modification. However, upon closer examination, there appears to be a slight pattern such that the effects of each antagonist appears to have greater effects in one time course model over the other. That is, the tendencies for Y₁ receptor antagonism with BIBP 3226 to alter DSS-induced effects more often occurred in the acute inflammatory model and were nearly nonexistent in the recovery model. Y₂ receptor antagonism on the other hand showed slight tendencies to alter DSS-induced effects in the recovery but not in the acute inflammatory model.

Experiment 7

Aim and Design

Based on these observations, the current studies were designed to replicate the potential effects of each antagonist given alone, but to also determine whether the effects would be enhanced if both antagonists were given in combination in the acute and recovery models. An additional experiment tested the combination of the Y₁ receptor antagonist given for the first 7 days and the Y₂ receptor antagonist given during the 3 recovery days, hereafter referred to as the mixed treatment. All animals in these studies received 4% DSS for 7 days. All measures assessed in the previous experiments were also collected for these studies. These three different studies were conducted concurrently
and each experimental design is depicted in the tables below. All statistical analyses for these experiments were computed as 2X2 factorial ANOVAs or as 3 way mixed Factorial ANOVAs (when adding a repeated measures variable) as appropriate.

### 7-Day Acute Inflammatory Model Combination Treatment

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### 10-Day Recovery Model Combination Treatment

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### 10-Day Recovery Model Mixed Treatment

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Results

**Y$_1$ and Y$_2$ Combined and Mixed Receptor Antagonism Treatments Do Not Significantly Improve DSS-induced Terminal Weight Loss**

All animals in these studies were exposed to DSS and showed the typical pattern of weight loss observed in previous experiments (see Figure 59). In the acute inflammatory model, animals treated with the Y$_1$ receptor antagonist had significantly more weight loss at the terminal point than animals receiving the dH$_2$O vehicle injection (F(1,35)=8.484; $p$=.006), an effect that may be driven by the co-administration of BIBP.
3226 with 12.5% DMSO as this group experienced the greatest weight loss among the groups. The Y$_2$ receptor antagonist on the other hand showed a protective effect in DSS-induced body weight loss when given alone which is in line with observations from previous experiments. Animals treated with the Y$_2$ receptor antagonist had lost significantly less weight at the terminal point of the acute model than animals treated with the 12.5% DMSO vehicle solution (F(1,35)=10.749; $p = .002$). The combination treatment of the two antagonists did not improved terminal body weight. Co-treatment with the Y$_1$ and Y$_2$ receptor antagonists ((F(1,30)=0.974; $p = .331$) or either antagonist-alone did not prevent or alleviate DSS-induced body weight loss at the terminal point in the recovery model((F(1,30)=0.038; $p = .846$ and (F(1,30)=1.445; $p = .239$) for BIBP 3226 and BIIE 0246 respectively). Similarly, in the recovery model, mixed antagonist treatment did not significantly improve terminal body weight (F(1,24)=0.113; $p = .740$). In this same model, animals treated with BIBP 3226 only during the acute inflammatory phase did not improve body weight (F(1,24)=0.007; $p = .933$). Similarly, animals only receiving BIIE 0246 treatment during the recovery phase did not show body weight improvement (F(1,24)=0.842; $p = .368$).
Figure 59. DSS induces significant body weight loss that is not attenuated by $Y_1$ and $Y_2$ receptor antagonist combination or mixed treatment. Mean percent body weight change across time in the acute inflammation model (A) and in the recovery model (B) with $Y_1$ and $Y_2$ receptor antagonist combination treatment and mixed treatment in the recovery model (C). Data presented as mean ± SEM.
Y₁ and Y₂ Combined and Mixed Receptor Antagonist Treatment Did Not Attenuate DSS-Induced Histopathological damage, Colon and Cecum Shrinkage or Weight Change

Y₁ and Y₂ combined in the acute F(1,39)=0.046; p = .831), recovery F(1,33)=0.001; p = .994) and mixed F(1,26)=1.826; p = .190) receptor antagonism does not attenuate DSS-induced histopathological damage. Combination treatment of BIBP 3226 and BIIE 0246 did not significantly improve DSS-induced colon shrinkage or weight change in either the acute inflammatory or recovery models (see Figure 60). However, animals treated with the Y₁ receptor antagonist has significantly larger colons than those not treated with the antagonists (control) (F(1,35)=6.451; p = .016). There was no interaction or significant main effects of either antagonist in either the combined or mixed treatments in the recovery model.
Figure 60. Treatment with the Y<sub>1</sub> receptor antagonist significantly attenuates DSS-induced colon shrinkage in the acute inflammatory model. Mean colon length in the acute inflammatory model with antagonist co-treatment (A) and colon length and weight in the recovery model with antagonist co-treatment (B,C) and mixed treatment (D, E).
Data presented as mean ± SEM. $ (p<.05), indicates a significant main effect of BIBP 3226.

**Neither Combined nor Mixed Y₁ and Y₂ Receptor Antagonism Significantly Alter DSS-Induced Colon Muscle Wall Thickness or Mucosa Crypt Length**

The combination or mixed treatment with BIBP 3226 and BIIE 0246 in either the acute inflammatory or recovery models did not significantly alter DSS-induced changes in colon muscle thickness and crypt lengths (see Figures 61-64). However, the Y₁ receptor antagonist alone did have significant effects on these measures in both the combined and mixed treatments in the recovery model. In the recovery model where animals were co-administered BIBP 3226 and BIIE 0236, animals treated with the Y₁ receptor antagonist had significantly shorter colon crypts (F(1,28)=7.508; $p =.011$). A slight statistical tendency in the same direction was observed with BIIE 0246 treated mice (F(1,28=3.165; $p =.086$) in the same model, however, the individual effects of these treatments did not interact to produce a synergistic effect (F(1,28)=1.054; $p =.313$). A similar tendency for the Y₁ receptor antagonist to decrease crypt length was also observed in the acute inflammatory model (F(1,34=3.085; $p =.088$). This negative effect of the Y₁ receptor antagonist was not observed in any previous experiments as well as in the mixed recovery model. A major difference between the experiments where the antagonist had detrimental effects from the other experiments not showing this effect is the co-administration of the antagonist with 12.5% DMSO. It is therefore speculated that BIB6 3226 can exacerbate DSS-induce mucosal crypt damage when administered along with 12.5% DMSO.
Co-administration of the antagonists at either the acute inflammatory or recovery models did not significantly alter DSS-induced colon muscle wall thickening. In the recovery model mixed antagonist treatment however, treatment with the Y₁ receptor antagonist resulted in significantly thicker colon walls (F(1,22)=8.341; p < .009). Analysis of the two muscular layers making up the colon wall revealed that this effect was observed in both muscles but only significantly increased in the circular layer (F(1,21)=8.949; p < .007) with strong statistical trend in the same direction for the longitudinal muscle (F(1,22)=4.009; p = .058), see Figure 55.

![Combined receptor antagonist treatment in the acute inflammatory model does not attenuate DSS-induced colon tissue damage.](image)

**Figure 61.** Combined receptor antagonist treatment in the acute inflammatory model does not attenuate DSS-induced colon tissue damage. Representative micrographs (10X) of a control animal receiving both vehicles (A), an animal co-treated with the Y₁ receptor antagonist and 12.5% DMSO (B), an animal co-treated with the Y₂
receptor antagonist and dH₂O (C) and an animal co-treated with the Y₁ and Y₂ receptor antagonist (D). Scale bar = 100µm.

![Combined receptor antagonist treatment in the recovery model does not attenuate DSS-induced colon tissue damage.](image)

**Figure 62.** Combined receptor antagonist treatment in the recovery model does not attenuate DSS-induced colon tissue damage. Representative micrographs (10X) of a control animal receiving both vehicles (A), an animal co-treated with the Y₁ receptor antagonist and 12.5% DMSO (B), an animal co-treated with the Y₂ receptor antagonist and dH₂O (C) and an animal co-treated with the Y₁ and Y₂ receptor antagonist (D). Scale bar = 100µm.
Figure 63. **Mixed receptor antagonist treatment in the recovery model does not attenuate DSS-induced colon tissue damage.** Representative micrographs (10X) of a control animal receiving dH₂O for the first 7 days post DSS treatment and then 12.5% for the last 3 days (A), an animal treated with the Y₁ receptor antagonist for the first 7 days and then 12.5% DMSO for the next 3 days (B), an animal receiving dH₂O for the first 7 days followed by 3 day treatment with the Y₂ receptor antagonist (C) and an animal treated with the Y₁ receptor antagonist for the first 7 days and then treated with the Y₂ receptor antagonist for the next 3 days (D). Scale bar = 100µm.
Figure 64. Treatment with the Y$_1$ receptor antagonist does not attenuate DSS-induced colon wall thickening or colon mucosal shortening. Mean length for the full muscle, circular muscle layer, longitudinal muscle layer and colon crypt for the antagonist co-treatment in the acute inflammatory model (A,B,C,D), recovery model (E,F,G,H) and the mixed antagonist treatment in the recovery model (I,J,K,L). Data presented as mean ± SEM. $ (p<.05)$, indicate a significant main effect of BIBP 3226.

Y$_1$ and Y$_2$ Receptor Antagonist Combined But Not Mixed Treatment Significantly Reduces DSS-Induced MPO Expression

In the acute inflammatory model, treatment with the Y$_2$ receptor antagonist alone and in combination with the Y$_1$ receptor antagonist but not the Y$_1$ receptor antagonist alone significantly regulated DSS-induced MPO activity in the distal colon (see Figure
65). Treatment with the Y$_1$ receptor antagonist did not significantly reduce DSS-induced distal colon MPO protein expression in either of the combination treatment studies, potentially as a result of 12.5% DMSO being co-administered, especially since it did decrease MPO activity in the mixed model in which DMSO was not present at the same time as this antagonist. In the acute inflammatory model with antagonist co-treatment, Y$_1$ receptor antagonism did not significantly reduce DSS-induced MPO activity in the distal colon (F(1,31)=1.123; $p$ = .298) while treatment with BIIE 0246 significantly lowered colon MPO as previously observed (F(1,31)=12.800; $p$ < .001). Additionally, there was a statistically significant interaction between BIBP 3226 and BIIE 0246 (F(1,31)=20.310; $p$ < .001) such that the combination of the two antagonists resulted in lower levels of distal colon MPO expression than control, however, not to the same extent as when the Y$_2$ antagonist was given alone, potentially due to the interaction of BIBP 3226 with the diluent of the Y$_2$ antagonist, DMSO. This pattern was not observed for the combination treatment in the recovery model where neither Y$_1$ receptor antagonism (F(1,25)=0.271; $p$ = .608), Y$_2$ receptor antagonism (F(1,25)=1.788; $p$ = .193) nor the combination of the antagonist (F(1,25)=2.281; $p$ = .144) significantly lowered DSS-induced colon MPO increases. Mixed antagonist treatment in the recovery models showed a different pattern from the combination treatment. In this model, treatment with BIBP 3226 significantly reduced DSS-induced distal colon MPO (F(1,20)=5.600; $p$ = .028) while BIIE 0246 did not significantly reduce MPO (F(1,20)=0.773; $p$ = .390) and no statistical interaction was observed between the treatments (F(1,20)=0.025; $p$ = .876).
Figure 65. Y1 and Y2 receptor antagonism significantly decreased DSS-induced MPO expression in the acute inflammatory combination and recovery mixed treatment models. Mean distal colon MPO protein concentration expressed as ng/mg of total sample protein in the acute inflammatory combined treatment (A) and recovery models (B) and in the mixed treatment recovery model (C). Data presented as mean ±
SEM. $ (p<.01)$, indicate a significant main effect of BIBP 3226, ## $(p<0.05)$ indicates a significant main effect of BIIE 0246, && $(p<.01)$ indicates a significant interaction.

**Y₁ and Y₂ Receptor Antagonism Significantly Alters DSS-Induced Cytokine Expression in The Different Models Assessed**

In line with previous experiments, the most consistently overexpressed cytokines as a result of DSS treatment across all three studies presented here were IL-1β, IL-6 and IL-12 with all other cytokines assessed showing detectable expression by some but not all animals across groups (see Tables 7-9). Those cytokines not reliably detected were not further considered for statistical analysis. Y₁ receptor antagonism in the acute inflammatory model significantly decreased IL-1β $(F(1,34)=14.250; \ p < .001)$, IL-6 $(F(1,32)=8.752; \ p < .005)$ and IL-12 $(F(1,34)=11.320; \ p < .001)$ and IFN-γ $(F(1,33)=4.228; \ p = .048)$ while Y₂ receptor antagonism did not significantly alter these parameters and no statistically significant interactions were observed. In the combined treatment recovery model, neither antagonist alone significantly altered DSS-induced cytokine increases however, the combination treated group showed significantly higher levels of IL-12 than the BIBP 3226 only treated group $(F(1,29)=5.619; \ p = .025)$. A similar pattern was observed in the mixed treatment recovery model where the group receiving the two antagonists treatment showed significantly higher levels of IL-6 $(F(1,22)=4.837; \ p = .039)$ and IL-4 $(F(1,21)=5.190; \ p = .033)$ than the BIBP 3226 only group.
Cytokine Profile for the Combined Antagonist Treatment in the Acute Inflammatory Model

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</tr>
<tr>
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<td>0.2551826</td>
<td>0.2588252</td>
<td>0.08318856</td>
<td>0.1107706</td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
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<td>14.4209</td>
<td>10.31475</td>
<td>7.780679</td>
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</tr>
<tr>
<td>SEM ±</td>
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<td>2.711801</td>
<td>1.811472</td>
<td>1.09863</td>
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</tr>
<tr>
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<td>9.020656</td>
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<td>0.5370187</td>
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<tr>
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<td>4.178279</td>
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<td>0.3493075</td>
<td>0.2487418</td>
<td>0.3016238</td>
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Table 7. DSS induces high expression of proinflammatory cytokines, an affect attenuated by Y1 receptor antagonism. Mean distal colon cytokine protein expressed in pg/mg of total sample protein. Y1 receptor antagonism significantly reduced the DSS-induced expression of IL1-β, IL-6, IL-12 and IFN-γ. $ (p<.05) indicates a main effect of BIBP 3226.
### Cytokine Profile for the Combined Treatment in the Recovery Model

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>dH2O-12.5% DMSO</th>
<th>12.5% DMSO-BIBP 3226</th>
<th>dH2O-12.5% DMSO-BIIE 0246</th>
<th>BIBP 3226-BIIE 0246</th>
<th>p Value</th>
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<tr>
<td>IL-1β</td>
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<td>3.11536</td>
<td>3.193594</td>
<td>3.895256 &amp;</td>
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<tr>
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</tr>
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<td>0.3444654</td>
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<td>IL-4</td>
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<td>SEM ±</td>
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</tr>
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<td>0.1921186</td>
<td>0.1504859</td>
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</tr>
</tbody>
</table>

@ indicates a statistically significant treatment interaction

**Table 8.** DSS induced IL-12 overexpression is reduced by Y₁ and Y₂ individual antagonism but not by the combined treatment. Mean distal colon cytokine protein expressed in pg/mg of total sample protein. & (p<.05) indicates a statistically significant interaction between treatments.
Table 9. Y_1 and Y_2 receptor antagonist co-treatment increases the DSS-induced expression of IL-6 in the recovery mixed treatment model. Mean distal colon cytokine protein expressed in pg/mg of total sample protein. & (p<.05) indicates a statistically significant interaction between treatments.
$Y_1$ and $Y_2$ receptor antagonism did not significantly alter distal colon NPY protein expression in either one of the recovery models but significantly changed this expression in the combined treatment acute inflammatory model (see Figure 66). Neither BIBP 3226 ($F(1,31)=0.574; \ p = .454$) nor BIIE 0246 ($F(1,31)=1.300; \ p = .263$) alone significantly altered NPY protein expression in the distal colon. However, these treatments showed a statistically significant interaction such that the $Y_2$ antagonist significantly increased NPY protein expression but not when co-administered with the $Y_1$ antagonist ($F(1,31)=7.910; \ p < .008$).
Figure 66. $Y_2$ receptor antagonism increased distal colon NPY expression but not when co-administered with the $Y_1$ receptor antagonist. Mean distal colon NPY protein
concentration expressed in ng/mg of total sample protein. Data presented as mean ± SEM. *(p<.01)*, indicate a significant interaction.

**Y₁ and Y₂ Combined Receptor Antagonism Significantly Alters Pain-Related Behavior in the Recovery Model**

Combined Y₁ and Y₂ receptor antagonism does not significantly alter DSS-induced behavioral alterations in general movement and locomotion. However, Y₂ receptor antagonism did significantly alter basic movements and X-Y ambulation in the mixed treatment recovery model. Y₂ receptor antagonism significantly decreased total counts of basic movements (F(1,20)=4.595; *p* =.045) and X-Y ambulation (F(120)=6.744; *p* =.017). Combined receptor antagonist treatment in the mixed antagonist treatment recovery model did not result in significant alterations to DSS-induced pain-related behaviors whereas antagonist treatment at either time point changed visceral pain-related behaviors (see Figures 67-76).

In the acute inflammatory model, treatment with the Y₁ receptor antagonist significantly reduced total rearing time (F(1,34)=5.557; *p* =.024), a finding that is in opposition to previous observations and may be due to a potentially detrimental effect of BIBP 3226 co-administered with 12.5% DMSO as was observed for other measures. Y₂ receptor antagonism in this same model increased time spent per rear (F(1,34)=13.220; *p* <.001).

In the combined treatment recovery model, treatment with the Y₂ receptor antagonist significantly increased total rearing time (F(1,26)=6.079; *p* =.021) and a statistically significant interaction was observed such that the combination treatment
group spent significantly more time rearing than the group only treated with the Y\textsubscript{1} receptor antagonist (F(1,26)=10.470; \( p < .003 \)). Normalized rearing behavior was not significantly altered in this model. Mixed receptor antagonist treatment did not change DSS-induced visceral-pain measures in the recovery model.

Figure 67. Y\textsubscript{1} and Y\textsubscript{2} receptor antagonism does not alter DSS-induced general movement profile in any model. Mean basic (A,B,C) and fine movement (D,E,F) total counts in the acute inflammatory and recovery models. Data presented as mean ± SEM.
Figure 68. \( Y_1 \) and \( Y_2 \) receptor antagonism does not alter DSS-induced general movement profile in any model. Mean basic (A,B,C) and fine movement (D,E,F) per 5-minute block counts in the acute inflammatory and recovery models. Data presented as mean ± SEM.
Figure 69. Y₂ receptor antagonism did not change most DSS-induced effects in locomotion but it did decrease ambulation in the mixed model. Mean total X-Y ambulation (A,B,C) and distance traveled (D,E,F) in the acute inflammatory and recovery model. Data presented as mean ± SEM.
Figure 70. DSS-induced locomotion is not altered by Y$_1$ or Y$_2$ receptor antagonism.

Mean X-Y ambulation (A,B,C) and distance traveled (D,E,F) per 5-minute block counts in the acute inflammatory and recovery models. Data presented as mean ± SEM.
Figure 71. DSS-induced increased immobility time is not significantly altered by either antagonist. Mean total rest time (A,B,C) and time spent immobile (D,E,F) in the acute inflammatory and recovery models. Data presented as mean ± SEM.
Figure 72. DSS-induced immobility time is not altered by either antagonist. Mean rest time (A,B,C) and time spent immobile (D,E,F) per 5-minute block counts in the acute inflammatory and recovery models. Data presented as mean ± SEM.
Figure 73. Combined $Y_1$ and $Y_2$ receptor antagonism significantly alter DSS-induced rearing duration decreases. $Y_1$ receptor antagonism resulted in significantly shorter rears in the acute inflammatory model while $Y_1$ and $Y_2$ receptor antagonism co-treatment significantly increased rearing time in the recovery model. Mean total rears (A,B,C) and rearing time (D,E,F) in the acute inflammatory and recovery models. Data presented as mean ± SEM. $ (p<.05)$, indicate a significant main effect of BIBP 3226.
Figure 74. Combined Y₁ and Y₂ receptor antagonism significantly alter DSS-induced decreased rearing duration decreases. Mean rears (A,B,C) and rearing time (D,E,F) per 5-minute block counts in the acute inflammatory and recovery models. Data presented as mean ± SEM.
Figure 75. \( Y_1 \) receptor antagonism significantly decreases normalized rearing and rearing duration in the acute inflammatory model. Mean total rears (A,B,C) and rearing time (D,E,F) normalized to ambulation and total rears respectively in the acute inflammatory and recovery models. Data presented as mean ± SEM. $ (p<.05)$, indicate a significant main effect of BIBP 3226, ## (p<.05) indicates a significant main effect of BIIE 0246.
Figure 76. Y₁ receptor antagonism significantly decreases normalized rearing and rearing duration in the acute inflammatory model. Mean rears (A,B,C) and rearing time (D,E,F) normalized to ambulation and total rears respectively per 5-minute block counts in the acute inflammatory and recovery models. Data presented as mean ± SEM.
Specific Aim 5: To assess DSS-induced gene expression changes, identify a DSS-induced genetic meta-signature from multiple independent studies and compare this to genetic profiles generated from human IBD patient samples.

The DSS experimental model of human IBD is often used for its simplicity and similarity to human pathology for the development of potential therapeutic targets. Although the clinical manifestations are similar between the model and human disease, it is necessary to determine if these similarities also translate to the genetic level. In order to address these questions, distal colon samples from four independent DSS studies (samples from Experiments 3 and 6) including from animals treated with the Y₁ and Y₂ receptor antagonists, were genetically profiled. Each analysis yielded more than 2500 genes that were significantly altered in animals exposed to DSS. These genetic profiles along with those from two other independent DSS studies conducted at Regeneron Pharmaceuticals, Inc. were cross-referenced to generate a list of genes that are reliably changed by DSS exposure. The resulting gene list represents the DSS-induced gene expression meta-signature. This gene expression meta-signature was then correlated to the gene profile from human IBD patient colon samples to gain clues about the validity of the DSS model for human IBD. In addition, the DSS meta-signature was correlated with normalized rearing measures collected from Experiments 3 and 6 to determine if a relationship exists between DSS-induced gene alterations and visceral pain.
Y₁ and Y₂ Receptor Antagonism Results in Significant Distal Colon Receptor Gene Upregulation

Exposure to DSS does not induce significant RNA changes for NPY nor for Y₁ and Y₂ receptors in the distal colon (see Table 10). Treatments with the specific antagonists do not change NPY gene expression but significantly change the gene expression for their own target receptor at specific time points. Y₁ receptor antagonism with BIBP 3226 resulted in a nearly doubled expression of the NPY receptor 1 gene in the recovery model but not in the acute model. Similarly, Y₂ receptor antagonism resulted in nearly doubled expression of the NPY receptor 2 gene in the acute inflammatory model but not during the recovery model. These data provide some validation that the two small molecule receptor antagonists used for these experiments are specific and reliably antagonizing the correct receptors in animals exposed to DSS.

<table>
<thead>
<tr>
<th>Gene</th>
<th>DSS No Treatment</th>
<th>Acute Inflammatory Model</th>
<th>Recovery Model</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td>BIBP 3226 Treatment</td>
<td>BIIE 0246 Treatment</td>
</tr>
<tr>
<td>Npy1r</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Npy2r</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

Table 10. Y₁ and Y₂ receptor antagonism results in significant receptor gene upregulation in the distal colon of DSS treated mice. Fold change values for the specific NPY receptors antagonized in Experiments 3 and 6.

DSS Significantly Alters Distal Colon Gene Expression

Exposure to DSS resulted in significant gene regulation in the distal colon encompassing on average over 2,500 genes altered including both up and down regulation of genes. Due to the vast number of DSS-induced gene alterations, only the top
25 genes significantly up and down-regulated for are presented in Tables 10-13. The effect of NPY receptor antagonism on DSS-induced gene expression for these 25 genes is included. Collectively analyzing the genes altered by DSS exposure between these experiments and 2 additional independent DSS studies conducted at Regeneron Pharmaceuticals, Inc. yielded a list of genes significantly expressed in all these studies, a DSS-induced genetic meta-signature. Using the publically available gene interaction mapping software GeneMania, the connectivity and functional networks of the top 25 DSS altered genes from each table below were analyzed. Top 25 upregulated genes from Experiment 3, 6 and the meta-signature list showed that these genes are highly co-expressed, some are co-localized and some have physical interactions. In addition, these genes often belonged to similar functional networks falling primarily into 7 functional categories: response to bacterium or molecules of bacterial origin, inflammatory responses, general chemotaxis, leukocyte chemotaxis, leukocyte migration, neutrophil chemotaxis and response to cytokine stimulus. Analysis of the top 25 down-regulated genes showed a lower degree of gene interaction, with those interacting being primarily involved in mitochondrial and organelle inner membrane functioning, lipid oxidation and fatty acid catabolic processes. See supplement for network depictions.
Top 25 Genes Upregulated by DSS From Experiment 3

<table>
<thead>
<tr>
<th>Gene</th>
<th>Acute Inflammatory Model</th>
<th>Recovery Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DSS Induced Vehicle</td>
<td>DSS Induced BIBP 3226</td>
</tr>
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</table>

Table 11. Top 25 genes significantly upregulated by DSS exposure. DSS altered genes are expressed as fold change from controls and are listed for both the acute inflammatory and recovery models. The effect of Y₁ receptor antagonism on gene fold change is provided for each model.
### Top 25 Genes Down-regulated by DSS From Experiment 3

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<th>Acute Inflammatory Model</th>
<th>Recovery Model</th>
</tr>
</thead>
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<td>DSS Induced BIBP 3226</td>
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**Table 12. Top 25 genes significantly downregulated by DSS exposure.** DSS altered genes are expressed as fold change from controls are listed for both the acute inflammatory and recovery models along with the effect of Y₁ receptor antagonism on gene fold change for each model.
Top 25 Genes Upregulated by DSS From Experiment 7

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<td>472.61</td>
<td>289.13</td>
</tr>
<tr>
<td>S100a9</td>
<td>453.85</td>
<td>180.21</td>
</tr>
<tr>
<td>Ngp</td>
<td>443.45</td>
<td></td>
</tr>
<tr>
<td>Chi3l3</td>
<td>428.80</td>
<td>225.31</td>
</tr>
<tr>
<td>Il6</td>
<td>350.48</td>
<td>1000.00</td>
</tr>
<tr>
<td>Saa3</td>
<td>325.12</td>
<td>612.84</td>
</tr>
<tr>
<td>Al747448</td>
<td>267.81</td>
<td>148.79</td>
</tr>
<tr>
<td>Tarm1</td>
<td>247.80</td>
<td></td>
</tr>
<tr>
<td>Clec4e</td>
<td>244.93</td>
<td>107.74</td>
</tr>
<tr>
<td>Retnlg</td>
<td>221.12</td>
<td>68.49</td>
</tr>
<tr>
<td>Gml</td>
<td>199.67</td>
<td>86.24</td>
</tr>
<tr>
<td>Marco</td>
<td>162.94</td>
<td>65.96</td>
</tr>
<tr>
<td>Irg1</td>
<td>155.02</td>
<td>80.07</td>
</tr>
<tr>
<td>Reg3b</td>
<td>122.39</td>
<td>900.22</td>
</tr>
<tr>
<td>Cxcl2</td>
<td>121.55</td>
<td>162.29</td>
</tr>
</tbody>
</table>

Table 13. Top 25 genes significantly upregulated by DSS exposure. DSS altered genes are expressed as fold change from controls are listed for both the acute inflammatory and recovery models along with the effect of Y2 receptor antagonism on gene fold change for each model.
### Top 25 Genes Down-regulated by DSS From Experiment 7

<table>
<thead>
<tr>
<th>Gene</th>
<th>Acute Inflammatory Model</th>
<th>Recovery Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DSS Induced Vehicle</td>
<td>DSS Induced BIIE 0246</td>
</tr>
<tr>
<td>2900026A02Rik</td>
<td>0.6670</td>
<td>0.5708</td>
</tr>
<tr>
<td>Trmt2b</td>
<td>0.6658</td>
<td>0.7174</td>
</tr>
<tr>
<td>Adi1</td>
<td>0.6658</td>
<td></td>
</tr>
<tr>
<td>Tug1</td>
<td>0.6655</td>
<td></td>
</tr>
<tr>
<td>Kdm5d</td>
<td>0.6654</td>
<td>0.6730</td>
</tr>
<tr>
<td>Odc1</td>
<td>0.6653</td>
<td></td>
</tr>
<tr>
<td>2810459M11Rik</td>
<td>0.6652</td>
<td>0.6681</td>
</tr>
<tr>
<td>Depdc6</td>
<td>0.6652</td>
<td>0.5936</td>
</tr>
<tr>
<td>Etdh</td>
<td>0.6651</td>
<td>0.7209</td>
</tr>
<tr>
<td>Pik3c2b</td>
<td>0.6649</td>
<td>0.6075</td>
</tr>
<tr>
<td>Zbtb7c</td>
<td>0.6647</td>
<td>0.5505</td>
</tr>
<tr>
<td>Uqcrh</td>
<td>0.6646</td>
<td></td>
</tr>
<tr>
<td>BC049349</td>
<td>0.6646</td>
<td></td>
</tr>
<tr>
<td>Coq2</td>
<td>0.6645</td>
<td></td>
</tr>
<tr>
<td>Pof1b</td>
<td>0.6645</td>
<td>0.7196</td>
</tr>
<tr>
<td>Ndufs1</td>
<td>0.6644</td>
<td>0.7010</td>
</tr>
<tr>
<td>Zfp54</td>
<td>0.6643</td>
<td></td>
</tr>
<tr>
<td>Scrn3</td>
<td>0.6641</td>
<td>0.6816</td>
</tr>
<tr>
<td>Abr</td>
<td>0.6640</td>
<td>0.6915</td>
</tr>
<tr>
<td>Casd1</td>
<td>0.6639</td>
<td>0.6431</td>
</tr>
<tr>
<td>Zfp606</td>
<td>0.6634</td>
<td></td>
</tr>
<tr>
<td>1110021L09Rik</td>
<td>0.6624</td>
<td></td>
</tr>
<tr>
<td>Hibch</td>
<td>0.6624</td>
<td>0.6483</td>
</tr>
<tr>
<td>Stard7</td>
<td>0.6624</td>
<td></td>
</tr>
<tr>
<td>Ppp1r3b</td>
<td>0.6623</td>
<td>0.4914</td>
</tr>
</tbody>
</table>

**Table 14. Top 25 genes significantly downregulated by DSS exposure.** DSS altered genes are expressed as fold change from controls are listed for both the acute inflammatory and recovery models along with the effect of Y2 receptor antagonism on gene fold change for each model.
DSS-Induced Genetic Meta-Signature

<table>
<thead>
<tr>
<th>Gene</th>
<th>Recovery Model</th>
<th>Recovery Model</th>
<th>Chronic DSS Model</th>
<th>Prolonged Recovery Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100a8</td>
<td>100.69</td>
<td>2056.82</td>
<td>145.44</td>
<td></td>
</tr>
<tr>
<td>Serpina3m</td>
<td>45.52</td>
<td>1000.00</td>
<td>47.64</td>
<td></td>
</tr>
<tr>
<td>Mmp8</td>
<td>230.05</td>
<td>483.85</td>
<td>117.65</td>
<td></td>
</tr>
<tr>
<td>S100a9</td>
<td>197.44</td>
<td>453.85</td>
<td>80.81</td>
<td>94.69</td>
</tr>
<tr>
<td>Chi3l3</td>
<td>174.44</td>
<td>428.80</td>
<td>50.62</td>
<td>4.09</td>
</tr>
<tr>
<td>Cxcl5</td>
<td>107.36</td>
<td>472.61</td>
<td>22.78</td>
<td></td>
</tr>
<tr>
<td>Saa3</td>
<td>96.78</td>
<td>325.12</td>
<td>63.79</td>
<td>39.30</td>
</tr>
<tr>
<td>Al747448</td>
<td>157.34</td>
<td>267.81</td>
<td>14.92</td>
<td>247.13</td>
</tr>
<tr>
<td>Clec4e</td>
<td>61.97</td>
<td>244.93</td>
<td>91.14</td>
<td></td>
</tr>
<tr>
<td>irg1</td>
<td></td>
<td>155.02</td>
<td>155.67</td>
<td>80.95</td>
</tr>
<tr>
<td>Retnlg</td>
<td>104.01</td>
<td>221.12</td>
<td>79.66</td>
<td>10.45</td>
</tr>
<tr>
<td>trem1</td>
<td>38.20</td>
<td>54.44</td>
<td>108.11</td>
<td></td>
</tr>
<tr>
<td>Reg3b</td>
<td>43.23</td>
<td>122.39</td>
<td>10.47</td>
<td>19.60</td>
</tr>
<tr>
<td>Nos2</td>
<td>133.76</td>
<td>43.74</td>
<td>18.77</td>
<td>74.78</td>
</tr>
<tr>
<td>fpr2</td>
<td>55.73</td>
<td>69.34</td>
<td>27.92</td>
<td></td>
</tr>
<tr>
<td>chi3l1</td>
<td>28.00</td>
<td>97.92</td>
<td>26.44</td>
<td></td>
</tr>
<tr>
<td>Il1b</td>
<td>61.63</td>
<td>68.37</td>
<td>46.71</td>
<td>16.77</td>
</tr>
<tr>
<td>Hp</td>
<td>31.00</td>
<td>110.61</td>
<td>15.48</td>
<td>4.43</td>
</tr>
<tr>
<td>Mmp3</td>
<td>40.26</td>
<td>81.16</td>
<td>25.61</td>
<td>8.79</td>
</tr>
<tr>
<td>Mmp10</td>
<td>74.13</td>
<td>21.64</td>
<td>13.34</td>
<td></td>
</tr>
<tr>
<td>Reg3g</td>
<td>46.34</td>
<td>51.27</td>
<td>12.82</td>
<td></td>
</tr>
<tr>
<td>Cxcl1</td>
<td>39.85</td>
<td>63.51</td>
<td>32.40</td>
<td>10.57</td>
</tr>
<tr>
<td>il1r2</td>
<td>29.73</td>
<td>50.90</td>
<td>18.94</td>
<td></td>
</tr>
</tbody>
</table>

Table 15. DSS-induced genetic meta-signature. Selected top genes significantly altered by DSS exposure in at least 3 out of 4 independent DSS studies. The recovery model data are from Experiments 3 and 7 of this dissertation. The chronic model consisted of 7 days of DSS exposure followed by 14 days of recovery and then re-exposure to DSS for 7 more days. The prolonged recovery model consisted of 3% DSS for 5 days followed by a 4 day recovery period.
The DSS-Model Genetic Meta-Signature Correlates with Human IBD Genetic Profiles

The computed DSS-induced genetic meta-signature was correlated against seven genetic studies of colon samples from human patients suffering from Ulcerative Colitis and Crohn’s disease. The human studies included colon samples from patients treated with infliximab, one of the leading treatments for IBD. Comparisons were made between colon samples pre- and post-treatment as well as between patients who responded or did not respond to treatment. In addition, the number of genes that were shared between the DSS meta-signature and the human studies are reported with the correlation direction and the correlation $p$ value.

As would be expected if the DSS-model was a valid model for human IBD, studies in which patients responded to treatment showed a negative significant correlation with the DSS meta-signature. That is, an effective treatment might be expected to decrease the expression of genes upregulated in the DSS meta-signature. Similarly, in studies for which treatment was ineffective or studies that compared inflamed versus non-inflamed tissue, elevated genes associated with IBD would be expected. In these studies, the DSS meta-signature showed significant positive correlations indicating that similar genes upregulated in IBD were upregulated in the DSS model, see Table 15. Together, these data provide strong validation for the DSS experimental model of human IBD.
The DSS Genetic Meta-Signature Correlates Well With Human IBD Studies

<table>
<thead>
<tr>
<th>Study Description</th>
<th>Specific Comparison</th>
<th>Correlation Direction</th>
<th>Common Genes</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1: Colon Samples from Ulcerative Colitis Patients Treated for 30 Weeks with Infliximab</td>
<td>Treatment Responders (10mg/kg) vs Baseline</td>
<td>-</td>
<td>434</td>
<td>2.00E-59</td>
</tr>
<tr>
<td></td>
<td>Treatment Non-responders (5mg/kg) vs Responders</td>
<td>+</td>
<td>261</td>
<td>1.40E-48</td>
</tr>
<tr>
<td></td>
<td>Treatment Non-Responses (10mg/kg) vs Responders</td>
<td>+</td>
<td>287</td>
<td>2.30E-45</td>
</tr>
<tr>
<td></td>
<td>Responder Crohn’s Patients After vs Before</td>
<td>-</td>
<td>331</td>
<td>1.60E-57</td>
</tr>
<tr>
<td></td>
<td>Crohn’s Patients Non-Responders vs Responders</td>
<td>+</td>
<td>433</td>
<td>1.40E-55</td>
</tr>
<tr>
<td></td>
<td>Crohn’s Patients Non-Responders vs Healthy Controls</td>
<td>+</td>
<td>497</td>
<td>8.90E-55</td>
</tr>
<tr>
<td>Study 2: Colon Mucosa Samples from IBD Patients’ First Infliximab Treatment</td>
<td>Ulcerative Colitis Patients Non-Responders Before Treatment vs Healthy Controls</td>
<td>+</td>
<td>520</td>
<td>2.50E-47</td>
</tr>
<tr>
<td></td>
<td>Crohn’s Patients Responders vs Healthy Controls</td>
<td>+</td>
<td>448</td>
<td>1.10E-40</td>
</tr>
<tr>
<td></td>
<td>Responder Ulcerative Colitis Patients After vs Before</td>
<td>-</td>
<td>362</td>
<td>6.60E-39</td>
</tr>
<tr>
<td></td>
<td>Ulcerative Colitis Patients Non-Responders vs Responders</td>
<td>+</td>
<td>289</td>
<td>3.10E-38</td>
</tr>
<tr>
<td></td>
<td>Responder Ulcerative Colitis Patients Before Treatment vs Healthy Controls</td>
<td>+</td>
<td>414</td>
<td>5.20E-36</td>
</tr>
<tr>
<td>Study 3: Colon Biopsy Samples from IBD Patients</td>
<td>IBD Patient vs Healthy Control</td>
<td>+</td>
<td>464</td>
<td>1.80E-54</td>
</tr>
<tr>
<td>Study 4: Colon Mucosa Sample from IBD Patients</td>
<td>IBD Patient Inflamed Mucosa vs Healthy Control</td>
<td>+</td>
<td>406</td>
<td>4.10E-50</td>
</tr>
<tr>
<td>Study 5: Inflamed and Non-Inflamed Colon Samples (descending) from Crohn’s Patients</td>
<td>Crohn’s Patients Inflamed vs Non-Inflamed</td>
<td>+</td>
<td>303</td>
<td>6.40E-44</td>
</tr>
<tr>
<td>Study 6: Colon Mucosa and Isolated Colonocytes samples from Ulcerative Colitis Patients</td>
<td>Patient Samples vs Healthy Controls</td>
<td>+</td>
<td>393</td>
<td>3.00E-40</td>
</tr>
<tr>
<td>Study 7: Colon Biopsy Samples from Ulcerative Colitis Patients</td>
<td>Patient Samples vs Healthy Controls</td>
<td>+</td>
<td>297</td>
<td>8.00E-40</td>
</tr>
</tbody>
</table>

Table 16. DSS genetic meta-signature correlated with human IBD studies.
DSS-induced Gene Changes are Associated with Mouse Pain-Related Measures in the Open Field

Genes that were significantly altered by DSS exposure in Experiments 3 and 6 are significantly associated with the four pain-related measures collected from the same animals in the open field, namely, rearing, rearing time, normalized rearing and mean rear duration. As is depicted in Table 16, there were a large number of genes that showed strong correlation, defines as a Pearson r > .6, with these pain measures. Due to the fact that total rearing and total rearing time can be affected by the animals overall activity levels and may be more representative of sickness behavior rather than pain, all subsequent tables are shown only for the normalized behavior which is proposed to be a more accurate measure of pain.

DSS-Induced Genes Correlate with Pain-Related Behaviors

<table>
<thead>
<tr>
<th>Experiment/Model</th>
<th>Total Rearing</th>
<th>Total Rearing Time</th>
<th>Normalized Rearing</th>
<th>Normalized Rear Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/Acute Inflammatory Model</td>
<td>322 (161+/161-)</td>
<td>457 (295+/162-)</td>
<td>364 (215+/149-)</td>
<td>255 (203+/52-)</td>
</tr>
<tr>
<td>3/Recovery Model</td>
<td>146 (113+/33-)</td>
<td>171 (117+/54-)</td>
<td>89 (74+/15-)</td>
<td>246 (123+/123-)</td>
</tr>
<tr>
<td>6/Acute Inflammatory Model</td>
<td>745 (262+/483-)</td>
<td>807 (273+/534-)</td>
<td>521 (184+/337)</td>
<td>888 (289+/599-)</td>
</tr>
<tr>
<td>6/Recovery Model</td>
<td>341 (76+/265-)</td>
<td>354 (92+/262-)</td>
<td>185 (88+/97-)</td>
<td>314 (76+/238-)</td>
</tr>
</tbody>
</table>

Table 17. Genes that are significantly altered by DSS exposure strongly correlate with pain-related measures. For each study from Experiments 3 and 6, the total number of strongly correlated genes is shown as well as the specific number of genes in each direction.
### Top 25 DSS-Induced Genes Correlated with Normalized Rearing Behavior

<table>
<thead>
<tr>
<th>Gene</th>
<th>Acute Inflammatory Model</th>
<th>Recovery Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DSS</td>
<td>BIBP 3226</td>
</tr>
<tr>
<td>Fpr2</td>
<td>-0.99</td>
<td>-0.30</td>
</tr>
<tr>
<td>1700017B05Rik</td>
<td>-0.99</td>
<td>0.23</td>
</tr>
<tr>
<td>Gpr65</td>
<td>0.99</td>
<td>0.17</td>
</tr>
<tr>
<td>Accs1</td>
<td>0.99</td>
<td>-0.79</td>
</tr>
<tr>
<td>Ptges</td>
<td>-0.99</td>
<td>0.08</td>
</tr>
<tr>
<td>Ccr12</td>
<td>-0.99</td>
<td>0.63</td>
</tr>
<tr>
<td>Dpp4</td>
<td>0.98</td>
<td>0.60</td>
</tr>
<tr>
<td>Alpl</td>
<td>-0.98</td>
<td>-0.17</td>
</tr>
<tr>
<td>Nrg1</td>
<td>-0.98</td>
<td>-0.40</td>
</tr>
<tr>
<td>Agr3</td>
<td>0.97</td>
<td>-0.87</td>
</tr>
<tr>
<td>Fam13a</td>
<td>0.97</td>
<td>-0.85</td>
</tr>
<tr>
<td>H1f0</td>
<td>0.97</td>
<td>-0.27</td>
</tr>
<tr>
<td>Cpm</td>
<td>-0.96</td>
<td>-0.93</td>
</tr>
<tr>
<td>Ly6a</td>
<td>-0.96</td>
<td>0.59</td>
</tr>
<tr>
<td>Lama1</td>
<td>-0.96</td>
<td>-0.60</td>
</tr>
<tr>
<td>Acsl3</td>
<td>0.96</td>
<td>-0.78</td>
</tr>
<tr>
<td>1110017F19Rik</td>
<td>0.96</td>
<td>-0.30</td>
</tr>
<tr>
<td>Parp3</td>
<td>-0.95</td>
<td>0.62</td>
</tr>
<tr>
<td>Scml2</td>
<td>0.95</td>
<td>0.53</td>
</tr>
<tr>
<td>Zfp704</td>
<td>0.95</td>
<td>-0.56</td>
</tr>
<tr>
<td>A430033K04Rik</td>
<td>0.95</td>
<td>-0.70</td>
</tr>
<tr>
<td>Lirb4</td>
<td>-0.95</td>
<td>-0.56</td>
</tr>
<tr>
<td>Atp8b5</td>
<td>0.95</td>
<td>0.29</td>
</tr>
<tr>
<td>Hhip</td>
<td>0.95</td>
<td>-0.23</td>
</tr>
<tr>
<td>Zfp612</td>
<td>0.95</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table 18. Genes significantly altered by DSS exposure correlate strongly with visceral pain measures in the open field. The top 25 genes with the greatest significant correlation with normalized rearing are shown as well as the effect of the $Y_1$ and $Y_2$ receptor antagonists on these correlations.
Table 19. Genes significantly altered by DSS exposure correlate strongly with visceral pain measures in the open field. The top 25 genes with the greatest significant correlation with normalized rear duration are shown as well as the effect of the Y1 and Y2 receptor antagonists on these correlations.
Chapter 5.

DISCUSSION

The studies presented here were designed to determine the potential role of neuropeptide Y on peripheral organ inflammation during an acute inflammatory phase and following a recovery phase. Since the Y₁ and Y₂ receptors are the most widely expressed Y receptors in the immune system, these studies focused on the role of NPY signaling via these receptors on DSS-induced bowel inflammation.

The effects of DSS administration were robust and consistent from experiment to experiment. In vivo, the major landmarks of the disease were diarrhea, bloody stool and progressive significant weight loss throughout the duration of DSS treatment. DSS-induced behavioral changes in the open field were also very consistent across experiments and included significant decreases in general movements such as grooming, locomotion, and rearing, and perhaps more notably, a preferential decrease in rearing behaviors relative to general locomotion.

Post mortem tissue analysis also revealed a clear and consistent DSS profile. DSS treated mice had colons that were significantly shorter in size but generally did not differ in weight from control animals. The ceca of these animals also shrunk and weighed significantly less than that of controls. Microscopically, DSS caused mucosal structure damage including a significant infiltration of immune cells, decreased crypt lengths, and significant thickening of the two smooth muscle layers making up the colon wall, and increases in submucosal space widths which appear to be a result of edema. This
potential increase in tissue fluid may explain why the shorter colons from DSS treated mice did not differ in weight from controls.

The immunoassay profile of the DSS model was also consistent across experiments and was largely characterized by significant increases in colon protein expression of MPO and the proinflammatory cytokines IL-1β, IL-6, IL-12 and TNF-α as well as increases in the anti-inflammatory cytokines IL-2 and IL-4. Furthermore, colon protein levels of NPY were consistently elevated in DSS treated mice to levels that were significantly greater than control mice.

Inhibition of NPY signaling via its Y₁ receptor resulted in significant regulation of some DSS-induced inflammatory and disease parameters. DSS treated mice receiving the Y₁ antagonist showed a slower rate of weight loss and lost significantly less weight overall compared to mice treated with DSS and vehicle. In addition, Y₁ receptor inhibition significantly decreased DSS-induced colon MPO protein expression and the expression of the proinflammatory cytokine IL-1β. In contrast, Y₁ receptor antagonism did not significantly alter DSS-induced tissue shrinkage, colon mucosa damage, muscle wall thickening or any behavior assessed in the open field.

Similar to Y₁ antagonism, inhibition of NPY signaling via its Y₂ receptors resulted in a slower rate of weight loss in DSS treated mice and significantly less weight loss at terminal points. Y₂ receptor antagonism also significantly decreased colon MPO protein expression but did not attenuate changes in cytokine expression in DSS-treated mice. In addition, and unlike Y₁ receptor antagonism, inhibition of Y₂ receptor signaling significantly increased the average rear duration in DSS treated mice, suggesting a
possible relief of pain-related behavioral changes. Y₂ antagonism did not, however, prevent DSS-induced tissue shrinkage, colon mucosa damage or colon muscle wall thickening, similar to Y₁ antagonism.

In light of the findings that Y₁ receptor antagonism inhibits the DSS-induced inflammatory cascade but does not alleviate visceral pain-related behavior and Y₂ antagonism only weakly attenuates the inflammatory response but significantly alleviates visceral pain-related behavior, particularly in the recovery period, the effect of co-administration and mixed treatment with the antagonists was assessed. Co-administration of the antagonists in the acute inflammatory model did not result in an additive protective effect that was different from the effects of each antagonist alone for any of the measures assessed. A similar pattern was observed with co-administration of the antagonists in the recovery model. None of the measures assessed improved significantly in response to the antagonist co-administration except for the total time spent rearing. This measure did not improve significantly over Y₂ antagonism alone, however, suggesting no advantage to concurrent treatment with both Y receptor antagonists.

The inability of these antagonists to produce a positive additive effect may be explained by the mechanism of action of each receptor. That is, inhibition of the Y₂ autoreceptor alone is expected to increase NPY levels by preventing the negative feedback signals from action on this receptor. Similarly, inhibition of the Y₁ receptor is expected to prevent post-synaptic signaling via this receptor, which may cause displacement of unbound NPY to its other post-synaptic receptors including the Y₂ and Y₅ receptors. However, concurrent inhibition of the Y₁ and Y₂ receptors can potentially prevent both the negative feedback mechanism and Y₁ post-synaptic signaling possibly
diverting most NPY signaling to another receptor type. Because Y₅ is the other receptor type often observed on immune cells and implicated in the gastrointestinal system, it is possible that concurrent inhibition of the Y₁ and Y₂ receptors results in enhanced Y₅ receptor signaling, leading to the observed detrimental effects. More work in this area is needed to test this hypothesis.

The DSS model is one of the most commonly used animal models for IBD because of its simplicity and similarity in gross pathology to human IBD. That is, after about four days of oral exposure to a 4% DSS solution in place of drinking water, animals begin to demonstrate marked weight loss, diarrhea, bloody stool and signs of visceral discomfort, which is very similar to the human clinical manifestation of IBD. In addition to this model’s face validity, the distal colon gene profiling data presented in this dissertation supports the use of the DSS model as a potentially valid animal model of human IBD. Specifically, the high consistency of the RNA regulation profile in response to DSS across several experiments conducted independently leading to the DSS meta-signature attests to the reliability of the model. Furthermore, the fact that this meta-signature shows strong correlations with genetic data from human studies using colon tissue from UC and Crohn’s disease patients further reinforces the validity of this model.

In line with the observed genetic profile similarity between the DSS model and human IBD, the gene profile data presented here also supports the idea that rearing behavior in the open field may be a valid measure to assess visceral pain in mice. It was observed that all four rearing measures showed strong and statistically significant correlations with DSS-induced gene alterations, an observation that was stronger when rearing and rearing time were normalized to ambulation and total rears respectively.
Taken together, these results suggest that DSS treatment induces an RNA signature that is similar to what is observed in human patients and that this signature strongly correlates with DSS-induced visceral pain-related measures in the open field.

In addition to providing a reliable model for human IBD, the DSS model may also be an appropriate model for the study of the cross talk between the CNS and immune system. The greatest DSS-induced inflammation was observed in the distal colon, an area rich in autonomic innervation, both parasympathetic and sympathetic. Sympathetic fibers innervate the two plexuses in the distal colon, the Meissner (or submucosal) plexus and the Auerbach (or myenteric) plexus. These plexuses have many properties similar to those of the CNS including the presence of synaptic mechanisms, interneurons, immune cells and a small extracellular space for neurotransmitter-mediated communication and peptide regulation. This anatomical and functional organization provides the necessary platform for a bidirectional communication system between the CNS and the immune.

Some support for this idea is provided from the current experiments. NPY, an abundant neuromodulator often co-released with norepinephrine from sympathetic fibers was observed to be significantly increased in response to DSS treatment. Furthermore, inhibition of NPY signaling via its Y_1 and Y_2 receptors significantly toned down the immune response to DSS in the colon including decreases in MPO activity and cytokine expression suggesting a potential relationship between the sympathetic nervous system and the immune system.

Though the data presented here provide some evidence for an interaction between the sympathetic nervous system and the immune system during DSS-induced
inflammatory disease, there is still a need to further elucidate the nature of this relationship. That is, it is still necessary to understand what is causing the upregulation of colon NPY and also what mechanisms the $Y_1$ and $Y_2$ antagonists are using to suppress inflammatory signals such as MPO activity and cytokine expression as well as weight loss. Similarly, it is of importance to unveil the mechanism by which the $Y_2$ antagonist may be alleviating visceral pain in mice.

Finally, the unexpected and often detrimental effects of co-treatment with both antagonists should be further studied. That is, if each antagonist on its own has the ability to decrease DSS-induced inflammatory signals, it might be expected that co-treatment would have at least an additive effect and perhaps even potentiate the anti-inflammatory effects of each antagonist. However, an unexpected detrimental effect was observed for some measures in the current studies when both antagonists were combined. One potential explanation for this seemingly contradictory result comes from the anecdotal observations that DMSO at high concentration increases the vulnerability of mice to DSS, potentially making these animals less responsive to the specific antagonists. Furthermore, it is also possible that DMSO directly or indirectly interfered with the effects of the $Y_1$ receptor antagonist via alterations in immune cell infiltrate caused by this chemical.

Based on the findings presented here, it appears that peripheral NPY may play a role in the regulation of colon inflammatory processes, particularly via $Y_1$ receptor signaling. These observations are in line with a growing body of literature that identifies peripheral NPY signaling as proinflammatory, an effect mainly mediated via its $Y_1$ receptor. This area of research is, however, an area that still needs scrutiny as there are
also reports suggesting anti-inflammatory properties of NPY itself. The observation that peripheral $Y_1$ receptor antagonism significantly downregulates DSS-induced inflammatory markers including MPO and proinflammatory cytokines provides support for the former theory rather than the latter.

Similarly, NPY signaling via the $Y_2$ receptor has been implicated in the regulation of immune responses. The findings that $Y_2$ receptor antagonism inhibits neutrophil activity as determined by decreased expression of MPO provide support for a growing body of evidence indicating a direct involvement of $Y_2$ signaling in inflammatory responses. Furthermore, the unexpected finding that peripheral $Y_2$ receptor antagonism alleviates DSS-induced visceral nociception in mice is contradictory to the well-documented anti-nociceptive properties of NPY via this receptor in the CNS. Though much work has been done on the role of this receptor in centrally mediated nociception, its peripheral effects have received little attention. A better understanding of the role of this receptor in peripheral afferents is needed to more accurately interpret the findings presented here.

Taken together, the data presented here provide evidence for the direct involvement of NPY in the neuroimmune crosstalk during inflammatory disease. The observation that NPY protein levels are elevated in inflamed colons is in line with similar observation in the serum of human patients suffering from inflammatory disease. In addition, the observation that inhibiting NPY signaling via its $Y_1$ and $Y_2$ receptors reduces the inflammatory profile and has anti-nociceptive properties further supports the involvement of NPY in inflammatory processes.
Hence, it can be theorized that activation of an immune response in this case by administration of DSS leads to increases in sympathetic responses as determined by increases in NPY expression, which exacerbates the immune response via its peripheral Y₁ and to a lesser extent, via the Y₂ receptors. This increased inflammatory response can then be recognized by intrinsic systems including afferent autonomic fibers found in the myenteric and mesenteric plexuses, which in turn can relay these inflammatory signals back to the CNS completing an inflammatory loop. In the case of the DSS model, this loop will continue to remain active in the presence of this irritant therefore contributing to the acute inflammation observed in this model. This is particularly supported by observations that upon DSS withdrawal, animals begin to gain back weight and the tissue pathology profile begins to reverse, indicating what can potentially be an inhibited inflammatory loop in the absence of the irritant.

In sum, this series of experiments were designed to assess the potential role of NPY signaling via its Y₁ and Y₂ receptors in DSS-induced inflammation and pathology. It was observed that Y₁ and Y₂ receptor inhibition significantly slowed down DSS-induced weight loss, Y₁ inhibition significantly suppressed DSS-induced inflammatory mediators including MPO and proinflammatory cytokines, and Y₂ inhibition suppressed MPO, albeit to a lesser extent than Y₁ inhibition. In addition, Y₂ signaling inhibition attenuated DSS-induced visceral pain-associated behaviors. These findings suggest that inhibition of these receptors could cause some alleviation of components of this inflammatory pathology albeit to a limited extent. Based on the gene expression signature data showing correspondence between DSS and human IBD signatures, these may be useful particularly for the treatment of human IBD.
# Hematoxylin and Eosin (H&E) Staining Protocols

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 3 Changes of Xylenes</td>
<td>3 Minutes each</td>
</tr>
<tr>
<td>2. 2 Changes of 100% Ethanol</td>
<td>2 Minutes each</td>
</tr>
<tr>
<td>3. 1 Change of 95% Ethanol</td>
<td>1 Minute</td>
</tr>
<tr>
<td>4. 1 Change of 70% Ethanol</td>
<td>1 Minute</td>
</tr>
<tr>
<td>5. 1 Change of 50% Ethanol</td>
<td>1 Minute</td>
</tr>
<tr>
<td>6. Running Tap Water Wash</td>
<td>1 Minute</td>
</tr>
<tr>
<td>7. Distilled Water Wash</td>
<td>1 Minute</td>
</tr>
<tr>
<td>8. Carazzi’s Hematoxylin</td>
<td>11 Minutes</td>
</tr>
<tr>
<td>9. Running Tap Water Wash</td>
<td>10 Minute</td>
</tr>
<tr>
<td>10. 1 Change of 95% Ethanol</td>
<td>1 Minute</td>
</tr>
<tr>
<td>11. Eosin/Phloxine Solution</td>
<td>1 Minute</td>
</tr>
<tr>
<td>12. 3 Change of 95% Ethanol</td>
<td>1 Minute each</td>
</tr>
<tr>
<td>13. 3 Changes of 100% Ethanol</td>
<td>1 Minutes each</td>
</tr>
<tr>
<td>14. 2 Changes of Xylenes</td>
<td>2 Minutes each</td>
</tr>
<tr>
<td>15. Coverslip in Permount Medium</td>
<td></td>
</tr>
</tbody>
</table>

**Protocol obtained from Histoserve, Inc, Maryland, USA**
Hematoxylin and Eosin (H&E) Staining Protocols
Established in Paraffin Embedded Tissue (Colon and Skin)

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>1. Xylenes, 3X</td>
<td>1. Xylenes, 3X</td>
</tr>
<tr>
<td></td>
<td>2min each</td>
</tr>
<tr>
<td>2. 100% Alcohol</td>
<td>2. 100% Alcohol</td>
</tr>
<tr>
<td></td>
<td>10 Dips</td>
</tr>
<tr>
<td>3. 95% Alcohol, 2X</td>
<td>3. 95% Alcohol, 2X</td>
</tr>
<tr>
<td></td>
<td>10 Dips each</td>
</tr>
<tr>
<td></td>
<td>Rinse OH off</td>
</tr>
<tr>
<td>5. Acidified Harris</td>
<td>5. Harris Hematoxylin</td>
</tr>
<tr>
<td>Hematoxylin*</td>
<td>10-15min</td>
</tr>
<tr>
<td></td>
<td>10 Dips Each</td>
</tr>
<tr>
<td>7. 0.25% Ammonia Water</td>
<td>7. 0.25% Ammonia Water</td>
</tr>
<tr>
<td>(dH20)**</td>
<td>(dH20)**</td>
</tr>
<tr>
<td></td>
<td>~20-30sec</td>
</tr>
<tr>
<td>8. Tap Water, 2X</td>
<td>8. Tap Water Rinse</td>
</tr>
<tr>
<td></td>
<td>Rinse Acid</td>
</tr>
<tr>
<td>9. Eosin</td>
<td>9. 0.25% Ammonia Water</td>
</tr>
<tr>
<td></td>
<td>(dH20)**</td>
</tr>
<tr>
<td>10. 70% Alcohol</td>
<td>10. Tap Water, 2X</td>
</tr>
<tr>
<td></td>
<td>10 Dips</td>
</tr>
<tr>
<td>11. 95% Alcohol</td>
<td>11. Eosin</td>
</tr>
<tr>
<td></td>
<td>10 Dips</td>
</tr>
<tr>
<td>12. 100% Alcohol, 3X</td>
<td>12. 70% Alcohol</td>
</tr>
<tr>
<td></td>
<td>10 Dips</td>
</tr>
<tr>
<td>13. Alcohol:Xylene</td>
<td>13. 95% Alcohol</td>
</tr>
<tr>
<td></td>
<td>10 Dips</td>
</tr>
<tr>
<td>14. Xylenes, 3X</td>
<td>14. 100% Alcohol, 3X</td>
</tr>
<tr>
<td></td>
<td>10 Dips</td>
</tr>
<tr>
<td>15. Alcohol:Xylene</td>
<td>15. Alcohol:Xylene</td>
</tr>
<tr>
<td></td>
<td>10 Dips</td>
</tr>
<tr>
<td>16. Xylenes, 3X</td>
<td>16. Xylenes, 3X</td>
</tr>
<tr>
<td></td>
<td>10 Dips</td>
</tr>
</tbody>
</table>

Notes:
* Acidified Harris Hematoxylin—add 4mL of glacial acetic acid to 96 mL of hematoxylin (e.g. 10mL of glacial acetic acid added to 240mL of hematoxylin)
** 250 μL of ammonia hydroxide in 100mL of distilled water (e.g. 625μL of ammonia hydroxide in 250 mL of dH20)
*** Add 1mL of hydrochloric acid to 100mL 70% alcohol (e.g. 2.5mL HCl added to 250 mL of 70% alcohol)
DSS Meta-Signature Top 25 Upregulated Gene Network
Experiment 3 Top 25 DSS Upregulated Gene Network

- **Co-expression** 70.29%
- **Predicted** 11.64%
- **Physical interactions** 10.28%
- **Co-localization** 7.00%
Experiment 7 Top 25 DSS Upregulated Gene Network

- Co-expression: 72.94%
- Physical interactions: 13.01%
- Co-localization: 7.04%
Experiment 3 Top 25 DSS Downregulated Gene Network

- Co-expression: 80.56%
- Co-localization: 17.25%
Experiment 7 Top 25 DSS Downregulated Gene Network

Co-expression: 79.41%
Co-localization: 17.45%
Distal Colon General Anatomy

Muscularis Mucosa
Mesenteric/Meissner’s Plexus
Mucosa
Circular Muscle
Longitudinal Muscle
Myenteric/Auerbach’s Plexus
Muscularis Propria/Colon Muscle Wall
Chapter 7.

REFERENCES


dysfunction and inflammatory disease? *Journal of Neuroimmunology, 175*(1–2), 118-127.


