

McMaster University

**VISCERAL PAIN RESPONSES TO COLORECTAL DISTENTION IN RATS  
THAT HAVE RECOVERED FROM A BOUT OF COLITIS**

By  
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**TITLE:** Visceral Pain Responses to Colorectal Distention in Rats that Have Recovered From a Bout of Colitis

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## **ABSTRACT**

Increased visceral pain is often seen in patients with gastrointestinal (GI) inflammation. Some studies, however, have suggested that such pain may persist after resolution of damage or inflammation. Despite the debilitating pain associated with GI inflammation, and its significant impact on affected individuals, few studies have addressed this issue. We hypothesized that altered visceral pain responses would persist after resolution of a bout of colitis in an animal model of colitis. We studied the pain responses to colorectal distention in Wistar rats with dinitrobenzene sulfonic acid (DNBS)-induced colitis, using changes in heart rate as an index of pain. Colonic inflammation had resolved by day 15 after DNBS administration. The assessment of colonic inflammation was based on histological scores, colonic tissue pro-inflammatory cytokine levels and myeloperoxidase activity. Rats examined at 15 days post-DNBS administration exhibited diminished pain responses to colorectal distention as compared to healthy rats. This was associated with significant increases in colonic tissue levels of IL-4 and IL-10 as compared to healthy rats, indicating a possible role for these anti-inflammatory cytokines in counteracting the generation of pain and hyperalgesia. We also studied the effects of hydrogen sulfide (H<sub>2</sub>S) in our animal model, by administering inhibitors of two of the key enzymes involved in the production of H<sub>2</sub>S. Our results demonstrated that inhibition of H<sub>2</sub>S production did not significantly alter the pain responses observed in rats at 15 days post-DNBS administration. In summary, our results demonstrate altered autonomic

responses to colorectal distension following resolution of colitis. Further research on the role of anti-inflammatory cytokines and H<sub>2</sub>S may help to determine the mechanism underlying this effect.

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## LIST OF ABBREVIATIONS AND SYMBOLS

©	Copyright
°C	Degrees Celsius
3MST	3-mercaptopyruvate sulfurtransferase
α-KG	Alpha-ketoglutarate
CHH	Carboxymethyl hydroxylamine hemihydrochloride
CAT	Cysteine aminotransferase
CBS	Cystathionine β-synthesis
CD	Crohn's disease
cm	Centimeter
CRD	Colorectal distention
CSE	Cystathionine γ-lyase
DNBS	Dinitrobenzene sulfonic acid
DSS	Dextran sodium sulfate
ECG	Electrocardiogram
FeCl <sub>3</sub>	Ferric (III) chloride
FG	Fulguration
g	Gram
GI	Gastrointestinal
h	Hour (s)
H <sub>2</sub> O	Water
H <sub>2</sub> S	Hydrogen sulfide
HCl	Hydrochloric
IBD	Inflammatory bowel disease
IL	Interleukin
i.p.	Intraperitoneally
K <sub>max</sub>	Maximum kinetic energy
kHz	Kilohertz

kg	Kilogram
L-PAG	L-propargylglycine
min	Minute (s)
mg	Milligram
mL	Milliliter
mM	Millimolar
mmHg	Millimeters mercury
MPO	Myeloperoxidase
n	Number(s)
NaCl	Sodium chloride
NaHS	Sodium hydrosulfide
nm	Nanomolar
NNDP	N,N'- dimethyl-p-phenylenediamine
P5P	Pyridoxal-5'- phosphate
PBS	Phosphate buffered saline
pg/mg	Picograms per milligram
rpm	Revolutions per minute
SEM	Standard error of mean
TCA	Trichloroacetic acid
TM	Trade mark
TNBS	Trinitrobenzene sulfonic acid
TNF- $\alpha$	Tumor necrosis factor alpha
U	Units
UC	Ulcerative colitis
umol/g/h	Micromolar per gram per hour
w/v	Weight per volume

## **DECLARATION OF ACADEMIC ACHIEVEMENT**

Experiments were conceived and designed by Jessica Sessenwein and John L. Wallace. Lu Wang contributed to the conception and design of the visceral pain protocol. Kyle Flannigan assisted with the hydrogen sulfide assay and analysis. Janice Kim assisted with the cytokine assay and analysis. Jessica Sessenwein and Lu Wang performed all other experiments. Jessica Sessenwein wrote this dissertation with contributions from John L. Wallace.

## **1.0 GENERAL INTRODUCTION**

### **1.1 Overview of Inflammatory Bowel Disease**

Inflammatory bowel disease (IBD) is a group of chronic inflammatory disorders of the gastrointestinal (GI) tract, which have been defined by clinical, pathological and endoscopic features (Podolsky, 2002; Silverberg et al., 2005). The histopathological features of ulcerative colitis (UC) and Crohn's disease (CD), the two forms of IBD, include a significant increase in the number of neutrophils within the lamina propria and the intestinal crypts, as well as depletion in the number and function of goblet cells (Xavier and Podolsky, 2007). In 2012, the estimated prevalence of Canadians with IBD was 1 in 150 (129,000 with CD and 104,000 with UC) (CCFC, 2012). Canada has one of the highest incidences of CD and UC in the world (Molodecky et al, 2012; [www.health.nih.gov](http://www.health.nih.gov)). IBD severely impacts quality of life through ongoing debilitating symptoms, impairment of physical and occupational functions, psychological state, social interaction, as well as pain (Sorensen et al., 1987; Ferry, 1999).

CD and UC have several distinct pathological features. CD is generally localized to the ileum and colon, but can affect any region of the GI tract. On the other hand, UC is localized to the colon. In CD, the inflammation is often transmural (affecting all layers of the colon), whereas in UC the inflammation is typically confined to the mucosa (Abraham and Medzhitov, 2012). Due to the

inflammation and ulceration of the mucosa in IBD, there can be dysfunction of the tissue, leading to symptoms such as diarrhea and abdominal pain.

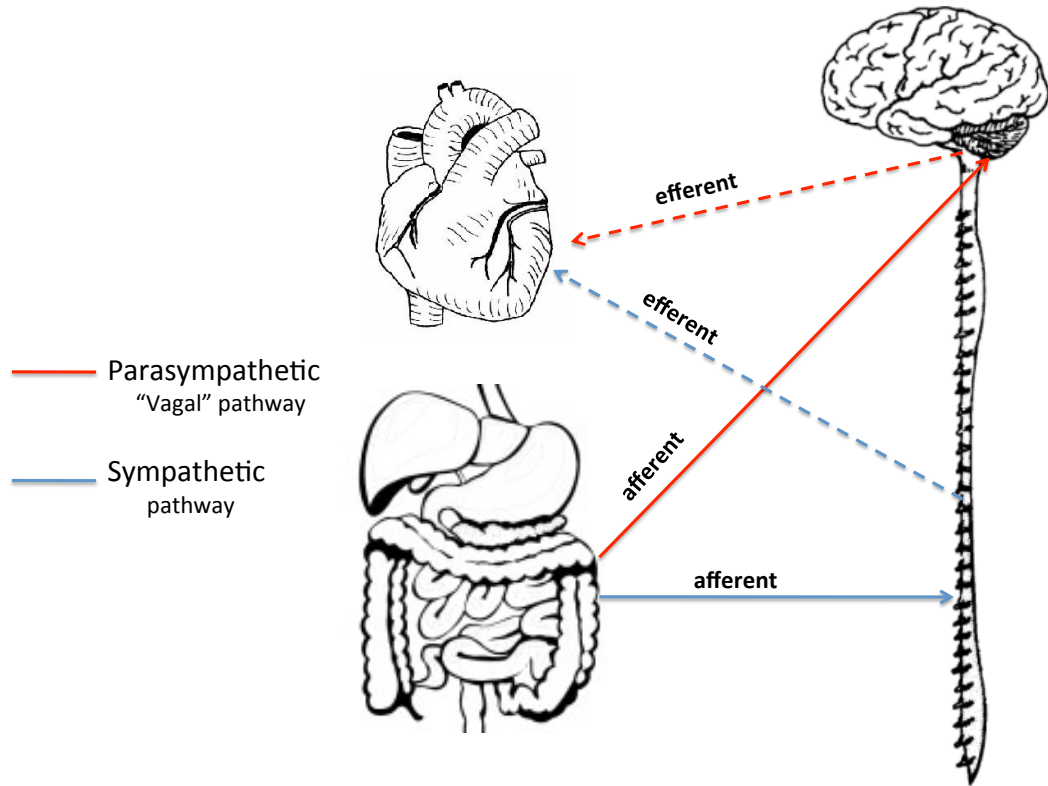
The causes of CD and UC remain unknown, however there is growing evidence suggesting that there is a combination of genetic and environmental factors that inappropriately activate the GI immune system (Loftus et al., 2004; Baumgart and Carding, 2007; Molodecky and Kaplan, 2010).

## **1.2 Pain associated with IBD**

Nociception is defined as "the neural processes of encoding and processing noxious stimuli" (Loeser and Treede, 2008). This includes visceral pain, which originates from the internal organs, and is regulated by both the sympathetic and parasympathetic pathways of the autonomic nervous system. Visceral pain is initiated by nociceptors (pain receptors), which can detect mechanical, thermal or chemical changes above a set threshold. Stimuli include distention of hollow organs, inflammation, ischemia, and spasm of smooth muscle (Gebhart, 1993; Payne and Poulton, 1923; Goligner et al., 1951). Visceral pain is often difficult to localize and quantify and is generally associated with motor and autonomic involvement (Collins, 2004). The viscera of the GI tract are innervated by both intrinsic (neurons within the enteric nervous system) and extrinsic neurons (neurons within the central nervous system). The extrinsic



innervations of the viscera serve both efferent (central nervous system ->viscera) and afferent (viscera-> central nervous system) functions, but the primary recognized function of these neurons is the afferent component (Vergnolle, 2010). Afferent neurons are the primary link between the nervous system and the peripheral environment. The vagus nerve is the major neuron that is the primary link between the nervous system and the peripheral environment. It is one of the cranial nerves that extends from the brain stem to the viscera, and is a medium for bidirectional signaling between the brain and the viscera (O' Mahoney et al., 2009). The majority of vagal nerve fibers (70-90%) are afferent fibers that collect signals from the GI tract and transmit them to the central nervous system through the parasympathetic pathway of the autonomic nervous system (Sengupta and Gebhart, 1994). Fibers travelling with the parasympathetic vagus nerve are thought to be primarily involved in autonomic regulation (Goyal and Hirano, 1996). As the vagus nerve is primarily involved in autonomic regulation, it is not surprising that its main function is to help regulate heart rate and ventilation, control muscle movement, and transmit a variety of neurotransmitters throughout the body (Pavlov and Tracey, 2005). Furthermore, it is also responsible for regulating peristaltic activity in the stomach and intestines, controlling the pH of stomach, as well as regulating food intake (Schwartz, 2000). For this project, the autonomic process of interest is the cardioautonomic regulation, see figure 1.1.



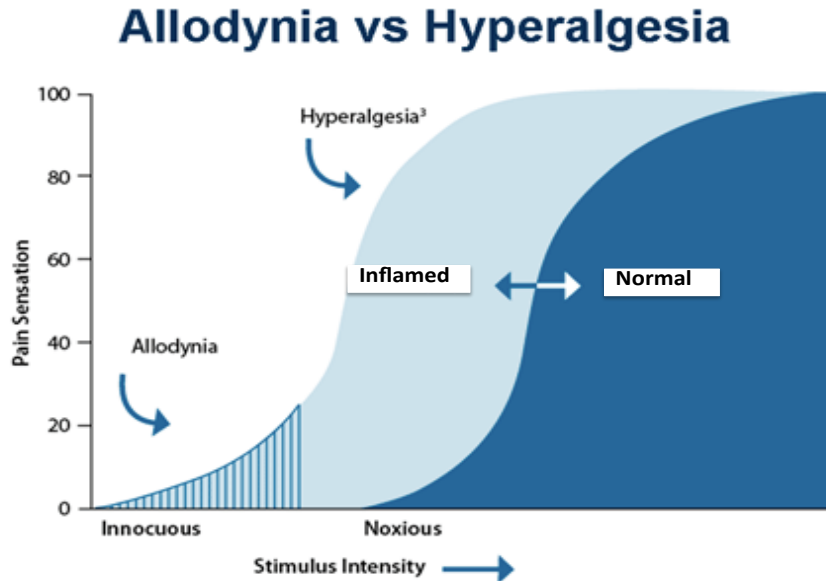
**Fig 1.1 Extrinsic innervations of the lower GI tract-** Afferent nerve fibers collect signals from the lower GI tract and transmit them to the brainstem (parasympathetic pathway) or the spinal cord (sympathetic pathway). Efferent nerve fibers then transmit signals to the heart, which produces a cardioautonomic response.

In the GI tract, sensory nerve endings innervate the serosal and mucosal layers. In the large intestine, sensory nerves mostly consist of small, thinly myelinated (A $\delta$  fibers) or un-myelinated (C-fibers) fibers. These afferent fibers

have low mechanical thresholds, enabling them to code normal physiological stimuli (i.e., non-noxious), as well as stimuli in the noxious range (Sengupta and Gebhart, 1994; Wood 2002; Cenac et al., 2007). A $\delta$  fibers respond predominantly to mechanical stimuli, whereas C-fibers respond to mechanical, thermal, and chemical stimuli. C-fibers are only excited by stimuli in the noxious range and are therefore referred to as high threshold neurons, whereas A $\delta$  fibers can respond to both low and high threshold stimuli (Waxman, 1999; Grundy and Scratcherd, 2011). Upon tissue injury, damaged cells and infiltrating inflammatory cells release numerous mediators that can elicit various effects on the terminals of afferent neurons innervating the tissue.

Most visceral pain experienced by patients with GI inflammation is referred to as visceral hypersensitivity. Visceral hypersensitivity (fig 1.2) consists of hyperalgesia (an enhanced response to a painful stimulus) and allodynia (a painful response to an innocuous stimulus). Hyperalgesia, and to some extent allodynia, are frequent symptoms of various diseases and thought to be useful adaptations for better protection of injured tissues. However, increased sensitivity to pain may persist long after the initial cause for pain has subsided (Sandkuhler, 2009). Hyperalgesia is mainly due to sensitization of nociceptive nerve endings, and altered processing in the central nervous system (Hucho and Lavine, 2007; Woolf, 2007). Allodynia on the other hand refers largely to pain evoked by low-

threshold fibers, such as A $\delta$ - fibers discussed above (Campbell and Gunn, 1988; Gracely et al., 1992).



**Fig 1.2 Visceral hypersensitivity-** Visceral hypersensitivity consists of hyperalgesia and allodynia. At low/innocuous stimulus allodynia occurs, whereas with higher/noxious stimulus, an inflammatory enhanced response to painful stimuli is observed. *Figure adapted from Vinik et al., 2008.*

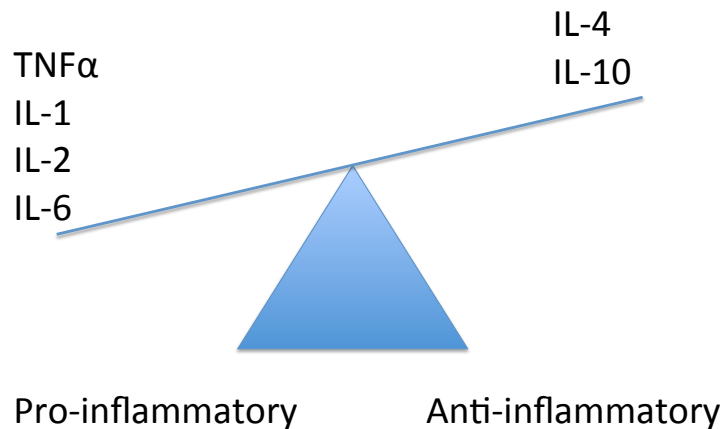
Pain is one of the hallmark symptoms of inflammation, it should therefore not be surprising that pain is one of the presenting symptoms in approximately 50%–70% of patients experiencing the initial onset or exacerbations of IBD

(Aghazadeh et al., 2005; Wagtmans et al., 1998). Moreover, subgroups of IBD patients without evidence of active inflammation, obstruction, or other physiological abnormalities, may continue to experience pain (Bielefeldt and Levinthal, 2009; Vergnolle, 2010; Marshall et al., 2010; Barbara et al., 2007). Up to one-sixth of IBD patients are chronically treated with opioids, a type of narcotic, to alleviate their pain (Cross et al., 2005; Edwards, 2001; Lichtenstein, 2006). However, there are significant concerns about opioid use, including the potential for abuse, as well as significant adverse effects on GI motility (Cross et al., 2005; Kaplan and Korelitz, 1988). Despite the debilitating pain associated with IBD, and its prevalence and impact on affected individuals, few studies have addressed pain management in IBD patients. Further research is thus necessary to determine the underlying mechanisms of pain generation, which will potentially lead to development of effective new therapies.

### **1.3 Role of Cytokines in IBD**

Cytokines are small molecules that are predominantly produced by immune cells. They facilitate communication between cells, stimulate the proliferation of memory (adaptive) immune cells, and mediate local and systemic inflammation (Neuman, 2007). Moreover, cytokines play a central role in the modulation of the intestinal immune system, and can have both pro-inflammatory and anti-inflammatory functions (Alex et al., 2009). Thus, an imbalance in the

ratio of anti-inflammatory and pro-inflammatory cytokines is in part what initiates the onset and drives the exacerbation and recurrence of IBD, see figure 1.3. Since cytokines may play a pivotal role in IBD, numerous inflammatory cytokines have been tested in animal models of colonic inflammation and in clinical trials of patients with IBD (Sanchez-Munoz et al., 2008).



**Fig 1.3 Cytokine imbalance in IBD-** Imbalances in the ratios of the anti and pro-inflammatory cytokine may be in part what initiates the onset, drives the exacerbation and recurrence of IBD. Listed above are some cytokines that may be implicated in IBD.

Recent evidence has supported a role for cytokines in linking the immune and nervous system, and their involvement in the generation of pain and hyperalgesia (Kress, 2010). With respect to visceral pain, inflammatory cytokines are known to sensitize afferent neurons, and recruit silent nociceptors (Bueno and Fioramonti, 2002). Pro-inflammatory cytokines interleukin (IL)-1, IL-2, IL-6, tumor necrosis factor alpha (TNF- $\alpha$ ), and anti-inflammatory cytokines IL-4 and IL-10 have been studied in IBD with regards to visceral pain. Both IL-1 and TNF- $\alpha$  are released early in an inflammatory reaction, and contribute to systemic responses to inflammation (Dinarello, 1988; Wallace and Ma, 2001). Elevated levels of IL-1 have been detected in plasma and tissue of patients with IBD, as well as in several experimental models of colitis (Wallace and Ma, 2001; Beck and Wallace, 1997). The main function of IL-6 is to activate the adaptive immune response (Baumann and Gauldie, 1994), whereas IL-2 regulates and stimulates proliferation of the adaptive immune response (Youngman et al., 1993). Previous studies have shown an increase in plasma IL-2 and IL-6 levels in TNBS-treated rats as compared to controls. This increase lasted up to day 14 post-TNBS administration (Adam et al., 2006). The same study also showed a correlation between increased levels of plasma IL-2/IL-6 and increased responses to pain. However, anti-inflammatory cytokines such as IL-4 and IL-10 have been shown to down-regulate and counteract these effects (Beck et al., 1994; Cominelli et al., 1993; Kam et al., 1995). IL-10 can down-regulate the inflammatory cascade in

IBD by suppressing the production of a number of pro-inflammatory cytokines, and enhancing the production of anti-inflammatory cytokines (Beck and wallace, 1997). The role of IL-10 within the immune system has been extensively studied, using both IL-10 knockout mice, which results in development of spontaneous colitis, and by IL-10 therapeutic efficacy in several animal models of colitis (Ribbons et al., 1997; Herfarth et al., 1996; Kuhn, 1993). IL-4 can down-regulate IL-1 and TNF- $\alpha$  production, as well as stimulate IL-10 synthesis, thus making it an important anti-inflammatory cytokine (Cominelli et al., 1993). It has been shown that monocytes from patients with UC and CD have a decreased responsiveness to IL-4 as well as a decrease in IL-4 receptor expression (Schreiber et al., 1995; Karttunen et al., 1994).

Inhibition of pro-inflammatory cytokines or the administration of anti-inflammatory cytokines reduces inflammation in several animal models of experimental colitis. Such models include the dextran sulfate colitis (DSS) model, the dinitrobenzene sulfonic acid (DNBS) model, and the IL-10 knockout model (Rogler and Andus 1998; Ribbons et al., 1996; Shen et al., 2006).

#### **1.4 IBD and hydrogen sulfide**

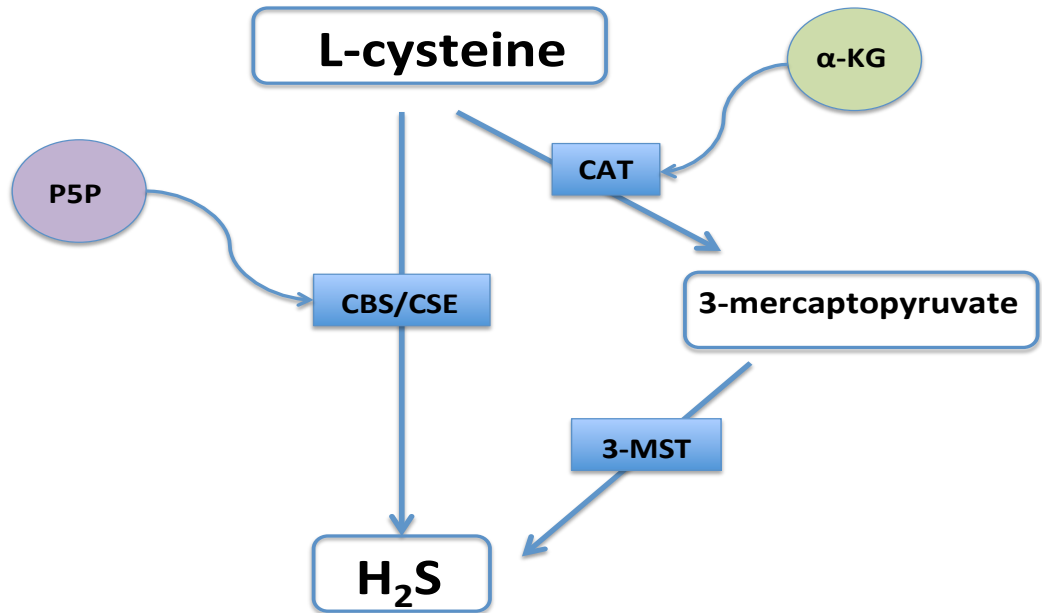
Hydrogen sulfide (H<sub>2</sub>S) is an endogenous gaseous mediator with important physiological roles in the digestive tract. Recent studies have highlighted a role for H<sub>2</sub>S in the regulation of blood flow, smooth muscle tone, and modulation of



epithelial secretion (Schicho 2006; Pouokam and Diener, 2011; Fiorucci et al., 2006). In addition, there is evidence that H<sub>2</sub>S can modulate the inflammatory processes in chemically induced colonic inflammation (Wallace et al., 2012; Duffton et al., 2012).

H<sub>2</sub>S is endogenously generated from L-cysteine through the activity of two pyridoxal-5'-phosphate-dependent enzymes, cystathionine  $\gamma$ -lyase (CSE) and cystathionine  $\beta$ -synthase (CBS). The activity of the CBS and CSE enzymes are dependent upon the presence of the biologically active form of vitamin B<sub>6</sub>, and pyridoxal-5'-phosphate (P5P) (Li et al., 2011; Wang, 2012). There exists an alternative pathway for H<sub>2</sub>S production, which occurs through two enzymes called cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3MST), see figure 1.4.

It is now known that CBS and CSE are differentially expressed throughout the mammalian body and are involved in the production of H<sub>2</sub>S in many organ systems (Wang, 2012). CBS and CSE are expressed in the colon and spinal cord (Distrutti et al., 2006). CSE is required for H<sub>2</sub>S production in peripheral tissues, and CBS is thought to be the main source of H<sub>2</sub>S in the central nervous system (Paul and Snyder, 2012). In the GI tract, CBS and CSE both make important contributions to H<sub>2</sub>S production (Martin et al., 2010; Wallace et al., 2009).



**Fig 1.4 Enzymatic production of colonic H<sub>2</sub>S** – Colonic H<sub>2</sub>S production via cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) is dependent on the cofactor pyridoxal-5'-phosphate (P5P). 3-mercaptopyruvate sulfurtransferase (3MST) produces H<sub>2</sub>S through a reaction involving the generation of 3-mercaptopyruvate from α-ketoglutarate (α-KG) by cysteine aminotransferase (CAT). *Figure adapted Kimura, 2011.*

### **1.5 Role of hydrogen sulfide in visceral pain**

H<sub>2</sub>S has been shown to play a key role in modulation of visceral hypersensitivity in experimental models of colonic inflammation (Fiorucci 2006 et al.). Distrutti et al. showed that following administration of a H<sub>2</sub>S donor, sodium hydrosulfide (NaHS), to either healthy rats or rats with colitis, pain responses to colorectal distention (CRD) were reduced in both groups. Moreover, the inhibitory effects of NaHS were abolished by pre-treating rats with DL-propargylglycine, an inhibitor of CSE (Distrutti et al., 2006a). Distrutti et al. studied the effect of a novel H<sub>2</sub>S-releasing derivative of mesalamine, ATB-429, on visceral pain. Administration of ATB-429 in healthy rats and rats with colitis, significantly reduced pain responses to CRD. They performed the same protocol using mesalamine, its administration was only marginally effective at reducing pain responses to CRD. Therefore, their hypothesis was that the diminished pain responses to CRD seen after ATB-429 administration was due to the release of H<sub>2</sub>S (Distrutti et al., 2006b).

On the other hand, Matsunami et al. demonstrated that after intrarectal administration of NaHS, mice showed enhanced visceral pain-related behavior in response to CRD (Matsunami et al., 2009). Xu et al. examined the change in responses to CRD after administration of hydroxylamine, a CBS inhibitor, in a rat model of colitis. Hydroxylamine treatment induced a significant decrease in pain

responses when compared with CRD prior to injection of hydroxylamine (Xu et al., 2009).

Therefore, there is growing evidence of the involvement of H<sub>2</sub>S in nociception. However, its role is likely to be complex as suggested by the contradictory results in the literature. Thus, it is sufficed to say that further experiments regarding the effects of the H<sub>2</sub>S signaling pathway on visceral pain are required.

## **1.6 General aims**

The responses to visceral pain stimulation after recovery from a bout of experimental colitis have not been examined. It is important to establish such a model in order to determine the mechanisms underlying the pain experienced by IBD patients who show no clinical signs of active inflammation.

Thus, the primary objectives of this project were to:

1. Design an animal model of post- inflammatory GI visceral hyperalgesia.
2. Explore possible correlations between cytokine levels, severity of colonic damage, and pain responses.

3. Explore the H<sub>2</sub>S pathway and its possible involvement in pain perception in response to visceral distention.

## **2.0 GENERAL MATERIALS AND METHODS**

In order to examine the effects of colonic inflammation on visceral pain perception, an experimental animal model of colitis was used. The same animal model of colitis was also used to investigate the role of hydrogen sulfide (H<sub>2</sub>S) in modulating visceral pain perception. This chapter describes the general material and methods used in this study, including, induction of colitis, assessment of colonic inflammation, induction of visceral pain, measurement of colonic tissue H<sub>2</sub>S synthesis, and cytokine profiling. Methods and materials pertinent to a specific experiment are detailed in the appropriate chapters.

### **2.1 Animals**

Male Wistar rats (200-225g) were housed in plastic cages and maintained under controlled temperature (20°C), humidity (60%-70%), and light cycle (12 h:12 h light-dark). Rats were fed standard laboratory chow and water *ad libitum*. Rats were fasted for 18 hours before experiments, but had unrestricted access to water. All experimental protocols were approved by the Animal Research Ethics Board at McMaster University, and adhered to the guidelines established by the Canadian Council on Animal Care.

## **2.2 Animal model of colitis**

Hapten-induced models of colitis are widely used to examine intestinal inflammation. The dinitrobenzene sulfonic acid (DNBS) model of colitis involves the use of ethanol to disrupt the colonic epithelial barrier, allowing DNBS to enter the lamina propria where it drives immune-mediated tissue injury and inflammation (Morris et al., 1989). Methods for the induction of colitis are outlined below.

## **2.3 Induction on colitis**

DNBS was administered using a plastic cannula made from a 5FG pediatric feeding tube fitted over a blunt 18-gauge needle. The cannula was inserted approximately 8 cm into the colon via the rectum. Colitis was induced by administering 0.5 mL of 60 mg/mL DNBS in 50% ethanol. Control groups received the same volume of saline. After injecting DNBS/saline, pressure was applied around the rectum of the rats for 30 sec to prevent leakage. Rats were lightly anaesthetized with isoflurane during the induction procedure.

## **2.5 Assessment of colonic microscopic damage**

Microscopic colonic damage was assessed using a protocol previously described (Fiorucci et al., 2002), outlined in table 2.1. In brief, a sample of colonic tissue was obtained, making sure to incorporate a margin of macroscopically

normal tissue. The tissue was then fixed in 10% buffered formalin phosphate, embedded in paraffin, sectioned, and stained with hematoxylin/eosin. The degree of inflammation on microscopic cross sections was scored blindly. Sections were graded from 0 to 4 based on cellular infiltration of inflammatory cells, vascular density, thickening of colon wall, and loss of goblet cells.

## **2.5 Measurement of myeloperoxidase activity**

Colonic tissue was obtained, making sure to incorporate a margin of macroscopically normal tissue. Samples were weighed, frozen on dry ice, and then stored at  $-80^{\circ}\text{C}$  until assayed. Tissue levels of myeloperoxidase (MPO) were determined using the technique previously described (Bradley et al., 1982). In brief, tissue samples were homogenized using a Polytron homogenizer (Kinematica, Bohemia, NY, USA), in hexadecyltrimethylammonium bromide buffer (50mg/ml). The homogenates were centrifuged (2min at 14,000rpm) and supernatants of each sample were used for the assay. MPO activity was assayed spectro-photometrically; supernatant were added to a mixture containing O-dianisidine dihydrochloride, distilled  $\text{H}_2\text{O}$ , potassium phosphate buffer, and 1% hydrogen peroxide. The change in absorbance was measured at a wavelength of 460 nm over 30 second intervals (SpectraMax M3, Molecular Devices Corp., Sunnyvale, CA, USA) and converted to a  $K_{\text{max}}$  value using SoftMax Pro 5.4



software. These values were then converted to average units of MPO activity per mg of tissue.

## **2.6 Visceral distention**

Rats were anaesthetized with a ketamine hydrochloride (75 mg/kg) and xylazine (10 mg/kg) mixture, given intraperitoneally (i.p.). A 5 cm length plastic balloon, affixed to a Teflon catheter with a length of approximately 20 cm, was inserted intrarectally into the distal colon via the rectum so that the tip was 6 cm from the anus. The catheter was connected to a barostat system composed of a flow meter and a pressure control program (Distender, G&J Electronic Inc, Toronto, Canada). The cardiac responses to colorectal distension were measured while inflating the balloon with air to pressures of 50, 80, and 100 mmHg for 60 seconds. In order to let the animal recover from the previous distension, 10 minutes rest was allowed before subsequent distension.

## **2.7 Cardioautonomic responses**

The cardiac response technique has been previously described (Tougas and Wang, 1999). In brief, continuous recordings of heart rate were taken through a surface electrocardiogram (ECG) obtained through three needle electrodes (23 gauge) applied to the left and right shoulders and the right leg of the animal. ECG signals were amplified and processed through a standard

clinical ECG amplifier (model 7807B, Hewlett-Packard) and CyberAmp 380 programmable signal conditioner (Axon Instruments, Foster City, CA), using a sampling rate of 1 kHz. The data were recorded on a personal computer using a commercial data acquisition program (Experimenter's Workbench, DataWave Technologies, Longmont, CO). Heart rate was recorded for 60 seconds before, during, and after distention. Changes in heart rate were expressed as percent changes in heart rate relative to the average heart rate in the 1-minute before the onset of distention in each animal.

## **2.8 Measurement of colonic tissue hydrogen sulfide production**

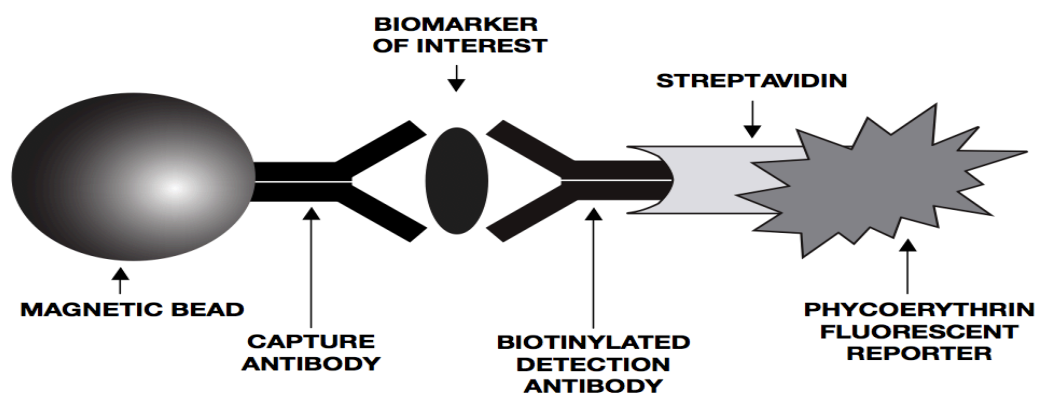
The capability of tissue to produce H<sub>2</sub>S was measured using a modified version of an assay first described by (Stipanuk and Beck, 1982). This method used zinc acetate to trap H<sub>2</sub>S which was then acidified with N,N'-dimethyl-p-phenylenediamine (NNDP) and ferric (III) chloride (FeCl<sub>3</sub>) to produce methylene blue. The color intensity of the methylene blue produced is directly proportional to the concentration of trapped H<sub>2</sub>S and was detected by spectrophotometry to provide relative levels of H<sub>2</sub>S. This assay was performed with colonic tissue homogenates homogenized in ice-cold 50 mM potassium phosphate buffer (pH 8.0; 12% w/ v), using a Polytron homogenizer (Kinematica, Bohemia, NY, USA). Tissue homogenates (0.5 mL), buffer (0.4 mL) and substrates of the H<sub>2</sub>S reaction were added to large scintillation vials resulting in a final volume of 1 mL. L-

cysteine (10 mM) and P5P (2 mM) or  $\alpha$ -ketoglutarate (0.1 mM) were added to the reaction mixture. 2-mL eppendorf tube containing a piece of filter paper (0.5 by 1.5 cm) soaked with zinc acetate (1%; 0.3 mL) were placed inside the larger vial. The larger vials were then flushed with nitrogen gas for 20 seconds and capped with an airtight serum cap. The vials were then transferred to a 37°C shaking water bath for 90 minutes. Samples were then transferred to wet ice for 10 minutes, after which trichloroacetic acid (TCA; 50%; 0.4 mL) was injected into the reaction mixture through the serum cap. The mixture was then transferred to a 50°C shaking water bath for 60 min to allow for the trapping of the produced H<sub>2</sub>S by the zinc acetate, making sure the zinc acetate used to trap H<sub>2</sub>S did not come into contact with the homogenized tissue during the reaction. The serum cap was then removed and NNDP (20 mM; 50  $\mu$ L) in 7.2 M HCl and FeCl<sub>3</sub> (30 mM; 50  $\mu$ L) in 1.2 M HCl were added to the inner tube containing zinc acetate. Samples were left to stand in the dark for 20 minutes. The absorbance was measured at a wavelength of 670 nm with a microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA). A calibration curve of absorbance vs. H<sub>2</sub>S concentration was obtained by using a NaHS solution of varying concentration as described previously (Qu et al., 2006), from which the H<sub>2</sub>S concentration was determined.

## **2.9 Measurement of colonic cytokines levels**

The Bio-Plex Pro™ cytokine, chemokine, and growth factor assay (Bio-Rad Laboratories Inc., Hercules, CA) is designed to measure multiple proteins in a minimal volume of matrix. The assay principle is fundamentally similar to that of a sandwich enzyme-linked immunosorbant assay. Capture antibodies directed against the desired biomarker are covalently coupled to magnetic beads (see fig. 2.9). The beads react with the specific biomarker in the samples. After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerythrin conjugate. Phycoerythrin serves as a fluorescent indicator.

Colonic tissue were collected and stored at -80 °C until assayed. Colonic tissue was homogenized in lysis buffer (1 M tris.HCl (pH 8)) = 50 uM, 5 M NaCl Triton X-100, and autoclaved dH<sub>2</sub>O). The Bio-Plex cytokine assay was performed according to manufacturer's instructions. In order to determine protein concentrations of each sample, the Bradford colorimetric assay was performed following a protocol previously described (Bradford et al., 1976); cytokine levels were then normalized to protein concentrations.



**Fig 2.1 Schematic diagram of the Bio-Plex sandwich immunoassay** (Bio-Rad instruction manual)

## 2.10 Statistical analysis

All data are expressed as means  $\pm$  SEM. Comparisons between two experimental groups were performed using unpaired Student's t test. Comparisons of two or more groups of data were performed using a one-way analysis of variance and Bonferroni multiple-comparison test. An associated probability (P value) of < 5% was considered significant.

## 2.11 Materials

Dinitrobenzene sulfonic acid (DNBS), L- cysteine, pyridoxal-5'-phosphate (P5P),  $\alpha$ -ketoglutarate, L-aspartate, potassium cyanide, trichloroacetic acid (TCA), ferric (III) chloride, N,N'-dimethyl-p-phenylenediamine sulfate salt (NNDP), acetate, L-propargylglycine (L-PAG), carboxymethyl hydroxylamine

hemihydrochloride (CHH), and NaHS were obtained from Sigma-Aldrich (Oakville, Ontario). Isoflurane was obtained from Abbott Laboratories (Montreal, Quebec). Bio-Plex Pro™ Assay kit was obtained from Bio-Rad Laboratories Inc., Hercules, CA).

**Table 2.1 Criteria for microscopic scoring of damage**

<b>Score</b>	<b>Description</b>
0	-No signs of inflammation
1	-Very low level of inflammation
2	-Low level of leukocyte infiltration
3	-High level of leukocyte infiltration, high vascular density, and thickening of the colon wall
4	-Transmural infiltrations, loss of goblet cells, high vascular density, and thickening of the colon wall

### **3.0 TIME-COURSE OF DNBS-INDUCED COLONIC INJURY**

#### **3.1 Introduction**

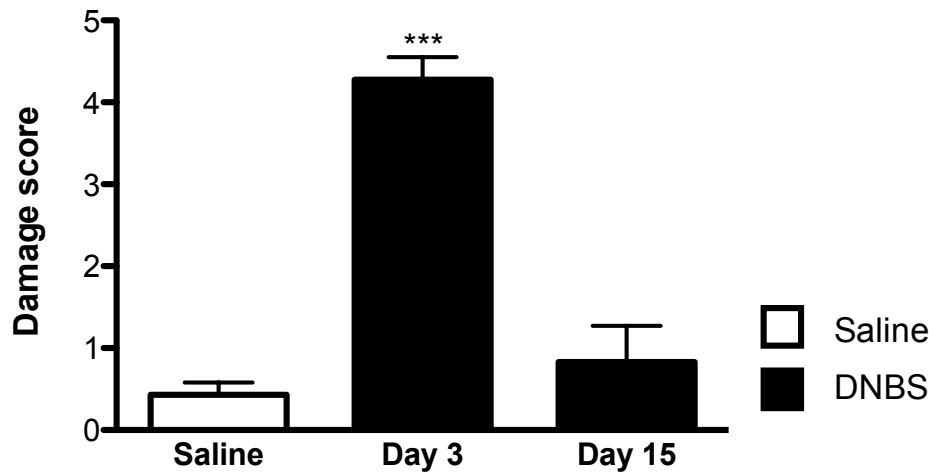
In order to study pain responses in the post-inflammatory state, an animal model was required. Thus, we performed experiments to determine a time point at which DNBS-induced colitis had resolved. Time points were chosen based on previous literature on DNBS-induced colitis (Wallace, 1995; Zhou et al., 2008).

#### **3.2 Materials & Methods**

Colitis was induced in male, Wistar rats (200-225g) by intrarectal administration of DNBS. The assessment of colonic inflammation was based on histological scores, colonic MPO activity, colonic tissue pro-inflammatory cytokine levels, as well as change in body weight. DNBS administration, scoring as well as MPO and cytokine assays were performed as described in chapter 2.

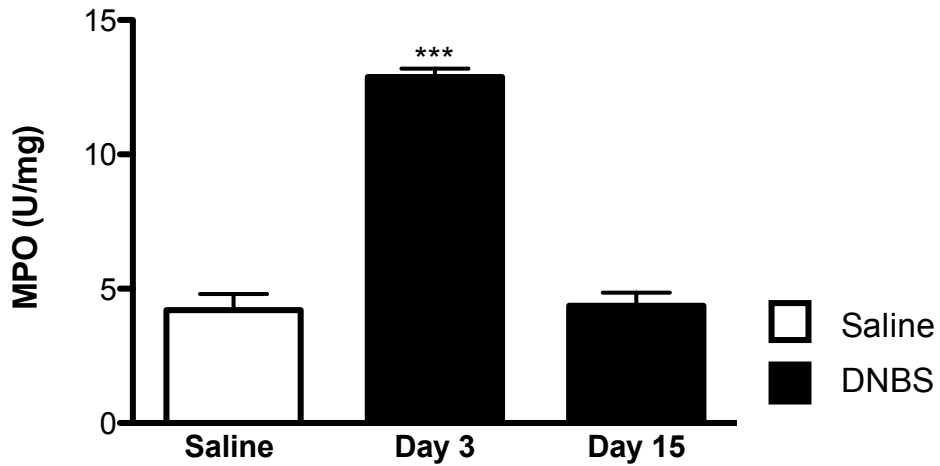
#### **3.3 Results**

Histological scores (fig 3.1), MPO activity (fig 3.2), colonic tissue pro-inflammatory cytokine levels (fig 3.3), and body weight (fig 3.4) at 15 days post-DNBS administration were back to levels comparable to healthy rats, demonstrating that there was resolution of colonic damage. Therefore, we chose day 15 post-DNBS administration as the time point for future experiments (unless indicated otherwise).



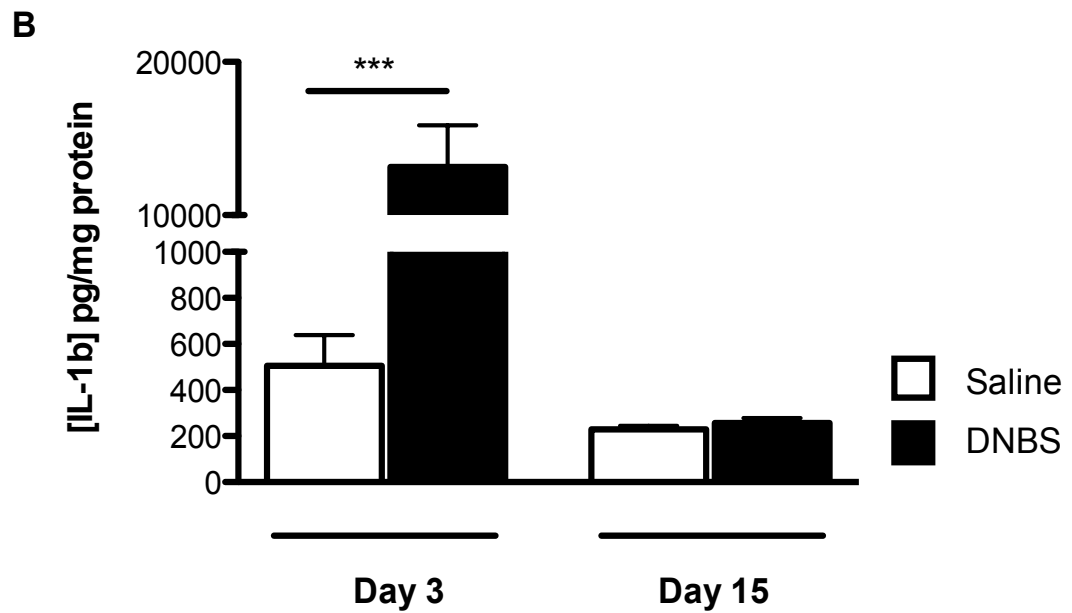
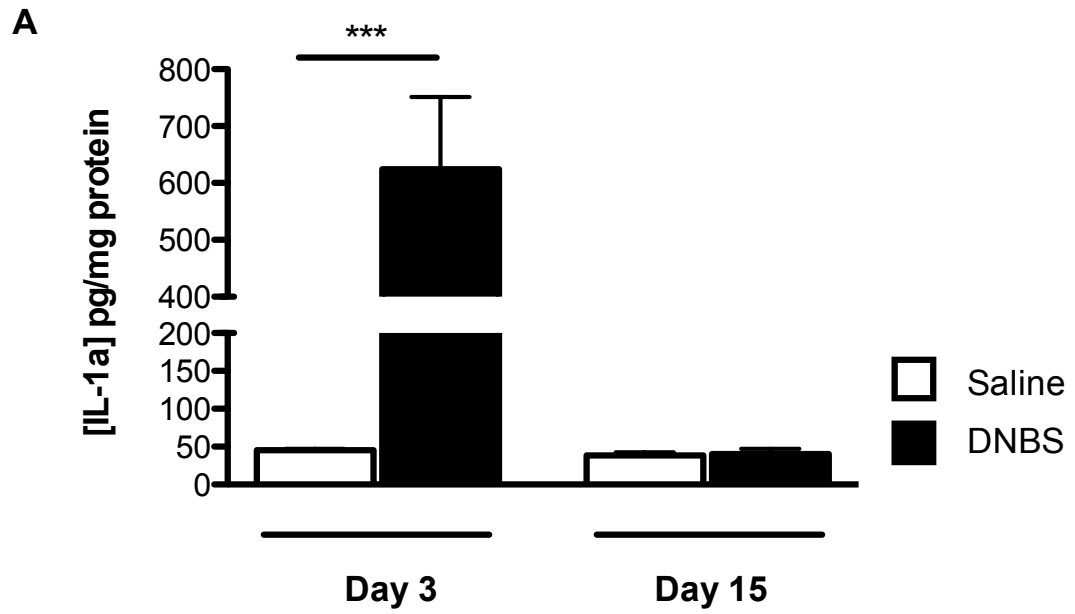
**Fig 3.1 Microscopic colonic damage in rats at 3 and 15 days post-DNBS administration-** Microscopic colonic damage score increased from  $0.43 \pm 0.149$  (n=12) in healthy rats to  $4.3 \pm 0.277$  (n=6) in rats at day 3 post-DNBS administration. \*\*\*p<0.001 (ANOVA and Bonferroni test)

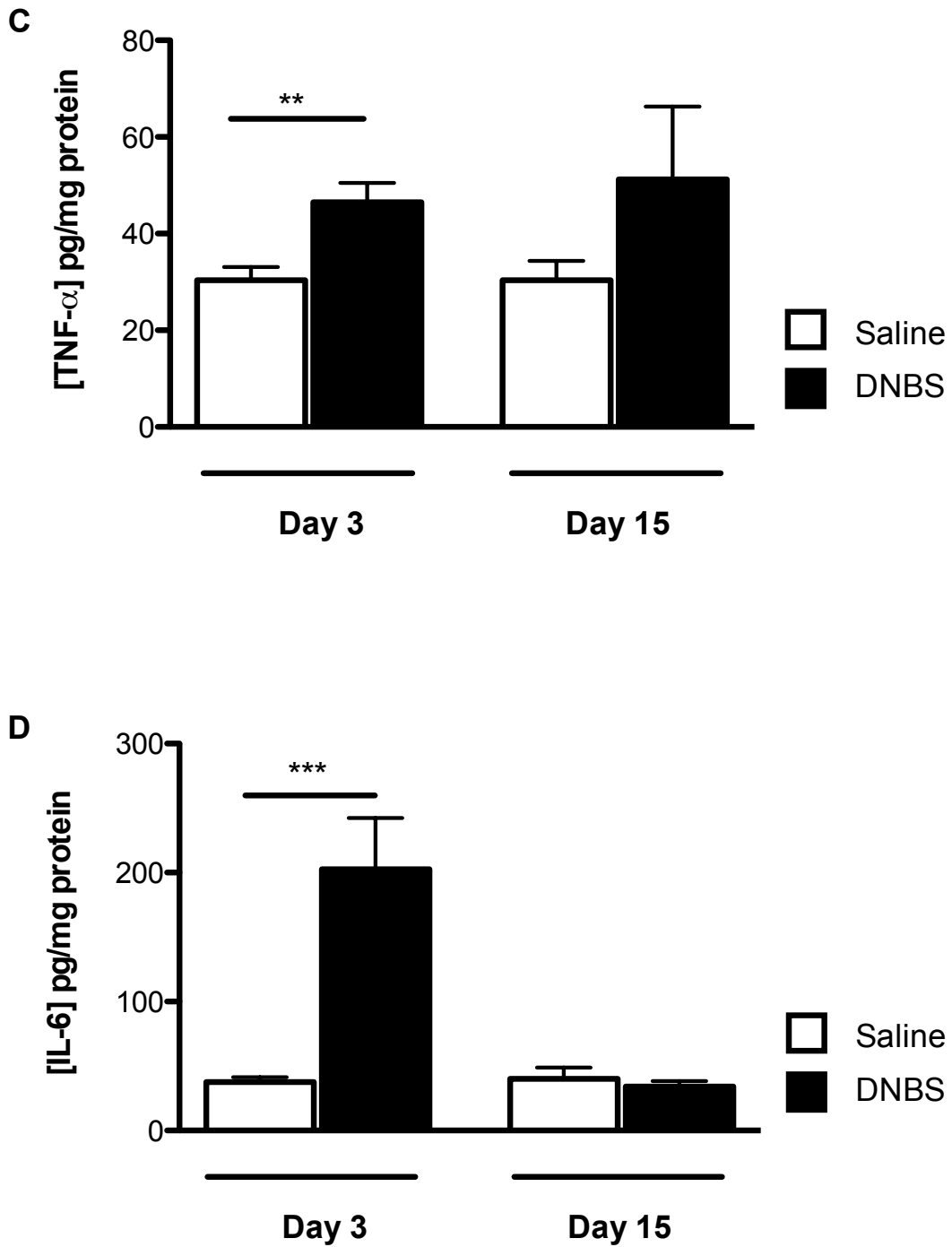




**Fig 3.2 Colonic tissues MPO activity in rats at 3 and 15 days post-DNBS administration-** Colonic MPO activity increased from  $4.2 \pm 0.589$  (n=12) in healthy rats to  $12.9 \pm 0.313$  (n=6) in rats at day 3 post-DNBS administration. \*\*\*p<0.001 (ANOVA and Bonferroni test)

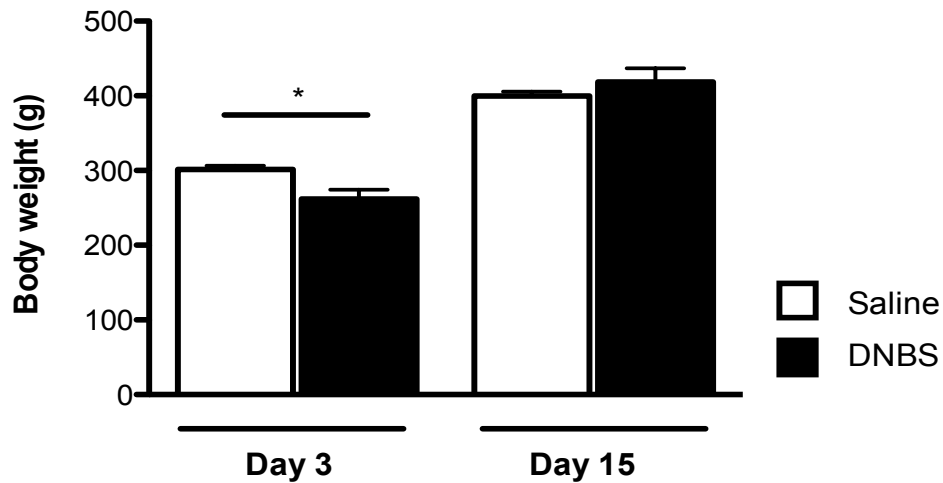
In order to look further into the severity of colonic injury, we investigated the levels of colonic tissue pro-inflammatory cytokines at each time point.





**Fig 3.3 Colonic tissue pro-inflammatory cytokine levels in rats at 3 and 15 days post-DNBS administration - Increased levels of colonic pro-inflammatory**

cytokines (a) IL-1a, (b) IL-1b, (c) TNF- $\alpha$ , and (d) IL-6 in rats at 3 days post-DNBS administration as compared to healthy rats. Each bar represents the mean  $\pm$  SEM (n=5-6). \*\*p<0.01, \*\*\*p<0.001 (ANOVA and Bonferroni test)



**Fig 3.4 Body weight of rats at 3 and 15 days post-DNBS administration.** Mean body weight decreased from 300 $\pm$  5.19 (n=6) in healthy rats to 260 $\pm$  12.3 (n=6) at day 3 post-DNBS administration. \*p<0.05 (ANOVA and Bonferroni test)

## **4.0 EFFECTS OF A BOUT OF COLITIS ON VISCERAL PAIN RESPONSES TO COLORECTAL DISTENTION IN RATS**

### **4.1 Introduction**

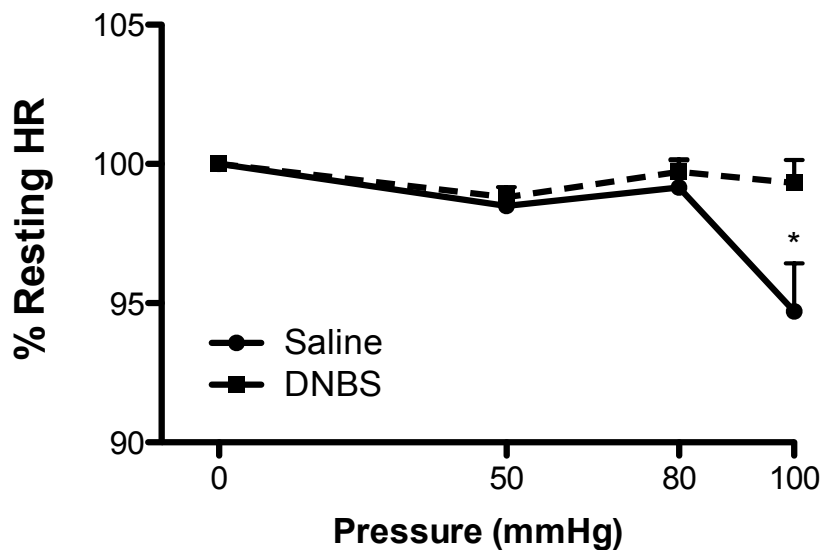
To study the effects of a bout of colitis on visceral pain responses, we employed the rat model of DNBS-induced colitis described in chapter 3. The cardioautonomic responses (changes in heart rate) were used to quantify the intensity of the pain response induced by colorectal distention.

### **4.2 Materials & Methods**

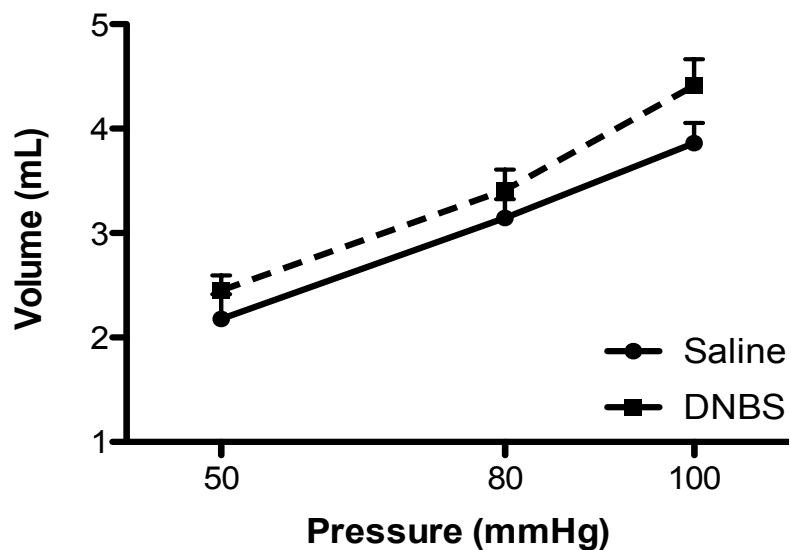
Colitis was induced in male, Wistar rats (200-225g) by intrarectal administration of DNBS. Colorectal distention and cardioautonomic responses were performed as described in chapter 2. Colonic muscle compliances were measured, as it is possible that DNBS-administration can induce damage to colonic mucosa as well as smooth muscle resulting in alterations of muscle compliance. Colonic muscle compliance is the ability of the colon to expand when a specific pressure is applied (pressure-volume relationship). A change in compliance might affect the pain responses. Therefore, we recorded the volume of air in the balloon at each pressure during colorectal distention.

### 4.3 Results

At 15 days post-DNBS administration rats had diminished pain responses to colorectal distention compared to healthy rats (fig 4.1). Differences in the visceral pain responses to colorectal distention were not attributable to a change in colonic muscle compliance (fig 4.2).



**Fig 4.1 Visceral pain responses to colorectal distention in rats at 15 days post-DNBS administration** - Mean responses to CRD in rats that had recovered from a bout of colitis changed to  $99.3 \pm 0.830$  (n=10) from  $94.7 \pm 1.732$  (n=9) in healthy rats, at distention pressure of 100mmHg. \*p<0.05 (ANOVA and Bonferroni test)



**4.2 Colonic muscle compliance in rats at 15 days post-DNBS administration-** Colonic muscle compliance was not altered by DNBS-administration. Each point represents the mean  $\pm$  SEM (n=9-10). (ANOVA and Bonferroni test)

## **5.0 EFFECTS OF A BOUT OF COLITIS ON VISCERAL PAIN RESPONSES TO GASTRIC DISTENTION IN RATS**

### **5.1 Introduction**

Inflammation has been shown to cause sensitization of nociceptive nerves at anatomically distant sites from the original tissue damage (Hobson et al., 2004; Ness and Gebhart, 1990; Jacobson et al., 1995). Therefore, we hypothesized that DNBS-induced colitis may have a significant effect on visceral perception in response to gastric distention.

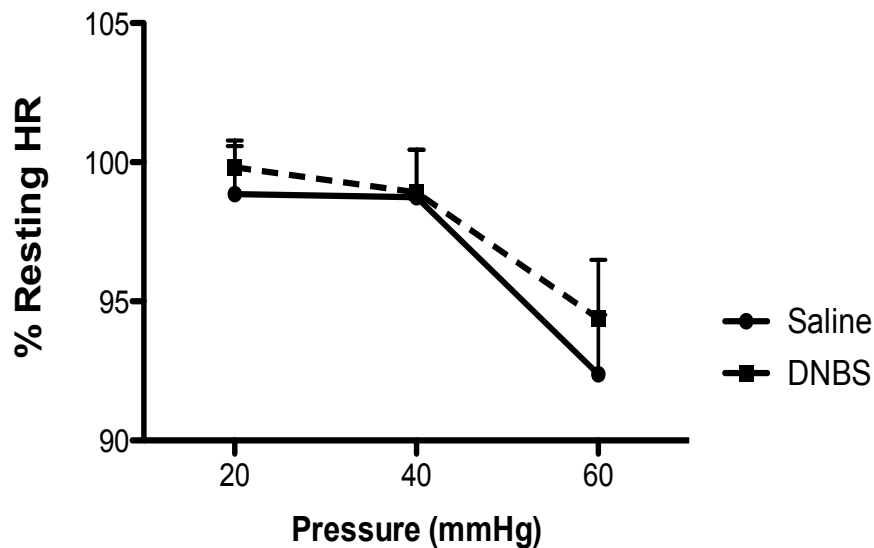
### **5.2 Materials & Methods**

Colitis was induced in male, Wistar rats (200-225g) by intrarectal administration of DNBS according to the method described in chapter 2. A midline laparotomy was performed, then a 3 × 3-cm plastic balloon affixed to a polyethylene tubing was inserted into the stomach through a small incision in the proximal duodenum and advanced, in a retrograde manner, through the pylorus and into the stomach. Cardiac responses to gastric distention were measured while inflating the balloon with air to 20, 40, and 60 mmHg for 1 minute each. In order to let the animal recover from the previous distension, 10 minutes of rest was allowed between each distension. Gastric muscle compliance was also taken into consideration, as it is possible that colonic administration of DNBS can cause a change in gastric compliance.

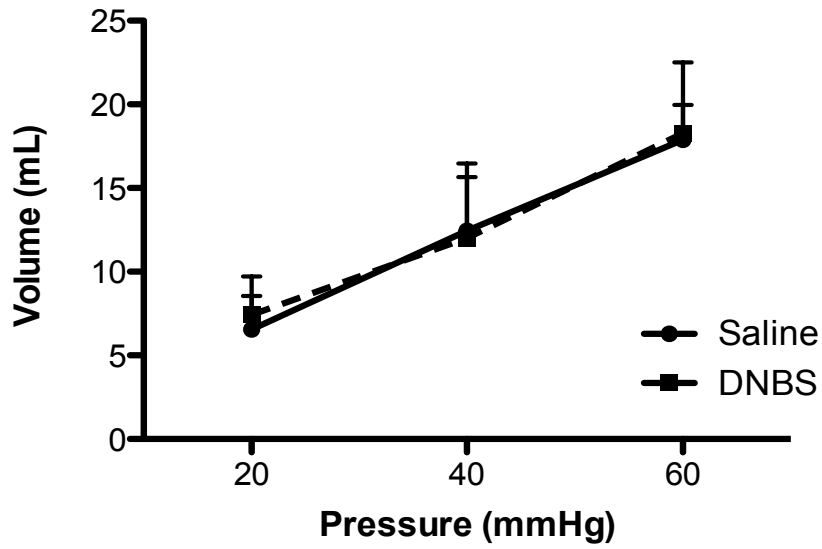


### 5.3 Results

At 15 days post-DNBS administration, rats showed no significant effect on visceral perception in response to gastric distention (fig 5.1). Differences in the visceral pain responses to gastric distention were not attributable to a change in gastric muscle compliance (fig 5.2).



**Fig 5.1 Visceral pain responses to gastric distention in rats at 15 days post-DNBS administration** - Responses to gastric distention in rats at 15 days post-DNBS administration were comparable to healthy rats. Each point represents the mean  $\pm$  SEM (n=7-9). (ANOVA and Bonferroni test)



**Fig 5.2 Mean gastric muscle compliance in rats at 15 days post-DNBS administration** - Gastric muscle compliance was not altered by DNBS-administration. Each point represents the mean  $\pm$  SEM (n=7-9). (ANOVA and Bonferroni test)

## **6.0 STRAIN-SPECIFIC EFFECTS OF A BOUT OF COLITIS ON VISCERAL PAIN RESPONSES TO COLORECTAL DISTENTION**

### **6.1 Introduction**

Pain responses in humans and animals often display considerable and unexplained inter-individual variability (Gralnek et al., 2004; Mogil, 1999). To determine if such variability existed at the strain levels in rats, we examined visceral pain responses to CRD in Wistar and Sprague-Dawley rats at 15 days post-DNBS administration.

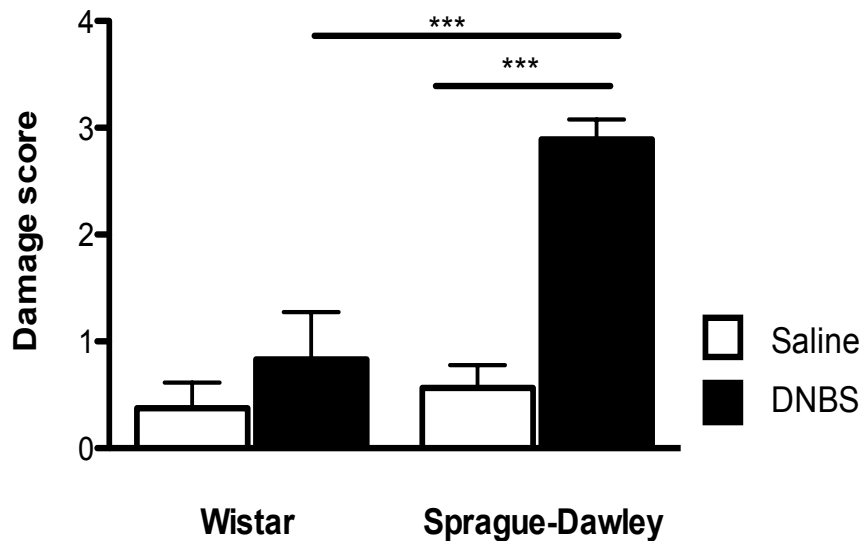
### **6.2 Materials & Methods**

Colitis was induced in male, Wistar and Sprague- Dawley rats (200-225g) by intrarectal administration of DNBS. DNBS-administration, histological scoring, MPO assay, colorectal distention, and cardioautonomic responses were performed as described in chapter 2. Colonic muscle compliance was taken into consideration, as it is possible DNBS-administration affects compliance in a strain-dependant manner.

### **6.3 Results**

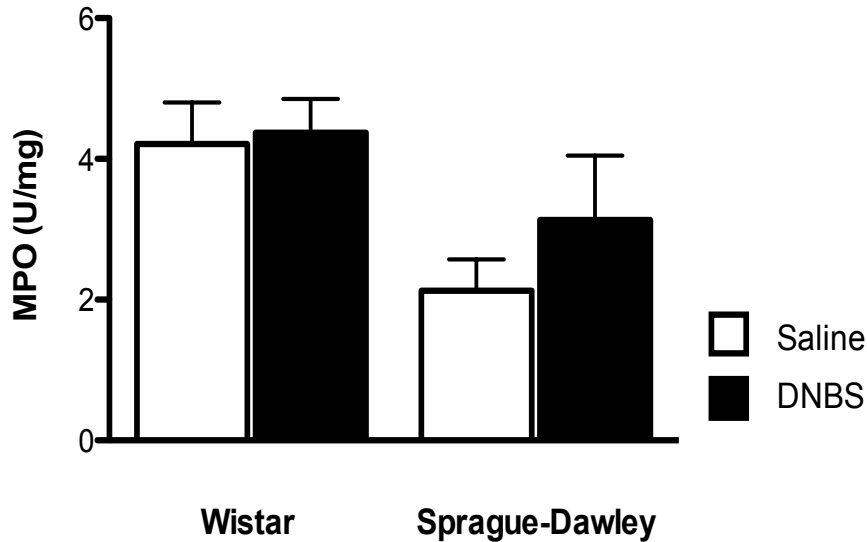
Our results showed that Sprague-Dawley rats had greater microscopic colonic damage scores (fig 6.1) at day 15 post-DNBS administration as compared to healthy rats. No significant differences were seen in colonic MPO

activity in Sprague-Dawley rats at 15 days post-DNBS administration as compared to healthy rats (fig 6.2). Sprague-Dawley rats at 15 days post-DNBS administration had similar responses to colorectal distention as healthy rats (fig 6.3), in contrast to what was seen in Wistar rats (fig 4.1). Colonic muscle compliance was not altered by DNBS-administration in Sprague-Dawley rats (fig 6.4).

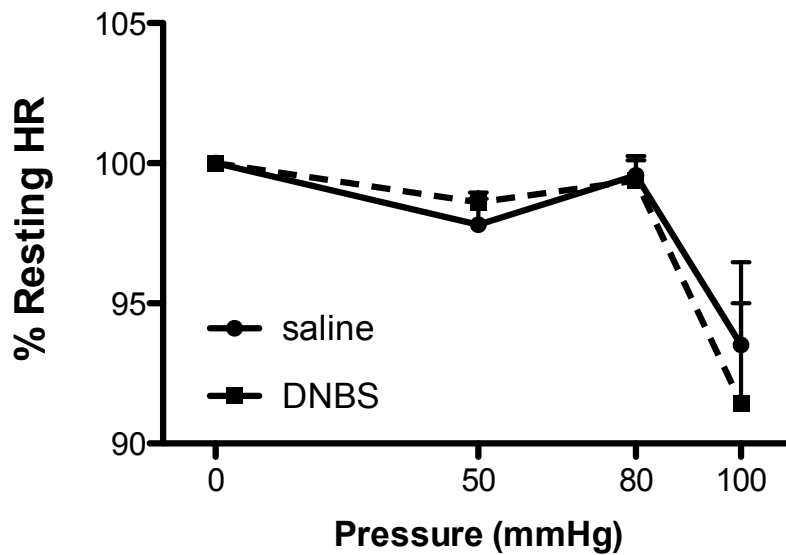


**Fig 6.1 Microscopic colonic damage in rats at 15 days post-DNBS administration-** Microscopic colonic damage score increased from  $0.56 \pm 0.212$  (n=6) in healthy rats to  $2.9 \pm 0.185$  (n=7) in Sprague-Dawley rats at 15 days post-DNBS administration. Microscopic colonic damage score increased from  $0.83 \pm$

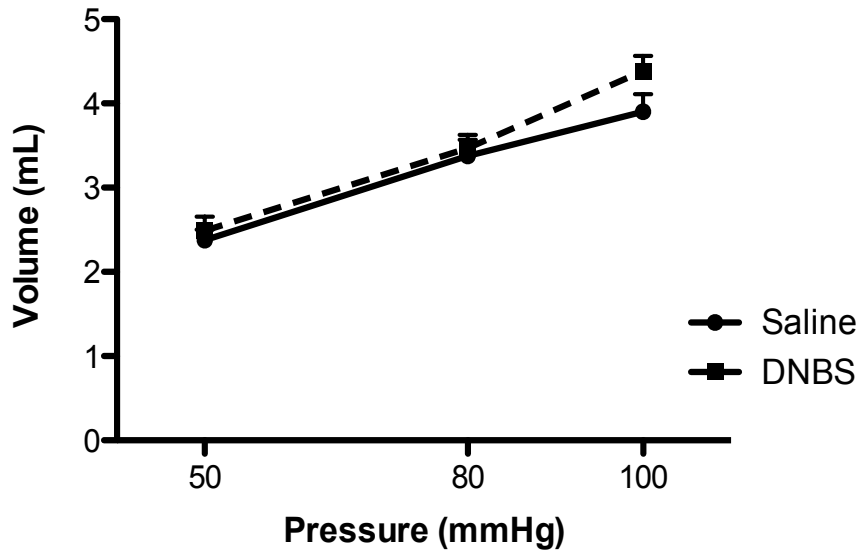
0.441 (n=3) in Wistar rats to  $2.9 \pm 0.185$  (n=7) in Sprague-Dawley rats at 15 days post-DNBS administration. \*\*\* $p < 0.001$  (ANOVA and Bonferroni test)



**Fig 6.2 Colonic tissue MPO activity in rats at 15 days post-DNBS administration-** Colonic MPO activity levels in both Wistar and Sprague-Dawley rats were comparable to healthy rats ( $p > 0.05$ ). Each bar represents the mean  $\pm$  SEM (n=6-9). (ANOVA and Bonferroni test)



**Fig 6.3 Visceral pain responses to colorectal distention in Sprague-Dawley rats at 15 days post-DNBS administration** – Pain responses in Sprague-Dawley rats were comparable to healthy rats. Each point represents the mean  $\pm$  SEM (n=9-13). (ANOVA and Bonferroni test)



**Fig 6.4 Mean colonic muscle compliance in Spague-Dawley rats at 15 days post-DNBS administration** - Colonic muscle compliance was not altered by DNBS-administration. Each group represents the point  $\pm$  SEM (n=9-13). (ANOVA and Bonferroni test)

## **7.0 ROLE OF ANTI-INFLAMMATORY CYTOKINES IN IBD**

### **7.1 Introduction**

Recently, evidence has emerged that cytokines link the immune and the nervous system and that they may be involved in the generation of pain and hyperalgesia (Kress, 2010). Pro-inflammatory cytokines are known to sensitize afferent neurons and recruit silent nociceptors, thereby increasing sensitivity to painful stimuli (Bueno and Fioramonti, 2002). However, anti-inflammatory cytokines such as IL-4 and IL-10 have been shown to down-regulate and counteract these effects (Beck and Wallace, 1994; Cominelli et al., 1993; Kam, 1995).

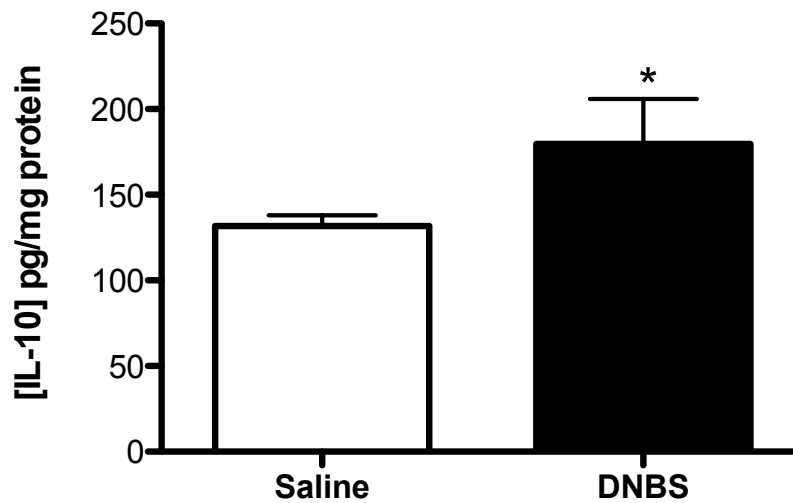
### **7.2 Materials & Methods**

Detailed materials and methods are outlined in section 2.10. The analyses were performed as followed; cytokine concentrations were quantified using a calibration or standard curve. 8- parameter logistic regression analyses were performed to derive an equation that allowed concentrations of unknown samples to be predicted and normalized to protein concentration.

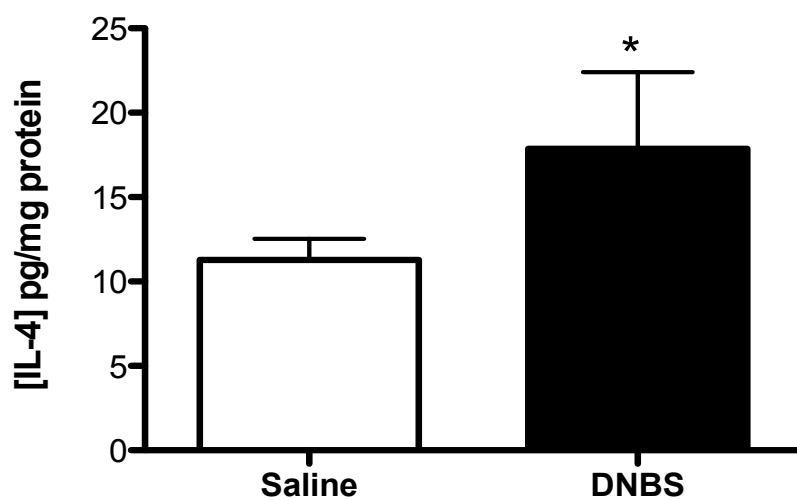


### 7.3 Results

Rats at 15 days post-DNBS administration had a significant increase in colonic tissue IL-10 (fig 7.1) and IL-4 (fig 7.2) levels as compared to healthy rats.



**Fig 7.1 Colonic tissue IL-10 levels in rats at 15 days post-DNBS administration** – Colonic tissue IL-10 concentration increased from  $131 \pm 6.22$  (n=6) in healthy rats to  $179 \pm 26.1$  (n=5) in rats 15 days post-DNBS administration. \*p<0.05 (unpaired Student's t test)



**Fig 7.2 Colonic tissue IL-4 levels in rats at 15 days post-DNBS administration** – Colonic tissue IL-4 concentration increased from  $11.3 \pm 1.24$  (n=6) in healthy rats to  $17.9 \pm 4.54$  (n=5) in rats 15 days post-DNBS administration. \* $p < 0.05$  (unpaired Student's t test)

## **8.0 ROLE OF HYDROGEN SULFIDE IN VISCERAL PAIN RESPONSES IN RATS THAT HAVE RECOVERED FROM A BOUT OF COLITIS**

### **8.1 Introduction**

Hydrogen sulfide (H<sub>2</sub>S) is an endogenous gaseous mediator with important physiological roles in the digestive tract. Recent studies have highlighted a role for H<sub>2</sub>S in regulation of blood flow, smooth muscle tone, and modulation of epithelial secretion (Pouokam and Diener, 2011; Fiorucci et al., 2006; Schicho et al., 2006). In addition, there is evidence that H<sub>2</sub>S can modulate inflammatory processes in hapten-induced colonic inflammation (Wallace et al., 2012; Dufton et al., 2012).

In chapter 4, we showed that visceral pain responses to colorectal distention were decreased at 15 days post-DNBS administration in rats. Thus, we hypothesized that inhibiting H<sub>2</sub>S production will result in increased pain responses in rats that have recovered from a bout of colitis.

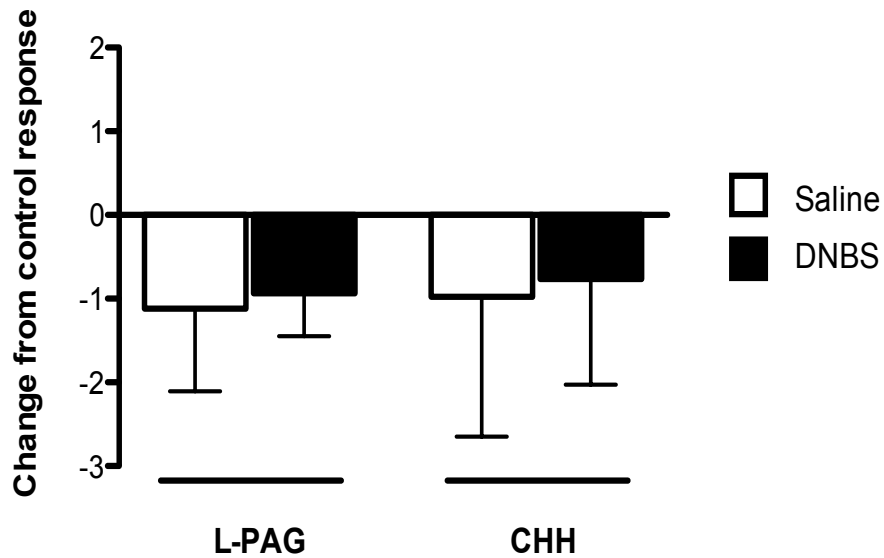
### **8.2 Materials & Methods**

Colitis was induced in male, Wistar rats (200-225g) by intrarectal administration of DNBS. DNBS administration and cardioautonomic responses were performed as described in chapter 2. To examine the role of H<sub>2</sub>S in visceral pain responses, we examined the changes in pain responses to colorectal

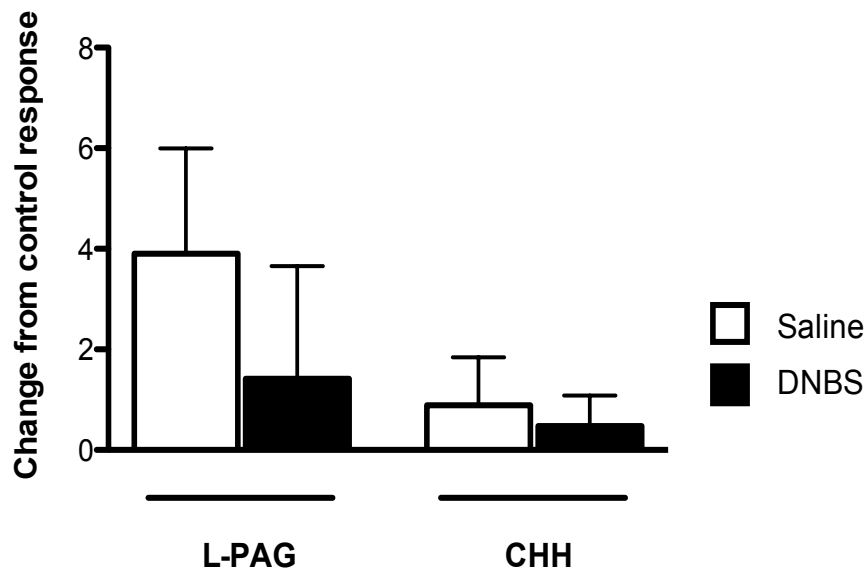
distension after inhibiting H<sub>2</sub>S production. We administered inhibitors (L-PAG and CHH), of the two key enzymes involved in the production of H<sub>2</sub>S. Dosing and colorectal distension protocols were adapted from Distrutti et al., 2006. Phosphate-buffered saline (PBS) was administered i.p. 15 minutes before colorectal distention. Immediately after the distention either L-PAG (50 mg/kg in PBS) or CHH (20 mg/kg in PBS) was administered i.p. to each rat. After 15 minutes, the second set of colorectal distention were performed. Changes in pain responses were calculated by taking the response from the first CRD (control response) and subtracting it from the response of the second CRD (after treatment) for each rat.

### **8.3 Results**

Administration of H<sub>2</sub>S inhibitors at 3 (fig 8.1) and 15 days (fig 8.2) post-DNBS administration did not alter the pain responses to colorectal distention as compared to healthy rats. However, we observed significant differences in the contributions of the two pathways involved in H<sub>2</sub>S production (fig 8.3), pathways represented in fig 1.4.



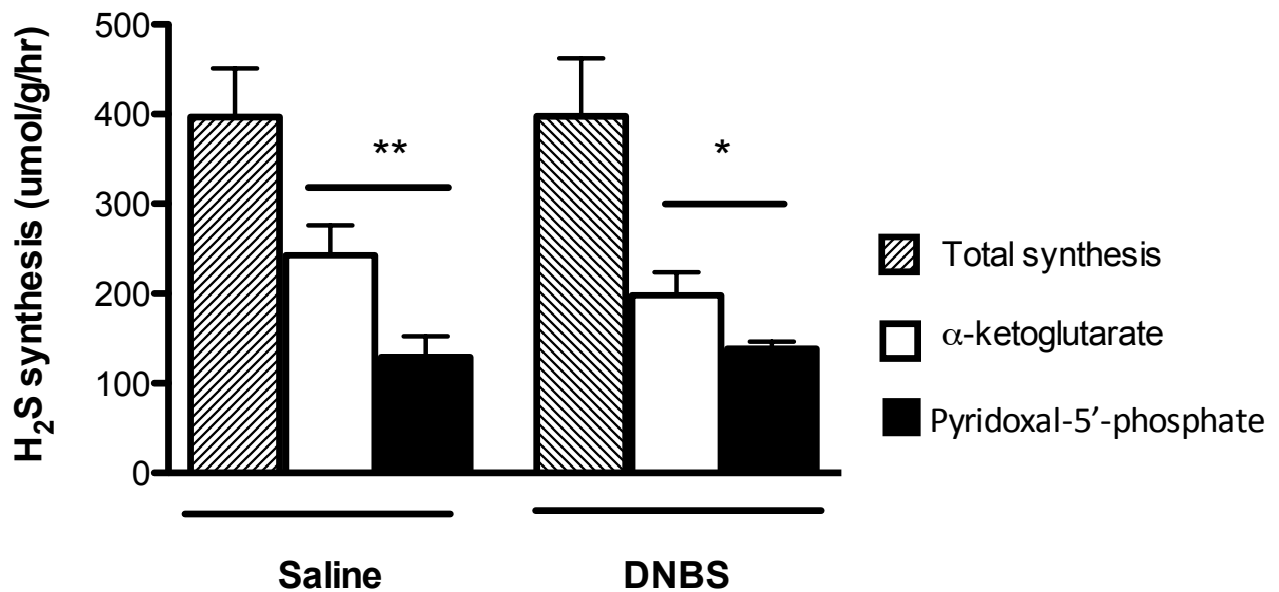
**Fig 8.1 Effects of inhibition of H<sub>2</sub>S production on visceral pain responses to colorectal distention in rats at 3 days post-DNBS administration** -No significant differences in the pain responses to colorectal distention after administration of CHH or L-PAG at 3 days post-DNBS administration as compared to healthy rats, at distension pressure of 100 mmHg. Each bar represents the mean  $\pm$  SEM (n=6-8). (ANOVA and Bonferroni test)



**Fig 8.2 Effects of inhibition of H<sub>2</sub>S production on visceral pain responses to colorectal distention in rats at 15 days post-DNBS administration** - No significant differences in the pain responses to colorectal distention at 15 days post-DNBS administration as compared to healthy rats, at distension pressure of 100 mmHg. Each bar represents the mean ± SEM (n=6-8). (ANOVA and Bonferroni test)

To examine the levels of colonic H<sub>2</sub>S synthesis in rats at 15 days post-DNBS administration, an additional group of rats were used to collect colonic tissue. Several techniques have been used to measure levels of H<sub>2</sub>S produced by biological specimens. One of the most commonly used methods is a zinc trapping- methylene blue formation assay (Hughes et al., 2009; Stipanuk and

Beck, 1982). This technique is reproducible and allows for comparison of the production of H<sub>2</sub>S from large sample sizes in a variety of situations. H<sub>2</sub>S synthesis was measured using the H<sub>2</sub>S assay protocol outlined in section 2.8.



**Fig 8.3 Colonic tissue H<sub>2</sub>S synthesis in rats at 15 days post-DNBS administration** - Total colonic H<sub>2</sub>S synthesis in rats at 15 days post-DNBS administration were comparable to healthy rats. The α-ketoglutarate pathway contributed 197± 25.9 (n=5) compared to P5P pathway, which contributed 138± 7.53 (n=5) in rats at 15 days post-DNBS administration. The α-ketoglutarate

pathway contributed  $242 \pm 33.2$  (n=6) compared to P5P, which contributed  $128 \pm 23.4$  (n=6) in healthy rats. \*p<0.05 \*\*p<0.01 (ANOVA and Bonferroni test)



## 9.0 GENERAL DISCUSSION

Pain is one of the hallmark symptoms in approximately 50%–70% of IBD patients (Aghazadeh et al., 2005; Wagtmans et al., 1998). Subgroups of IBD patients without evidence of active inflammation, obstruction, or other physiological abnormalities, may continue to experience pain (Bielefeldt and Levinthal, 2009; Vergnolle, 2010; Marshall et al., 2010; Barbara et al., 2007). Opioids are the conventional treatment for such symptoms. However, there are significant concerns about opioid use, including the potential for abuse, as well as significant adverse effects on GI motility (Cross et al., 2005; Kaplan and Korelitz, 1988). The aim of the present study was to determine, using an animal model, the underlying mechanisms of the pain that persists after resolution of colitis.

In the Wistar rat model of hapten-induced colitis used, colonic inflammation had resolved by day 15. The assessment of colonic inflammation was based on histological scores, colonic tissue pro-inflammatory cytokine levels, MPO activity, and change in body weight. Using this animal model of resolved colonic inflammation, we studied the pain responses to colorectal distention using changes in heart rate as an index of pain. Changes in heart rate are physiological responses, used to measure the intensity of perceived visceral stimuli (Woodworth and Sherrington, 1904). We showed that at 15 days post-DNBS administration, rats experienced diminished responses to colorectal distention as

compared to healthy rats. Differences in the visceral pain responses to colorectal distention were not attributable to a change in colonic muscle compliance.

Previous studies have shown that inflammation causes sensitization of nociceptive nerves at anatomically distant sites from the original tissue damage (Hobson et al., 2004; Ness and Gebhart, 1990; Jacobson et al., 1995). However, our results suggested that DNBS-induced colitis did not have significant effects on pain responses to gastric distention.

Pain responses in humans and animals often display considerable and unexplained inter-individual variability (Gralnek et al., 2004; Mogil, 2009). To determine if such variability existed at the strain levels in rats, we examined visceral pain responses to colorectal distention in Wistar and Sprague-Dawley rats. Our results showed that Sprague-Dawley rats at 15 days post-DNBS administration exhibited similar responses to colorectal distention as compared to healthy Sprague-Dawley rats (in contrast to what was observed in Wistar rats). In addition, we showed that Sprague-Dawley rats had greater microscopic colonic damage scores at 15 days post-DNBS administration as compared to Wistar rats, which may explain the difference in pain responses between the two strains of rats.

Recent evidence supports a role for cytokines in linking the immune and nervous systems, and their involvement in the generation of pain and hyperalgesia (Kress, 2010). However, anti-inflammatory cytokines such as IL-4 and IL-10 have been shown to down-regulate and counteract the effects of pro-inflammatory cytokines in IBD (Beck and Wallace, 1994; Cominelli et al., 1993; Kam et al., 1995). Thus, we assayed colonic tissue for levels of anti-inflammatory cytokines. There were significant increases in colonic tissue anti-inflammatory cytokine levels (IL-4 and IL-10) in rats at 15 days post-DNBS administration as compared to healthy rats. This indicates a possible role for these cytokines in counteracting the generation of pain and hyperalgesia. The mechanism underlying these effects remains to be determined.

Previous studies have shown that H<sub>2</sub>S can modulate inflammatory processes in hapten-induced colonic inflammation (Wallace et al., 2012; Dufton et al., 2012). In addition, it has been shown that H<sub>2</sub>S plays a key role in modulation of visceral hypersensitivity in experimental models of colonic inflammation (Fiorucci et al., 2006; Distrutti et al., 2006a, Distrutti et al., 2006b). Thus, we studied the effect of H<sub>2</sub>S in our animal model by administering inhibitors of the two key enzymes involved in the production of H<sub>2</sub>S (CSE and CBS). Our results showed that H<sub>2</sub>S production did not play a significant role in the decreased pain responses seen in rats at 15 days post-DNBS administration. These results, however, do not exclude a possible contribution of H<sub>2</sub>S to pain

responses. Unpublished data from our lab has shown that administration of the H<sub>2</sub>S precursor (L-cysteine) decreased pain responses in healthy rats, and the administration of an inhibitor of H<sub>2</sub>S synthesis counteracted this effect.

In summary, our results showed evidence of altered autonomic responses to colorectal distention following resolution of colitis. Further research on the role of anti-inflammatory cytokines and H<sub>2</sub>S may help to determine the mechanism underlying this effect.

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