MITIGATION OF TNBS-INDUCED COLITIS WITH THE GLUTAMINASE INHIBITOR, 6-DIAZO-5-OXO-L-NORLEUCINE

By

Rebekah John

Bachelor of Science in Biology

University of Oklahoma

Norman, OK

2013

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
Master of Science
May 2018

MITIGATION OF TNBS-INDUCED COLITIS WITH THE GLUTAMINASE INHIBITOR, 6-DIAZO-5-OXO-L-NORLEUCINE

Thesis Approved:

Dr. Kenneth E. Miller

Thesis Adviser

Dr. Gerwald A. Kohler

Dr. Frank R. Champlin

ACKNOWLEDGEMENTS

I would like to thank Dr. Miller for all the time and energy that you've put into making this project what it is. You are amazing!! A special thanks to Michael Anderson, Dr. Subhas Das, Dr. Zijia Zhang, and Brittany Bolt for all their help and advice. You all made completing this so much easier. To my family and friends and all who have sent prayers, love, and support my way, You da real MVP! And lastly, and most importantly, thank you Jesus! You've been so faithful and I couldn't have done this without You!

Name: Rebekah John

Date of Degree: MAY 2018

Title of Study: MITIGATION OF TNBS-INDUCED COLITIS WITH THE GLUTAMINASE INHIBITOR, 6-DIAZO-5-OXO-L-NORLEUCINE

Major Field: Biomedical Sciences

Abstract: Inflammatory bowel disease (IBD) is an idiopathic disorder characterized by chronic abdominal pain and, on occasion, organ pathology. It is initiated by a traumatic insult inducing acute colitis, i.e., colon inflammation, and, over time, develops into a chronic condition due to failure of the inflammatory cascade to self-regulate correctly. The etiologies of inflammatory bowel disease are poorly understood and pain management therapies are limited, so this work attempted to gain a deeper understanding of methods that may potentially affect current practices in the treatment of IBD. A key feature of IBD is the large increase in immune cells, granulocytes, and pro-inflammatory cytokines, which results in an expansion of the submucosal area. This project was born out of a desire to better understand the neurogenic component of the inflammatory process in 2,4,6-trinitrobenzene sulfonic acid (TNBS) -induced colitis. TNBS, dissolved in ethanol, is delivered by intracolonic infusion and the epithelial mucosal barrier is compromised over time producing a diffuse inflammation in the distal colon characterized by ulceration, edema, leukocyte infiltration, and pro-inflammatory cytokine production. Because 6-diazo-5-oxo-l-norleucine (DON), a glutaminase (GLS) inhibitor, is known to reduce neurogenic inflammation in a somatic model of inflammation, we hypothesized that colonic administration of DON prior to induction of TNBS colitis will reduce inflammation in the rat colon. We specifically aimed at evaluating the anti-inflammatory effect of DON in a colon that has received TNBS, determining its protective properties by analyzing TNBS-induced leukocyte infiltration, maintenance of epithelial integrity, and preservation of nerve fiber interaction with the lamina propria. Lastly, we studied the ability of DON to attenuate the increase in pro-inflammatory mediator mRNA during TNBS-induced colitis. Based on our results we accept our hypothesis, as we saw a decrease in edema, pro-inflammatory cell infiltration, and epithelial damage in colons that had been pre-treated with DON.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Introduction

According to the International Association for the Study of Pain, "Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Bonica, 1979). Pain is an important system that alerts the body to the presence of injury. Pain and accompanying reflex withdrawal, autonomic, and affective responses, make up the nociceptive reaction (Woolf and Ma, 2007). This reaction is generated by nociceptors which detect any stimuli that “in continuing to act would injure [the skin] still further” (Sherrington, 1903).

One subtype of pain is inflammatory pain, which occurs after an insult to the body has occurred. It usually is accompanied by heat, redness, and swelling, all characteristic symptoms of inflammation. These responses function to promote healing of injured tissue and to protect the injured area while healing is underway by causing a guarding reflex to minimize external contact. This occurs in somatic and visceral inflammation, such as colitis or colon inflammation, the topic of this thesis.

Dorsal Root Ganglion Neurons

Dorsal root ganglia (DRGs) are a collection of primary sensory neural cell bodies. DRG neurons are pseudounipolar with a single axon that bifurcates, with one axonal limb going to the periphery and one reaching centrally into the spinal cord (Martin, 2005). The peripheral axon terminates in the target tissue and has sensory receptors that detect and transmit signals along the DRG axon into the spinal cord (Figure 1). Sensory information that is initiated by the DRG neuron ultimately is transmitted to the cerebral cortex and other central nervous system sites (Martin, 2005, Lumpkin and Caterina, 2007).

Pain sensing neurons are termed nociceptors, noxious stimulus detectors (Woolf and Ma, 2007). Nociceptors express specific high threshold (noxious stimulus) sensory transducers that are found in the transient receptor potential (TRP) family. Examples of high threshold TRPS include TRP vanilloid 1 (TRPV1) and TRP ankyrin 1 (TRPA1). TRPV1 is a noxious heat transducer responding to temperatures above 42°C, tissue stretching, exogenous compounds such as capsaicin, and endogenous molecules such as hydroperoxyeicosatetraenoic acids (HPETEs), hydroxyoctadecadienoic acids (HODEs) and leukotrienes (Romac and Liddle, 2012; Alsalem et al., 2014). TRPA1 responds to exogenous environmental irritants, such as acrolein or isothiocyanates, and endogenous inflammatory molecules, such as 4-hydroxynonenal (4-HNE) and 4-oxononenal (4-ONE) (Bautista et al., 2013). Nociceptors also express specific voltage-gated sodium (Na_v) ion channels, in particular Na_v 1.8 (Gold et al., 2003) to promote conduction of unique action potentials along the axon from the periphery toward the spinal cord. Furthermore, the peripheral nerve terminals of nociceptors have the ability to release neurotransmitters or neuromodulators into the innervated tissue (Woolf and Ma, 2007; Miller et al., 2011).

Figure 1: Diagram of Dorsal Root Ganglion

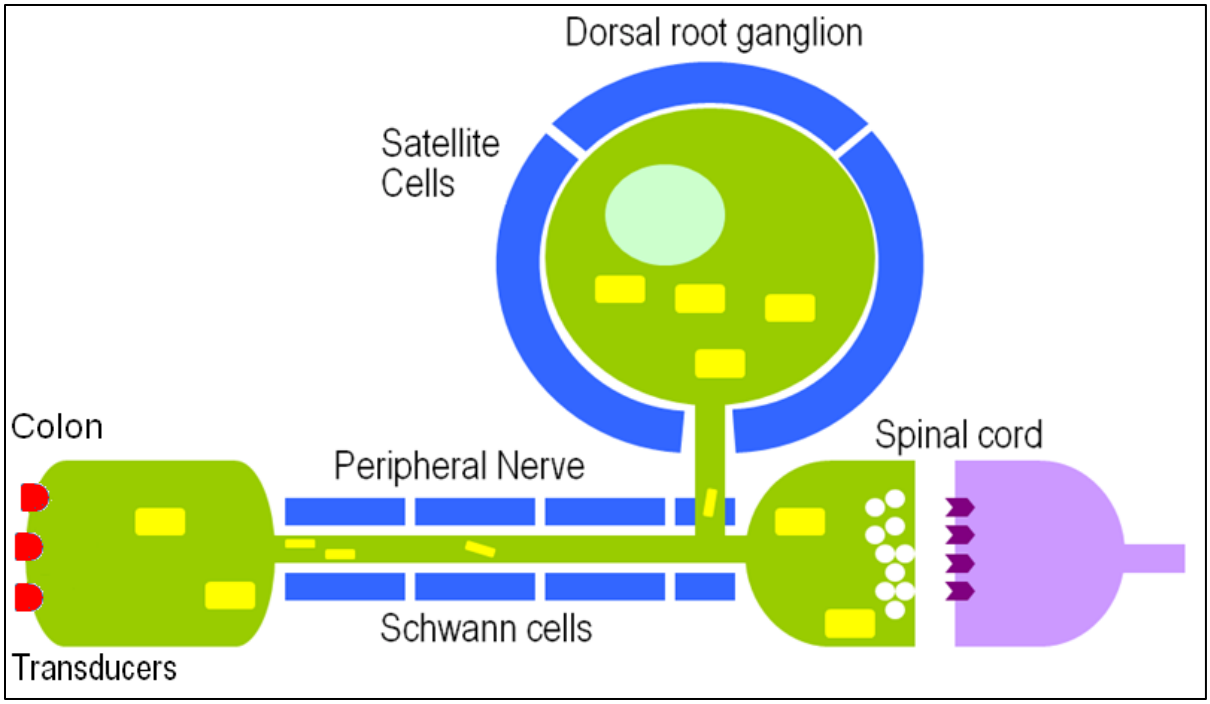

Figure 1. Dorsal root ganglia (DRG) neurons are pseudounipolar neurons with a single axon that bifurcates. One axonal limb goes to the periphery and one reaches centrally into the spinal cord. The peripheral axon terminates in the target tissue, i.e., colon, and has sensory receptors that detect and initiate the transmission of signals along the DRG axon into the spinal cord. Nociceptors express specific high threshold sensory transducers (red chevrons) that are found in the transient receptor potential (TRP) family.

Neurogenic inflammation

Some markers of inflammation, as mentioned above, are heat, redness, and pain and occur after nociceptors are stimulated in damaged tissue to release substances that produce neurogenic inflammation. Neurogenic inflammation stems from the release of pro-inflammatory mediators, such as glutamate, calcitonin gene-related peptide (CGRP), and substance P (SP), from nociceptor peripheral nerve terminals. CGRP and SP cause blood vessel dilation and increased permeability to produce plasma extravasation and inflammatory cell migration and infiltration (Figure 2).

The underlying mechanisms that produce neurogenic inflammation continue to be under investigation. For example, glutamate levels increase in inflamed tissues and initiate sensitization of peripheral nerve terminals, producing hyperalgesia and allodynia (Figure 3; Miller et al., 2011). Hyperalgesia is defined as increased pain from a stimulus that normally provokes pain and **allodynia is p**ain due to a stimulus that does not normally provoke pain (Classification of Chronic Pain, 1994). Glutamate, released from peripheral nerve terminals, stimulates glutamate receptors (GluRs) in an autocrine manner and on surrounding nerve terminals (Miller et al., 2011). Antagonism of GluRs on peripheral nerve terminals produces pain relief, i.e., reduces hyperalgesia and allodynia, during inflammation (Miller et al., 2011). The enzymes, aspartate aminotransferase (AST) and glutaminase (GLS), in nerve terminals are important for glutamate synthesis (Figure 4) and appear to have important roles in promoting neurogenic inflammation. For example, administration of 6-diazo-5-oxo-L-norleucine (DON), a glutaminase inhibitor, during rat hindpaw inflammation reduces edema, decreases spinal neuron activation from inflamed

Figure 2: Diagram of Neurogenic inflammation

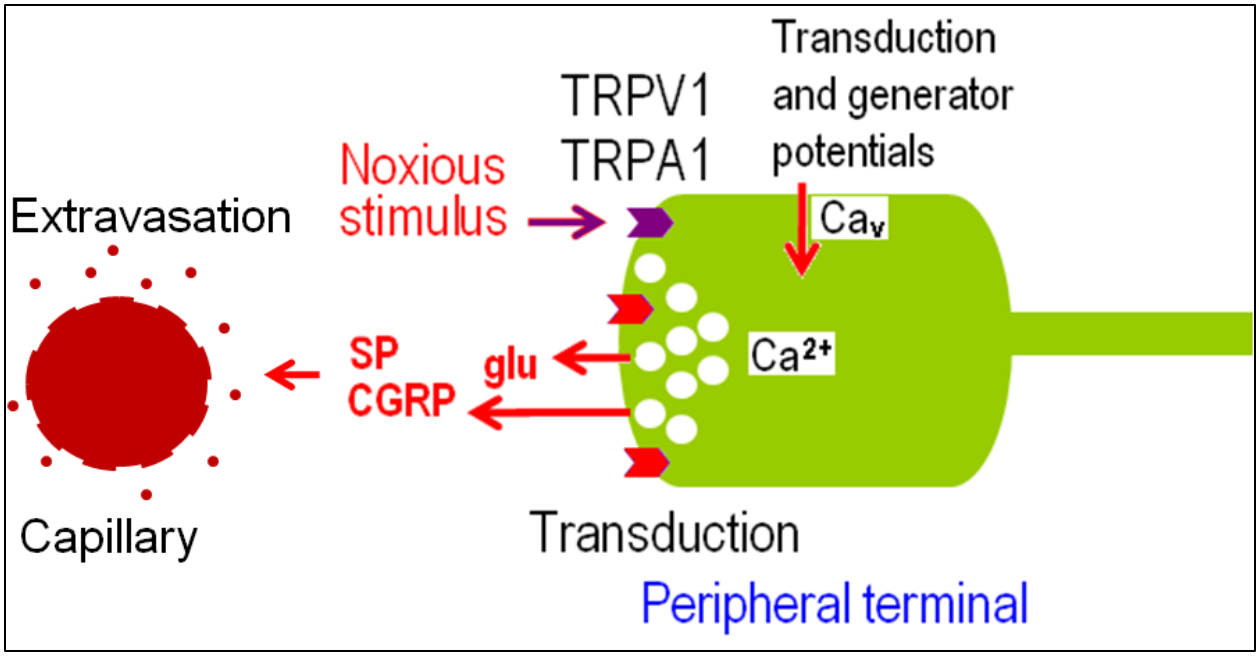

Figure 2. Noxious stimuli activate transient receptor potentials (TRPs; purple chevron) to activate voltage calcium channels (Ca_v) and allow calcium (Ca^{2+}) influx into peripheral nerve terminals. Calcium influx allows for the vesicular release of glutamate (glu), calcitonin gene-related peptide (CGRP), and substance P (SP) to induce neurogenic inflammation. CGRP and SP cause blood vessel dilation and increased permeability to produce plasma extravasation and inflammatory cell migration and infiltration. Glutamate interacts with glutamate receptors (red chevrons, GluRs) to cause peripheral sensitization of the peripheral terminals.

Figure 3: Peripheral sensitization

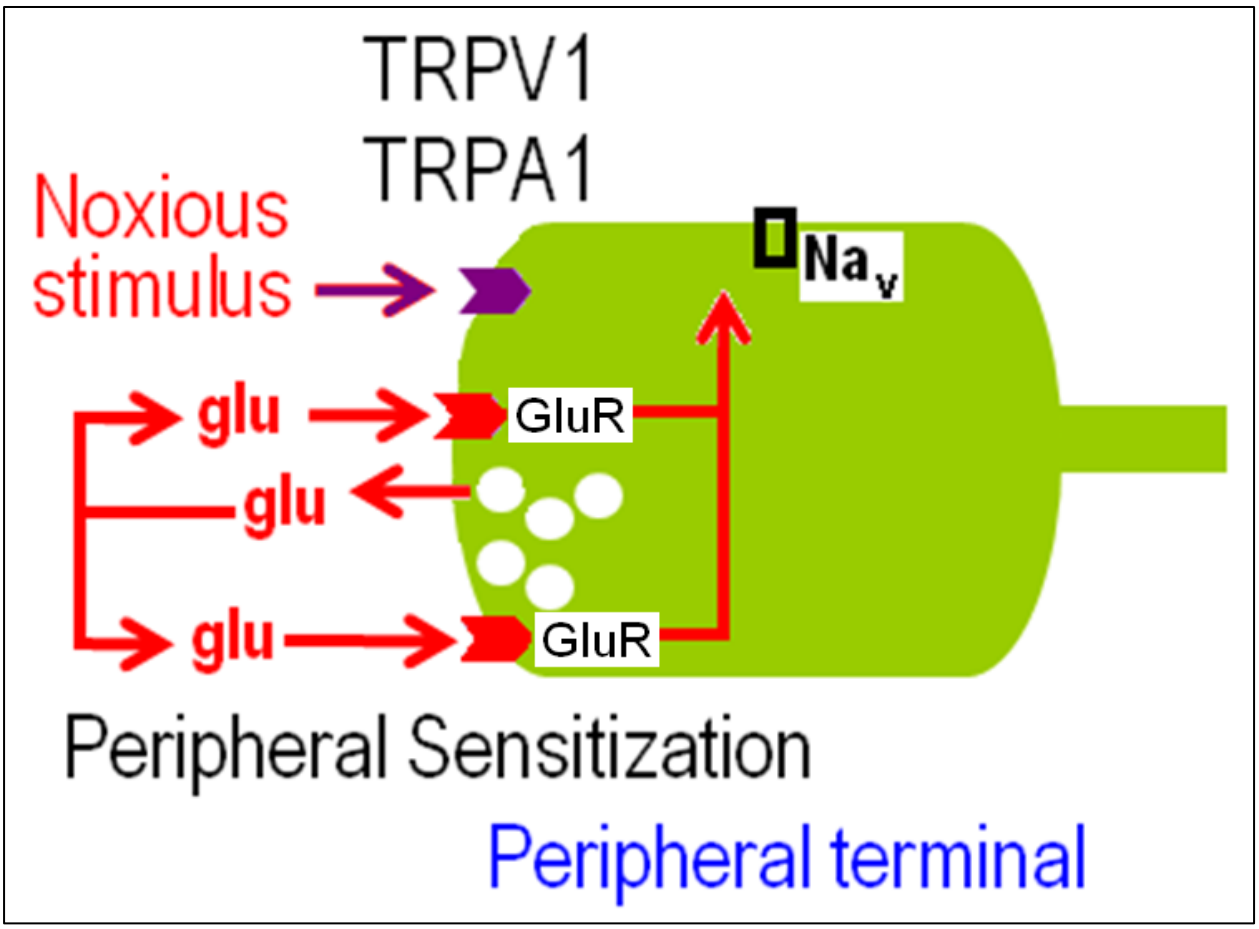

Figure 3. Noxious stimuli activate transient receptor potentials (TRPs; purple chevron) to cause vesicular release of glutamate (glu). Glutamate interacts with glutamate receptors (GluRs; red chevrons) to cause peripheral sensitization by lowering the threshold of TRPs and increasing the activation of voltage gated sodium channels (Na_v). Peripheral sensitization of peripheral terminals causes hyperalgesia and allodynia. Hyperalgesia is increased pain from a painful stimulus and allodynia is pain due to a nonpainful stimulus.

Figure 4: Glutamate synthesis and packaging

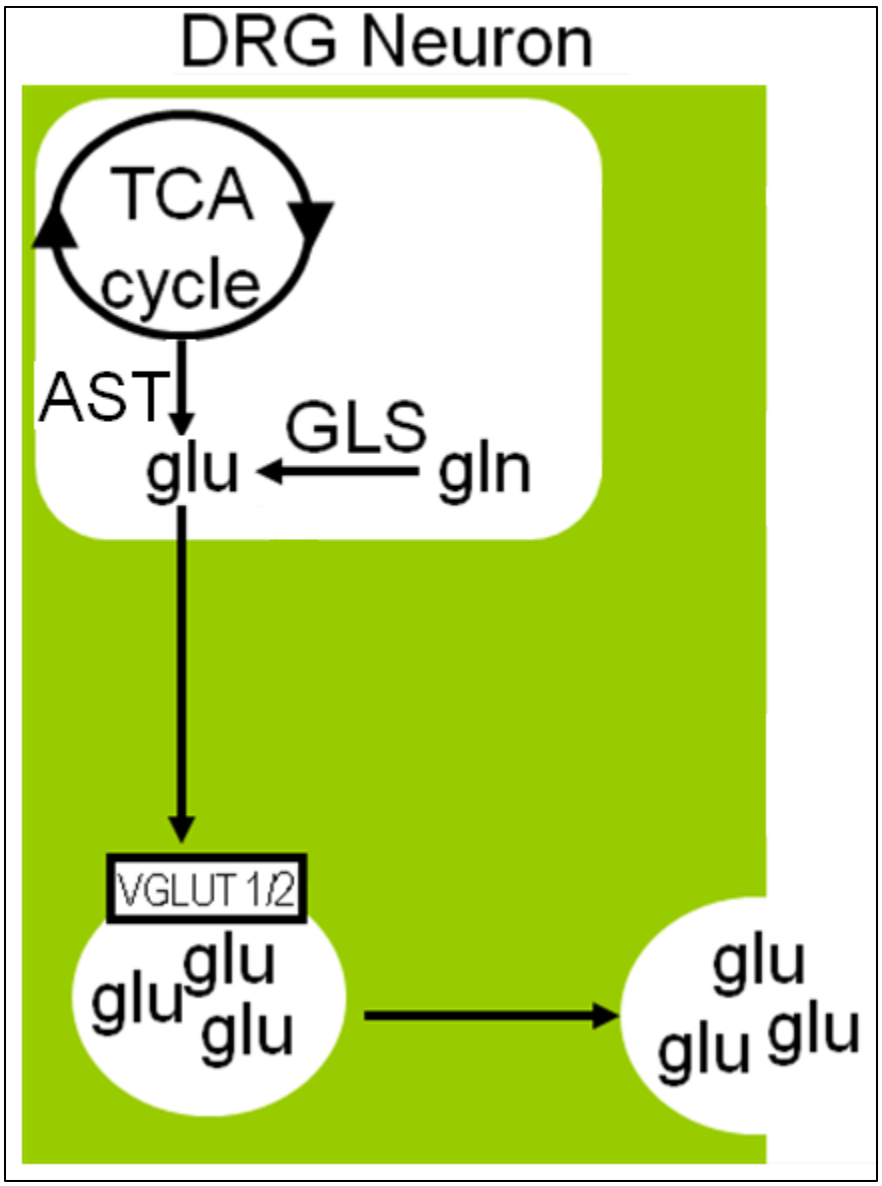

Figure 4. Glutamate (glu) is synthesized by two enzymes, glutaminase (GLS) and aspartate aminotransferase (AST). GLS converts glutamine (gln) to glutamate, whereas AST transaminates aspartate with 2-oxoglutarate (α-ketoglutarate) to produce glutamate and oxaloacetate. The tricarboxylic acid cycle (TCA) supplies 2-oxoglurate for the production of glutamate. Glutamate is packaged into synaptic vesicles by vesicular glutamate transporter 1 or 2 (VGLUT1/2) for storage and later release.

tissue, and provides pain relief (Hoffman and Miller, 2010, Miller et al., 2011, Miller 2007).

Inflammation

Following neurogenic inflammation, there is a migration of macrophages and granulocytes to the site of injury. These cells function in two main roles, as killing cells (granulocytes, macrophages) or as amplifiers (macrophages, T cells) of inflammation via inflammatory mediators (Hume, 2006). After peripheral trauma, inflammatory mediators augment inflammation including an ongoing sensitization of nociceptors, maintaining hyperalgesia and allodynia (Birklein and Shmelz, 2008). Some examples include release of interkeukin 6 (IL-6), a pro-inflammatory cytokine, from T cells and macrophages that is heavily involved in the inflammatory and pain cascades (De Jongh et al., 2003). Local and migrating mast cells increase expression and release of mast cell tryptase and histamine (Groneberg et al., 2005). The increase of histamine and tryptase into the extracellular milieu ultimately results in increased expression and release of tumor necrosis factor alpha (TNF-α) in macrophages and other inflammatory cells, which further sensitizes nociceptive terminals. Epithelial cells are stimulated to release nerve growth factor beta (NGF-β), a neurotrophin that sensitizes nociceptors via the receptors Neurotrophin Receptor K1 (NTRK1; also known as TRKA), and Low-Affinity Nerve Growth Factor Receptor (LNGFR or $p75^{NTR}$). After an initial release of NGF-β, epithelial cells increase production and exocytosis of NGF-β that is endocytosed by nerve terminals and retrogradely transported to DRG cell bodies causing altered protein production, such as increased production of GLS and AST (Woolf and Ma, 2007, Miller et al., 2011, 2012).

Thesis project

My project was developed out of a desire to better understand the neurogenic component underlying the inflammatory process in TNBS-induced colitis. The etiologies of inflammatory bowel disease are poorly understood and pain management therapies are limited, so this work represents an attempt to gain a deeper understanding of methods that may potentially change current practices in the treatment of IBD. Because DON, a GLS inhibitor, is known to reduce neurogenic inflammation in a somatic model of inflammation (Hoffman and Miller, 2010), I hypothesized that colonic administration of DON prior to induction of TNBS colitis would reduce inflammation in the rat colon (Figure 5). Three specific aims will address the hypothesis:

1. DON will reduce TNBS-induced redness and swelling in the colon. Qualitative evaluation of the gross morphology of the colon will be used for this aim.

2. Administration of DON will diminish TNBS-induced leukocyte infiltration, maintain epithelial integrity, and preserve nerve fiber interaction with the lamina propria. Tinctorial histochemistry and immunohistochemistry will be used to address this aim.

3. DON will attenuate the increase in pro-inflammatory mediator mRNA during TNBS-induced colitis. RT-PCR will be used in this aim to evaluate mRNA levels of pro-inflammatory mediators in the colon.

Figure 5: DON inhibition of GLS

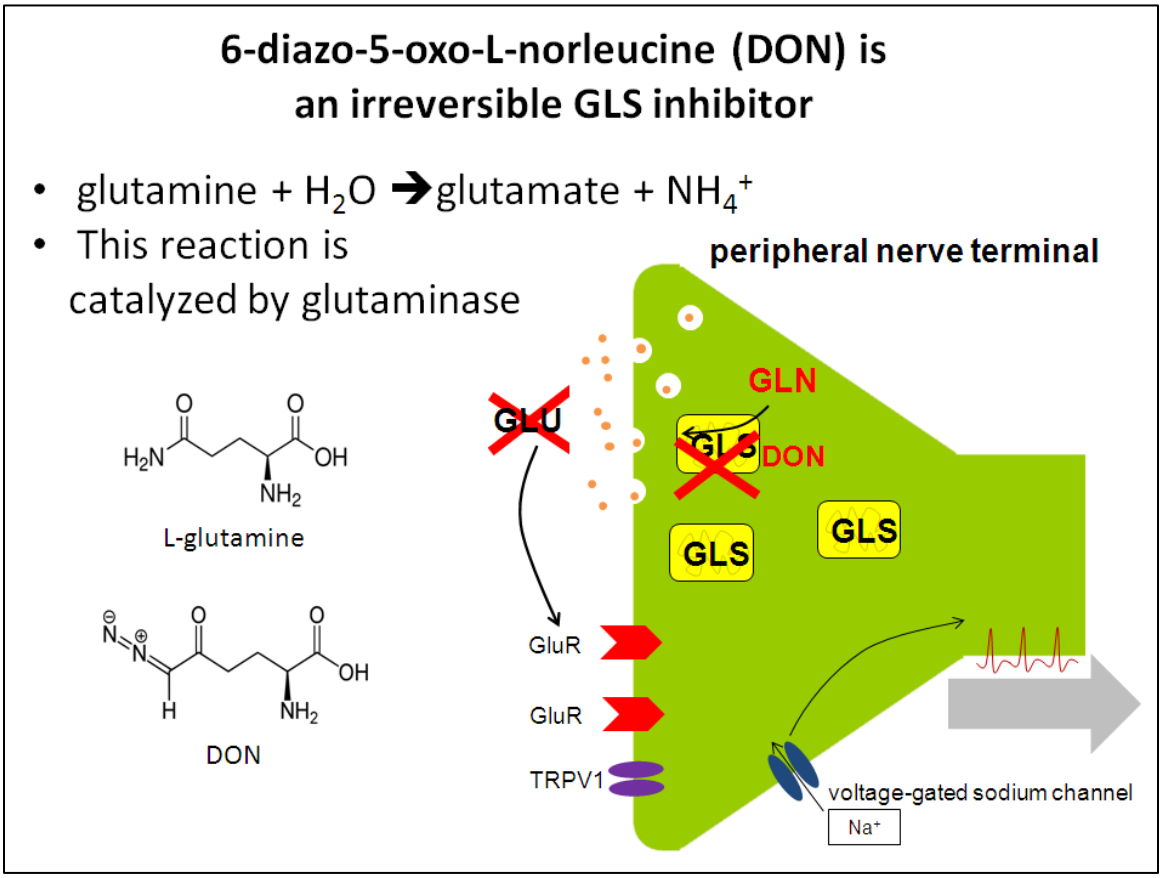

Figure 5. 6-diazo-5-oxo-L-norleucine (DON) is a glutamine (GLN) analogue that irreversibly inhibits glutaminase (GLS) enzyme. Inhibition of GLS results in decreased synthesis and levels of glutamate (GLU) in peripheral nerve terminals. Decreased levels of GLU mitigate neurogenic inflammation in peripheral target tissue by reducing extracellular GLU interaction with glutamate receptors (GluR, red chevrons). This project will use the application of DON to further determine the effects of neurogenic inflammation during colitis.

CHAPTER II

THESIS STUDY

Inflammatory bowel disease (IBD) is characterized by chronic abdominal pain and, in some cases, organ pathology (Greenwood-Van Meerveld et al., 2015). It is initiated by a traumatic insult inducing acute colitis, i.e., colon inflammation, and, over time, develops into a chronic condition due to failure of the inflammatory cascade to self-regulate correctly. A key feature of IBD is the large increase in immune cells, granulocytes, and pro-inflammatory cytokines. This infiltrate results in an increased expansion of the submucosal area. Clinical symptoms of IBD include: inflammation, pain, ulceration, and edema, among other unpleasant symptoms (Monavallian-Naeini et al., 2012).

Colitis and IBD are afflictions that have captured the interest of the medical and scientific world for decades because the exact causes are not known and, in most cases, treatment is inadequate (Greenwood Van-Meerveld et al., 2015). It has been proposed that instead of a single etiology, development of IBD is multifactorial. Dysregulation of the mucosal immune system, however, has been strongly implicated as a key factor in the pathogenesis of IBD (McCafferty et al.,1994). Despite the large amount of information available about this painful disease, there remains much that is unknown. It is of utmost

importance, therefore, to determine critical time periods and events in the development of this disease to be able to better regulate the pathology.

We have employed 2,4,6-trinitrobenzene sulfonic acid (TNBS) to provoke colitis in the rat colon (Velde et al., 2006). TNBS, dissolved in ethanol, is delivered by intracolonic infusion and the epithelial mucosal barrier becomes compromised over time (Randhawa et al., 2014). TNBS produces a diffuse inflammation in the distal colon characterized by ulceration, edema, leukocyte infiltration, and pro-inflammatory cytokine production (Isik et al., 2011; Rogler and Andus, 1998; Mascaraque et al., 2015). Some studies have suggested that TNBS-induced colitis has a neurogenic inflammation component (Engel et al., 2011). Denervation of the colon or TRPV1 antagonism prior to TNBS-induction reduces inflammatory processes in the colon (Takami et al., 2009; Miranda et al., 2007).

In the current study, a blockade of neurogenic inflammation was attempted by interference of the glutamatergic function of colonic peripheral nerve terminals. A previous study demonstrated that glutamate receptor antagonism decreased inflammatory activation in TNBS-induced colitis (Varga et al., 2010). We employed intracolonic pretreatment with DON, a glutaminase inhibitor (Hoffman and Miller, 2010). Previous studies in a somatic inflammatory model showed that DON pretreatment decreased edema during carrageenan-induced inflammation (Miller et al., 2011). My project, therefore, aimed to determine whether pretreatment with DON would mitigate the colonic inflammatory effects of TNBS. A variety of methods were used to examine colon morphology and determine expression levels of different cytokines. We used histological staining to visualize the extent of inflammatory cell infiltrate into the submucosa and qualitatively evaluated the results. Immunohistochemistry was employed to better

understand how acute inflammation affects the nerve fiber - lamina propria interface. Lastly, PCR and Real Time PCR were used to determine RNA levels of pro-inflammatory cytokines.

Materials and Methods

Experimental Animals

6-8 week-old female Sprague-Dawley rats (n=12) weighing between 200-250 g were bred and housed in a temperature controlled room with uniform humidity and lighting cycles. They had free access to food and water and were placed in plastic cages with wire lids and cedar bedding. Four experimental groups were used, with each group containing three rats: naïve control, DON-only, TNBS-only, and DON and TNBS. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Oklahoma State University Center for Health Sciences.

Administration of 6-Diazo-5-Oxo-l-Norleucine (DON) and Induction of 2,4,6-trinitrobenzenesulfonic acid (TNBS) –induced colitis

Rats were fasted for 24 hours prior to intracolonic infusion with DON. Rats were anesthetized with isofluorane (5% induction, 2% maintenance) and 100 µl of DON (Sigma-Aldrich, St. Louis, MO) at concentration of 20 µmol/100 µl sterile PBS was administered approximately 8 cm into the colon via a disposable catheter attached to a 1ml syringe. Rats were then placed back in their cages and were fasted for 20 more hours.

After 20 hours, rats were anesthetized again with isoflurane and infused with a second dose of 100 µl DON. Animals were allowed to recover and, one hour later, the rats were anesthetized with isoflurane and infused with 50mg/kg TNBS (Sigma-Aldrich) in 2ml 50% ethanol. The rats were kept in a Trendelenberg position during and after administration of the TNBS to avoid backflow. After infusion, the rats recovered from anesthesia and were returned to their cages with food and water. After 24 hours, the rats were euthanized with carbon dioxide and exsanguinated. The descending colon was excised quickly and processed for further analysis.

Tissue Preparation

The descending colon was excised, washed in 0.1M phosphate buffer (pH 7.2) and one 5.0 mm piece was cut and placed in fixative (0.2% paraformaldehyde and 0.8% picric acid in 0.1M phosphate buffer, pH 7.2) for morphological studies. The remaining portion of the colon was placed in an Eppendorf tube and frozen with liquid nitrogen. The tissue was stored at 80°C for later RTPCR processing.

Histological Examination (Immunohistochemistry and Tinctorial Staining)

The 5.0 mm tissue were removed from the fixative and the gross morphology was evaluated for each colon. Representative samples were photographed with a digital camera. The pieces were rinsed in phosphate buffered saline (PBS) (pH 7.2) and cut into 2.0 mm segments. Segments were placed in M-1 Embedding Matrix (Shandon-Lipshaw,

Pittsburgh, PA) and snap frozen in liquid nitrogen. The block containing the colon segment was placed in a cryostat and 14 μm and 20 μm sections were cut and thaw mounted on gel-coated glass slides (Thermo Fisher Scientific, Waltham, MA). The slides were placed on a slide warmer at 37°C for 60 minutes to dry the tissue and ensure sufficient adherence of the tissue to the slide.

Wright's Stain (Tinctorial Stain)

Wright's stain is a tinctorial, histologic stain of red (eosin) and methylene blue dyes that allows for evaluation of white blood cell types. Wright's stain working solution consisted of 0.2 g Wright's stain powder (Baker, Sanford, ME) in 100 ml methanol (Sigma-Aldrich) and was filtered before use on the day of experiment. Working solution (1 ml) was placed on each slide covering all colon tissues for 1 min. Distilled water (2 ml) was added to the undiluted solution on the slides for 2 min. Tissue sections were gently washed with running distilled water for up to one minute. Slides were blotted dry and observed under a brightfield microscope (Olympus BX51). Microphotographs were taken with 10X and 20X objectives using a SPOT RT740 camera (Diagnostic Instruments) and stitched together using ImageJ (Mosaic plugin).

Immunohistochemistry

A diluent of PBS with 0.3% Triton X-100 (PBS-T) containing 0.15 g of bovine serum albumin (BSA) and 0.15 g of polyvinylpyrolidone (PVP) was used for all antiserum dilutions. Two groups of slides that were analyzed: VGLUT2 and Peripherin and GLS and TRKA. (Table 1) All sections were incubated with primary antibodies for 16 hours

at 4° C. After incubation, the slides were washed in PBS three times for 10 minutes each and incubated in secondary antibodies for 1 hour at room temperature. The tissue was washed in PBS three times for 10 minutes each cycle and incubated in 300 nm DAPI (for nuclear localization) for 15 minutes. Sections were washed in PBS three times for 10 minutes each. Coverslips were apposed with Prolong Gold mounting medium and stored at room temperature in the dark for later analysis. Representative areas of the colon were photographed using a fluorescence microscope (Olympus BX51) with 40X objective and a SPOT RT740 camera (Diagnostic Instruments).

Polymerase Chain Reaction and Real Time Polymerase Chain Reaction

Extraction of total RNA

Colon samples were thawed and 200 µl of Trizol was added in a sterile Eppendorf tube. Tissue was homogenized to extract the total RNA. Another 800 µl of Trizol was added and the sample was incubated for 10 minutes at room temperature. After the addition of 0.2 ml of chloroform, the samples were vortex mixed and incubated at room temperature for 10 minutes. Aqueous and organic phase were separated by centrifugation at 12000 g for 10 minutes at 4°C. The aqueous phase was removed and placed in a sterile Eppendorf tube and 0.5 ml of 100% isopropanol was added. The sample was incubated at room temperature for 10 minutes and then centrifuged for 15 minutes at 12000 g at 4ºC. The supernatant was decanted and the pellet was washed with 1.0 ml of 70% ethanol and vortex agitated. The samples were centrifuged for 5 min at 7500 g at 4ºC. The supernatant was removed and the pellet was allowed to dry at room temperature for 5-10 minutes. The pellet was resuspended in50 µl of RNase-free molecular grade water and incubated at 55-60ºC for 5-10 minutes

Table 1: Antibodies (Primary & Secondary) and Concentrations

Antibodies

Target	1° Antibody	2° Antibody
VGLUT2/Peripherin	Rabbit antiVGLUT2 (1:2000)	Alexafluor Donkey antirabbit 555 (1:1500)
	Mouse antiperipherin (1:10,000)	Alexafluor Donkey antimouse 488 (1:1500)
GLS/TRKA	Rabbit antiGLS (1:10,000)	Alexafluor Donkey antirabbit 555 (1:1500)
	Mouse anti TRKA (1:1,000)	Alexafluor Donkey antimouse 488 (1:1500)

Table 1. Antibodies (Primary & Secondary) and Concentrations. PBS-T with BSA and PVP was used for all antiserum dilutions. Two groups were analyzed: VGLUT2 and Peripherin or GLS and TRKA. Peripherin labels peripheral sensory nerve axons, whereas VGLUT2 and GLS label glutamatergic nerve fibers. TRKA labels NGF responsive nerve fibers. All sections were incubated with primary antibodies, followed by washing in PBS and incubation in secondary antibodies.

on a heating block. The total RNA was quantified in a nanodrop spectrophotometer (Table 2) and the tube stored at -70° C for further analysis

Synthesis of cDNA (first strand) from extracted mRNA

We used the total RNA and M-MLV Reverse Transcriptase to synthesize cDNA. Total RNA (2 µg) was added to a 0.2 ml PCR tube and water was added to bring the total volume to 24µl.

Statistics

Table 2: RNA Quantification

Plots | Report

Test type: Nucleic Acid | 6/16/2015 9:51 AM | Exit

Report Name | Report Full Mode: Ignore

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
C1	Default	6/16/2015	9:46 AM	335.17	8.379	4.326	1.94	1.66	40.00	260	8.379	0.026
C2	Default	6/16/2015	9:46 AM	314.76	7.869	4.058	1.94	1.55	40.00	260	7.869	0.016
C3	Default	6/16/2015	9:47 AM	340.15	8.504	4.394	1.94	1.52	40.00	260	8.504	0.009
T1	Default	6/16/2015	9:47 AM	362.68	9.067	4.637	1.96	1.51	40.00	260	9.067	0.019
T2	Default	6/16/2015	9:48 AM	312.90	7.823	4.061	1.93	1.61	40.00	260	7.823	-0.014
T3	Default	6/16/2015	9:48 AM	520.26	13.007	6.585	1.98	1.25	40.00	260	13.007	0.277
D1	Default	6/16/2015	9:49 AM	372.84	9.321	4.854	1.92	1.57	40.00	260	9.321	-0.003
D2	Default	6/16/2015	9:49 AM	322.07	8.052	4.172	1.93	1.58	40.00	260	8.052	0.013
D3	Default	6/16/2015	9:50 AM	294.95	7.374	3.821	1.93	1.37	40.00	260	7.374	0.011
DT1	Default	6/16/2015	9:50 AM	315.76	7.894	4.134	1.91	1.19	40.00	260	7.894	0.030
DT3	Default	6/16/2015	9:50 AM	358.49	8.962	4.693	1.91	1.23	40.00	260	8.962	0.016

Table 2. The total RNA for the experimental groups was quantified with Nanodrop and stored at -70° C for further analysis. All values fell within normal ranges.

Mix 1 (6 µl, Table 3) was added to all the tubes and incubated at 65-70°C for 5 minutes. After incubation, the tubes were rapidly chilled on ice and Mix 2 (10 µl, Table 4) added. Samples, with a total volume of the 40 µl reaction mixture, were vortexed, followed by centrifugation. Samples were incubated according to the following schedule: 15 minutes at 25°C; 60 minutes at 42°C; 5 minutes at 95°C and stored at 4°C.

Amplification of cDNA

The synthesized cDNA was diluted to 20 ng/µl by adding 60 ul deionized water to each tube. A master mix was created according to the following table and primers for IL-10, p75, GLS, IL-6, TNF-α, NGF-β, and NTRK1 RNA added to each separate tube. (Table 5) cDNA (5 µl; 20x5=100 ng cDNA) was placed in each tube, bringing the total volume to 20 µl. Table 6 contains protocol used to amplify the cDNA fragment.

Agarose gel electrophoresis of amplified fragment

An agarose gel (2%) was made in TBE buffer (2.0 g of agarose in 100 ml 1x TBE buffer). (Table 7) The liquid was poured into a gel casting tray and allowed to polymerize for at least 30-45 minutes with the specific combs. Following polymerization, combs were removed and the chamber was filled with 1X TBE buffer. The samples were loaded into the wells and the gel run at 170V for 10-15 minutes. Following the run, the gel was stained with Sybr-safe stain (2 ul in 50ml of TBE buffer) for 15-20 min and scanned with a Typhoon scanner.

Table 3:

MIX 1	
Random Hexamer	4 µl
dNTP	2 µl
Total	**6 µl**

Table 4:

MIX 2	
5x RT buffer	8 µl
RNase Inhibitor	0.25 µl
Reverse Transcriptase (200U/ul)	1.0 µl
Deionized water	0.75 µl
Total	**10 µl**

Table 5:

Master mix	
2x Green Go Taq master mix	10 µl
Forward primer	1 µl
Reverse primer	1 µl
Deionized water	3 µl
cDNA	5 µl
Total	20 µl

Table 6:

Step	*Temperature/time*	*Cycle(s)*
Denaturation	94ºC/2 min	1 cycle
Denaturation	94ºC/15 sec	
Annealing	55ºC/15 sec	
Extension	68ºC/15 sec	30 cycles
Final extension	72ºC/5 min	1 cycle
End	4ºC	

Table 7:

TBE buffer	**10x/1L**
Tris-HCl	108 g
Boric acid	55 g
EDTA (0.5M, pH 8.0)	40 ml
Adjust the volume to 1000 ml	

Table 3. 6 µl of Mix 1 was added to all the tubes and incubated at 65-70°C for 5 minutes.

Table 4. After incubation, the tubes were chilled on ice and 10 µl of Mix 2 added. Samples were mixed with vortex agitation, followed by centrifugation.

Table 5. A master mix was prepared and primers for IL-10, p75, GLS, IL-6, TNF-α, NGF-β, and NTRK1 RNA added to each separate tube. 5.0 µl of cDNA was placed each tube, bringing the total volume to 20 µl.

Table 6. The amplification of the cDNA of IL-10, p75, GLS, IL-6, TNF-α, NGF-β, and NTRK1 was carried out in a thermocycler.

Table 7. The TBE buffer was prepared in a manner to preclude DNA deprotonation and nucleic acids degradation. The slightly basic conditions of the Tris-HCl buffer keeps DNA deprotonated and soluble in water. EDTA is a chelator of divalent cations and functions to protect the nucleic acids against enzymatic degradation by inactivating degrading enzymes.

Real Time PCR of IL-10, p75, GLS, IL-6, TNF-α, NGF-β, and NTRK1 at 24 Hours

Quantitative real-time PCR was used to measure IL-10, p75, GLS, IL-6, TNF-α, NGF-β, and NTRK1 mRNA transcription. After completing the thermocycler program, messenger RNA (mRNA) levels were measured from the colon at 24 hours and melt curves and threshold cycle (Ct) data generated. The cytokine and neutrophin expression level of rat colon was normalized with that of rat β-actin mRNA. Primers used for RT-q PCR were as follows: 5'-CCGATAGTGATGACCTGACC-3' for rat β-actin (BA).

Results

Gross Morphology

At 24 hours after rectal administration of TNBS, the gross morphology of the colon showed considerable inflammation and swelling compared to colons from naïve rats (Figure 6). The colons from the DON+TNBS group had less swelling and inflammation compared to TNBS treated colons, but was more distended than the control colons (Figure 6). The DON treated colons remained relatively unchanged when compared to the control colons (Figure 6).

Figure 6: Gross morphology of experimental colons

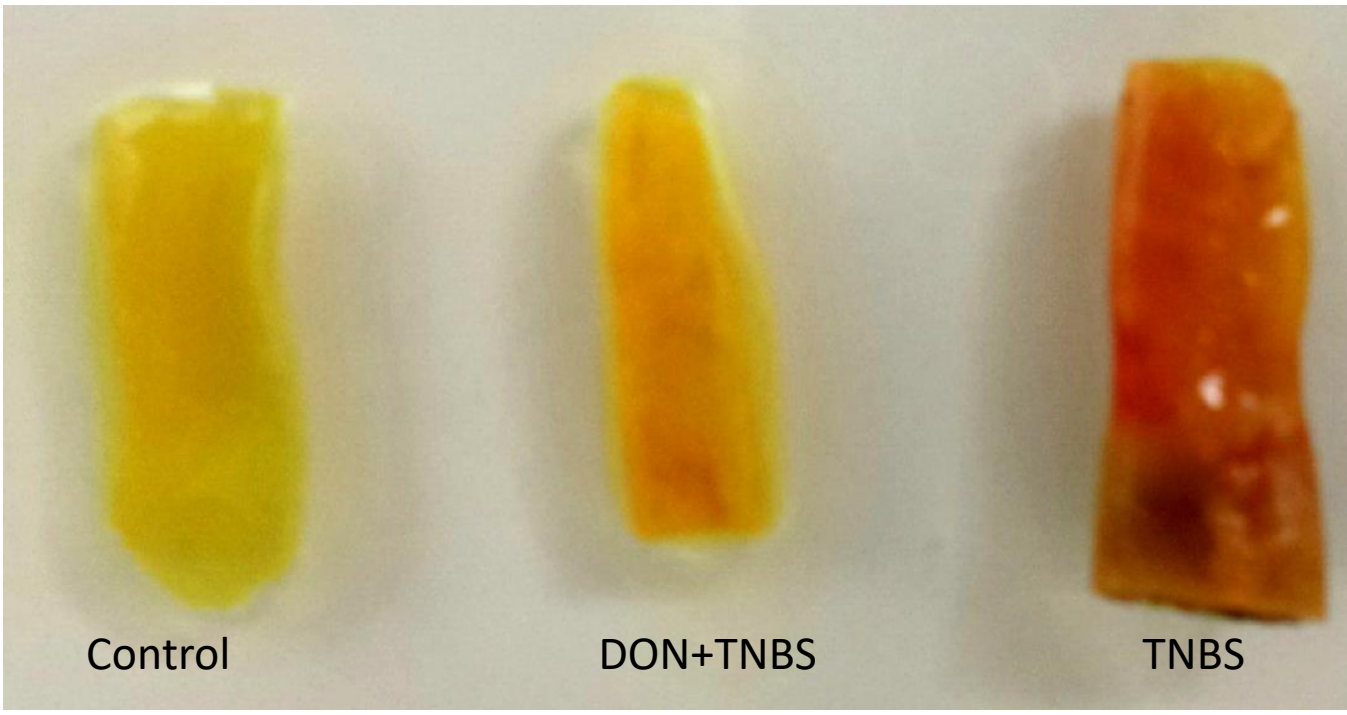

Figure 6. The colon on the left came from the control group. This group received no treatment of any kind. The tissue sample in the middle is from a DON+TNBS rat. There is some visible swelling and inflammation. The colon on the right is from the TNBS treated rat. There is obvious redness and swelling present in this section caused by administration of TNBS.

Wright's Stain

Under microscopic evaluation, there was no histological damage to the naïve control group (Figure 7A). In the TNBS group, the epithelium sustained severe damage and thinning (Figure 7C). There was edema and transmural inflammation present in all TNBS treated animals, with the presence of granulomas and submucosal granulocyte permeation (Figure 7C). Additionally, the lumen was noticeably larger than the lumen from the control group (Figure 7C). In the DON+TNBS group, the colon sections were edematous and had some submucosal infiltration by granulocytes, however not to the extent as the TNBS treated colons (Figure 7D). The DON-only colons appeared similar to control with a slight thickening of the submucosa (Figure 7B).

Immunohistochemistry

We stained for two groups: Peripherin + VGLUT2 and GLS + TRKA. In the Peripherin + VGLUT2 group, the nerve fibers were labeled with a green fluorophore for VGLUT2 and the peripherin was labeled with a red fluorophore (Figure 8). In the control, we saw intact crypts and nearby nerve fibers interacting with cells of the lamina propria. DON only sections were similar to the control. The TNBS group had an unstructured epithelial layer with degraded crypts. Nerve fibers in the lamina propria interacted with granulocytes and were in close proximity to the lumen. The DON + TNBS had light epithelial loss, but in most cases the damage was slight. Nerve fibers in lamina propria were comparable to controls. Similar results occurred in the GLS+TRKA group (data not shown).

Figure 7: Histological staining of cross sections of the colon

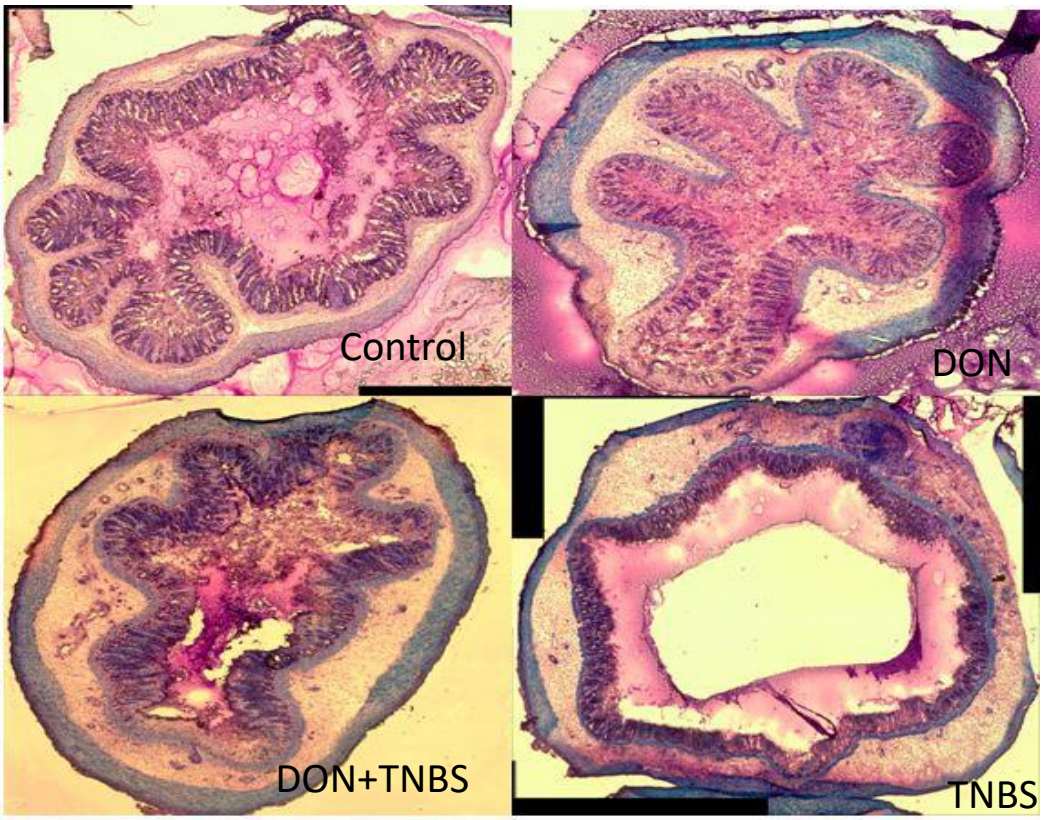

Figure 8: Immunohistochemical labeling of VGLUT2 and Peripherin

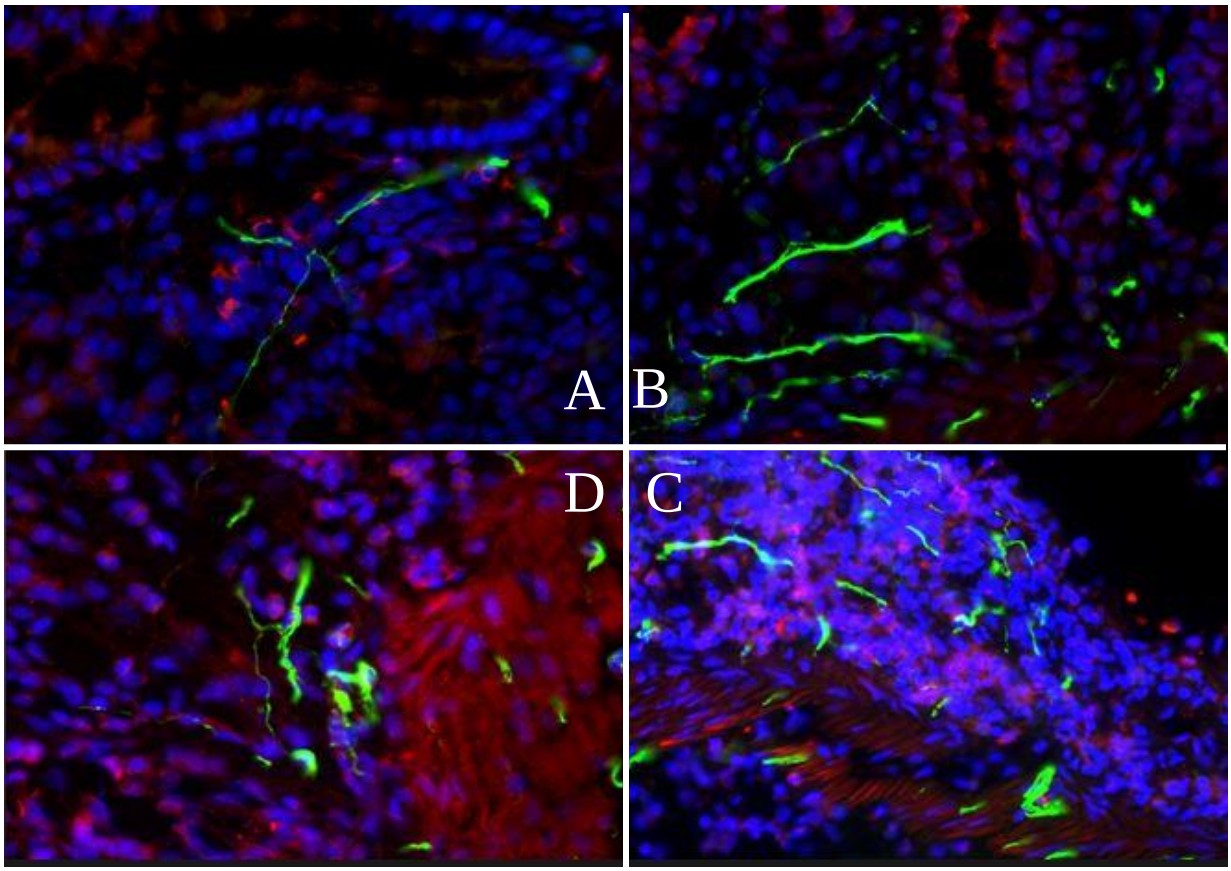

Figure 7. Groups are labeled as follows: Control (A), DON (B), TNBS, (C), DON+TNBS (D). There was no histological damage to the naïve group and little damage to the DON group. In the TNBS group, the epithelium sustained severe damage and the colon was edematous and inflamed with the presence of granulomas and submucosal granulocytic permeation. The lumen was larger than the control lumen. In the DON+TNBS group, the colon was edematous with some submucosal infiltration by granulocytes. The DON colon was similar in appearance to control, but the submucosa was slightly thicker.

Figure 8. Groups are labeled as follows: Control (A), DON (B), TNBS, (C), DON+TNBS (D) with Peripherin (red) + VGLUT2 (green). In controls and DON only colon sections, there were intact crypts and nerve fibers were in close contact with lamina propria cells. The TNBS colon had an unstructured epithelial layer and degraded crypts. The lamina propria had granulocyte infiltration and nerve fibers appear to interact with granulocytes. Nerve fibers in the DON + TNBS colons were comparable to controls.

PCR/RTPCR

β-actin was used as a loading control for RTPCR experiments and levels of β-actin remained constant in all experimental groups. In the TNBS group, there was a noticeable increase in IL-6, TNF-α, NGF-β, and NTRK1 mRNA as compared to the control. The DON+TNBS group had attenuated levels IL-6, TNF-α, NGF-β, and NTRK1 mRNA compared to the TNBS treated group. The DON group was similar to the naïve control group in mRNA levels for IL-6, TNF-α, NGF-β, and NTRK1. (Figures 9, 10, 11)

Discussion

There has been an increase in interest from researchers searching for various methods that may be used to treat IBD. The TNBS model provides a consistent colitis in rats and may be used to evaluate treatments for IBD in humans. In rats, TNBS is administered rectally and slowly disrupts the epithelium. This method has been commonly used to study the effects of IBD in an animal model (Morris et al., 1989) and TNBS administration results in symptoms that closely mimic characteristics of IBD in humans (Blumberg et al., 1999). Acute TNBS colitis is induced by T-helper 1 (Th1) cell response and propagated by release of Th1 and Th2 cytokine release (Dohi and Fujihashi, 2006).

Our study was designed to determine if GLS inhibition with 6-diazo-5-oxo-L-norleucine (DON) would have anti-inflammatory properties. When TNBS was administered rectally, there was considerable macroscopic inflammation and swelling in the colon, a change consistent with previous descriptions (Motavallian-Naeini et al., 2012). Microscopically, we saw increase in increase in edema and infiltration of submucosal granulocytes.

Figure 9: Results of Polymerase Chain Reaction

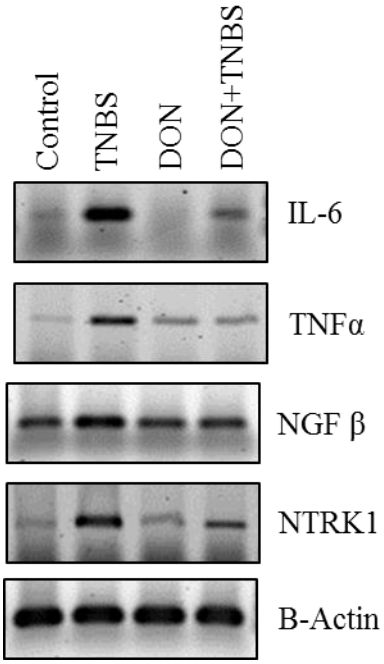

Figure 10: Relative expression of pro-inflammatory cytokines and neurotrophins-neurotrophic receptors

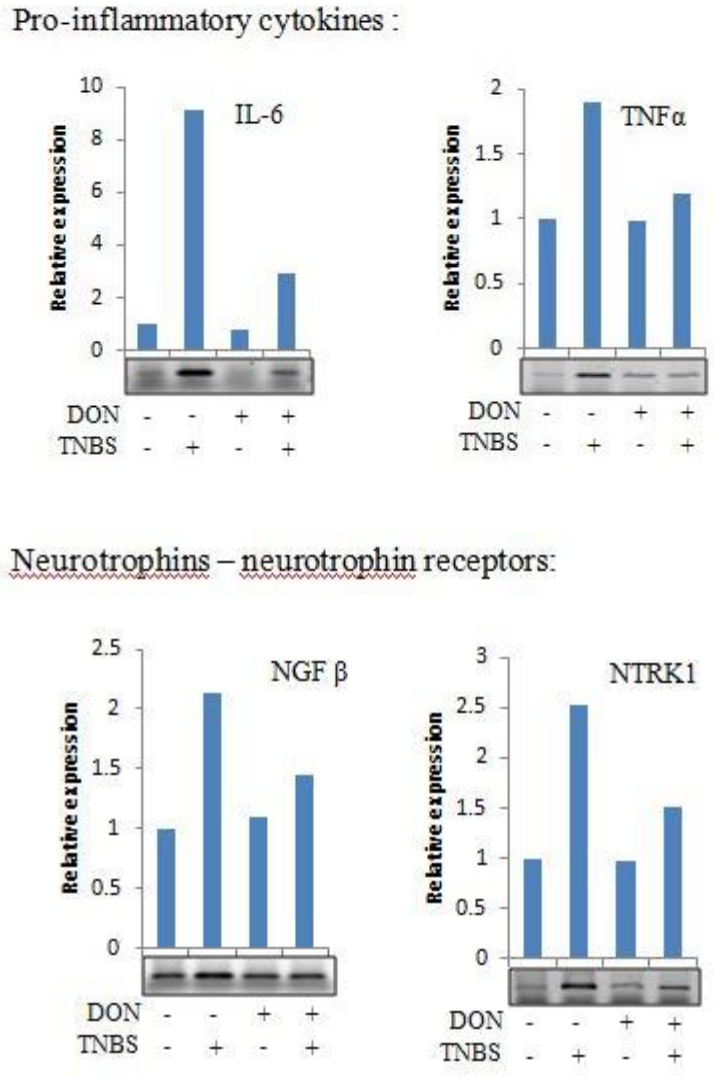

Figure 11: Results of Real-Time Reverse Transcriptase Polymerase Chain Reaction

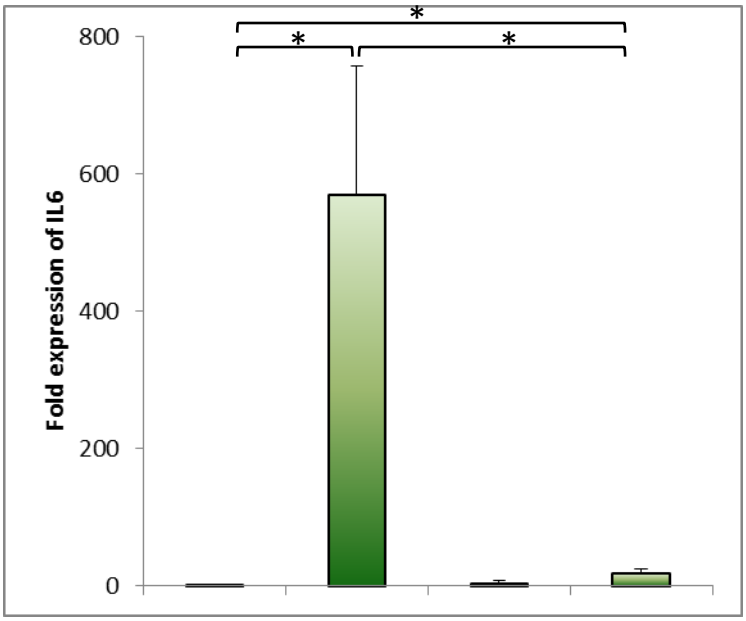

Figure 9. In the TNBS group, mRNA levels of IL-6, NTRK1, and TNF-α show a dramatic increase qualitatively. NGF-β mRNA levels increase slightly as compared to control levels, but the difference between groups is not as pronounced as with other mRNAs. β-actin mRNA was used as a loading control.

Figure 10. Quantitative evaluation of single RTPCR blots. All graphs illustrate an increase in mRNA for inflammatory cytokines, neurotrophins, or neurotrophic receptors in the TNBS group compared to controls. In the DON+TNBS group, there is a marked decrease in mRNA levels compared to TNBS alone. Relative expression of mRNAs for the control and DON groups are similar in all graphs. The IL-6 graph shows an almost 6-fold increase in the TNBS group compared DON+TNBS group and 8-fold increase compared to Control and DON groups. There is also a 3-fold increase in IL-6 mRNA in DON+TNBS group compared to and DON and Control. TNBS doubles mRNA for TNF-α, NGF-β, and NTRK1 compared to Control and DON groups and this alteration is attenuated in the DON+TNBS group.

Figure 11. Real-time RTPCR. A comparison of fold expression of IL-6 mRNA shows that there is a significant elevation in TNBS groups and DON+TNBS attenuates this increased expression.

An increase in edema and infiltration of submucosal granulocytes was also observed. Analyses of the pro-inflammatory proteins revealed greater levels of pro-inflammatory mediators and their receptors. The DON served as a protective agent and reduced the amount of swelling and redness. As was previously mentioned, DON is a glutaminase inhibitor that plays a role in decreasing inflammation by affecting the activity of peripheral neuronal fibers. The TNBS initiates neurogenic inflammation, which drives the development and persistence of colitis. DON acts upon the nerve fibers to reduce neuronal activity and thus, reduce inflammation. Similar studies have examined this phenomenon in a somatic model or with different pharmacotherapies (Hoffman and Miller, 2010; McCafferty et al., 1994), however, this work is novel in that RNA expression of inflammatory mediators in the inflamed colon at 24 hours has not previously been studied.

When TNBS is initially administered, the ethanol immediately begins to degrade the epithelium exposing the mucosa to luminal antigen. Although the etiology of induced IBD is not fully understood, there is work that suggests that the robust response is due to the mucosal immune system's failure to familiarize itself with luminal antigens (Brandtzaeg et al., 1997). An alternate hypothesis proposed that the pathology is due to T_h cells that fail to regulate cytokine levels resulting in an inflammatory response (Bouma and Strober, 2003). Regardless of the etiology, colonic infusion resulting in inflammation bears striking similarities to inflammatory bowel disease. The increase in the amount of macrophages and neutrophils present in the submucosa also seem to contribute to the symptoms seen in colitis. Macrophages produce cytokines such as IL-6 and TNF-α,

which may induce various factors that function to disrupt the extracellular matrix and vessel walls (MacDonald and Murch, 1994).

Colitis is the result of chronic inflammation in the colon and is accompanied by pain and discomfort. Pain and inflammation are often seen together after an injury is sustained, therefore, reducing inflammation will also reduce or relieve pain. Abdominal pain is a common cause for seeking medical attention; however, methods of treatment are mostly inefficient and usually are accompanied by many unpleasant side effects (Greenwood Van-Meerveld et al., 2015). In many cases, the pain goes beyond simple discomfort and enters the realm of crippling pain. This high incidence of visceral pain has resulted in an interest in methods of relieving pain and inflammation in IBD. A deeper understanding of the etiology of the disease has the potential to reveal better methods of prophylaxis and therapy. Additionally, determining what compounds can be utilized to provide anti-inflammation and pain relief will allow for more efficacious chemotherapy of patients who are afflicted with this disease.

In the future, we hope to better understand the course of IBD by studying it at various time points and by studying the effects of DON administration at different points. Another possible direction is the analysis of pain felt by the animal with and without the protective effects of DON and by analyzing this over a period of time. This study served as a stepping stone for future work that we expect will further explore inflammatory phenomena in this disease.

CHAPTER III

CONCLUSIONS

We hypothesized that administration of DON prior to colonic infusion of TNBS will reduce inflammation in the rat colon. Our results lead us to accept the hypothesis by virtue of the decrease in edema, pro-inflammatory cell infiltration, and epithelial damage seen in colons that had been pre-treated with DON. Aim 1 was to determine decreased redness and swelling in the colons of rats that received treatment with DON. This was seen macroscopically when analyzing gross morphology of the colons; there was a visible difference between the appearances of colons in different experimental groups. We also saw a decline in pro-inflammatory cytokine expression in rats who received DON. The RNA levels were significantly reduced in the DON + TNBS group when compared to the TNBS group. Finally, administration of DON resulted in less pro-inflammatory cell infiltration and a preservation of nerve fiber interaction with the lamina propria. Also, the structures of the crypts in the DON+TNBS group were preserved, unlike those of the TNBS group. In this experimental group, there was severe degradation of the structure of the epithelium and crypts.

In the future, we hope to better understand the course of IBD by studying it at various time points. This will serve to give us a more in-depth understanding of the inflammatory

process by allowing us to study cytokine and neurotrophin profiles to determine what compounds are present and active in the inflamed area. Repeating this experiment at longer intervals will also allow us to study the effects of DON administration at different points. It is possible that the effects of DON are different depending on what point the animal is in the development of colitis.

We did not study nociception in this project; however, it would be a valid addition to a future study. A possible future direction is the analysis of inflammation-induced nociception felt by the animal after induction of TNBS. It would be worthwhile to determine how pain levels change with and without the protective effects of DON administration. This could also be further expanded by studying effects of DON on nociception over a period of time and at various time points. Finally, a future study that involves examination of changes in protein levels and determination of the time difference between expression of protein and its corresponding RNA would yield a wealth of information about the time line of the development of colitis. This study served as a stepping stone for future work that we expect will further explore inflammatory phenomena in this disease.

REFERENCES

Alsalem, M., Wong, A., Millns, P., Arya, P. H., Chan, M. S. L., Bennett, A., . . . Kendall, D. A. (2013). The contribution of the endogenous TRPV1 ligands 9-HODE and 13-HODE to nociceptive processing and their role in peripheral inflammatory pain mechanisms. *British Journal of Pharmacology, 168*(8), 1961-1974. doi: 10.1111/bph.12092

Bautista, D. M., Pellegrino, M., & Tsunozaki, M. (2013). TRPA1: A gatekeeper for inflammation. *Annu Rev Physiol, 75*, 181-200. doi: 10.1146/annurev-physiol-030212-183811

Birklein, F., & Schmelz, M. (2008). Neuropeptides, neurogenic inflammation and complex regional pain syndrome (CRPS). *Neurosci Lett, 437*(3), 199-202. doi: 10.1016/j.neulet.2008.03.081

Blumberg, R. S., Saubermann, L. J., & Strober, W. (1999). Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. *Curr Opin Immunol, 11*(6), 648-656.

Bonica, J. J. (1979). The need of a taxonomy. *Pain, 6*(3), 247-248.

De Jongh, R. F., Vissers, K. C., Meert, T. F., Booij, L. H., De Deyne, C. S., & Heylen, R. J. (2003). The role of interleukin-6 in nociception and pain. *Anesth Analg, 96*(4), 1096-1103, table of contents.

Dohi, T., & Fujihashi, K. (2006). Type 1 and 2 T helper cell-mediated colitis. *Curr Opin Gastroenterol, 22*(6), 651-657. doi: 10.1097/01.mog.0000245545.80160.0f

Engel, M. A., Becker, C., Reeh, P. W., & Neurath, M. F. (2011). Role of sensory neurons in colitis: increasing evidence for a neuroimmune link in the gut. *Inflamm Bowel Dis, 17*(4), 1030-1033. doi: 10.1002/ibd.21422

Gold, M. S., Weinreich, D., Kim, C. S., Wang, R., Treanor, J., Porreca, F., & Lai, J. (2003). Redistribution of Na(V)1.8 in uninjured axons enables neuropathic pain. *J Neurosci, 23*(1), 158-166.

Greenwood-Van Meerveld, B., Prusator, D. K., & Johnson, A. C. (2015). Animal models of gastrointestinal and liver diseases. Animal models of visceral pain: pathophysiology, translational relevance, and challenges. *Am J Physiol Gastrointest Liver Physiol, 308*(11), G885-903. doi: 10.1152/ajpgi.00463.2014

Groneberg, D. A., Kindermann, B., Althammer, M., Klapper, M., Vormann, J., Littarru, G. P., & Doring, F. (2005). Coenzyme Q10 affects expression of genes involved in cell signalling, metabolism and transport in human CaCo-2 cells. *Int J Biochem Cell Biol, 37*(6), 1208-1218. doi: 10.1016/j.biocel.2004.11.017

Hoffman, E. M., & Miller, K. E. (2010). Peripheral inhibition of glutaminase reduces carrageenan-induced Fos expression in the superficial dorsal horn of the rat. *Neurosci Lett, 472*(3), 157-160. doi: 10.1016/j.neulet.2010.01.066

Hume, D. A. (2006). The mononuclear phagocyte system. *Curr Opin Immunol, 18*(1), 49-53. doi: 10.1016/j.coi.2005.11.008

Isik, F., Tunali Akbay, T., Yarat, A., Genc, Z., Pisiriciler, R., Caliskan-Ak, E., . . . Sener, G. (2011). Protective effects of black cumin (Nigella sativa) oil on TNBS-induced experimental colitis in rats. *Dig Dis Sci, 56*(3), 721-730. doi: 10.1007/s10620-010-1333-z

Lumpkin, E. A., & Caterina, M. J. (2007). Mechanisms of sensory transduction in the skin. *Nature, 445*(7130), 858-865. doi: 10.1038/nature05662

Mascaraque, C., Gonzalez, R., Suarez, M. D., Zarzuelo, A., Sanchez de Medina, F., & Martinez-Augustin, O. (2015). Intestinal anti-inflammatory activity of apigenin K in two rat colitis models induced by trinitrobenzenesulfonic acid and dextran sulphate sodium. *Br J Nutr, 113*(4), 618-626. doi: 10.1017/s0007114514004292

McCafferty, D. M., Sharkey, K. A., & Wallace, J. L. (1994). Beneficial effects of local or systemic lidocaine in experimental colitis. *Am J Physiol, 266*(4 Pt 1), G560-567.

Miller, K. E., Balbás, J. C., Benton, R. L., Lam, T. S., Edwards, K. M., Kriebel, R. M., & Schechter, R. (2012). Glutaminase Immunoreactivity and Enzyme Activity Is Increased in the Rat Dorsal Root Ganglion Following Peripheral Inflammation. *Pain Res Treat, 2012*.

Miller, K. E., Hoffman, E. M., Sutharshan, M., & Schechter, R. (2011). Glutamate pharmacology and metabolism in peripheral primary afferents: physiological and pathophysiological mechanisms. *Pharmacol Ther, 130*(3), 283-309. doi: 10.1016/j.pharmthera.2011.01.005

Miller, K.E., Method of alleviating chronic pain via peripheral glutaminase regulation, US Patent #7,288,246, 2007.

Miranda, A., Nordstrom, E., Mannem, A., Smith, C., Banerjee, B., & Sengupta, J. N. (2007). The role of transient receptor potential vanilloid 1 in mechanical and chemical visceral hyperalgesia following experimental colitis. *Neuroscience, 148*(4), 1021-1032. doi: 10.1016/j.neuroscience.2007.05.034

Morris, G. P., Beck, P. L., Herridge, M. S., Depew, W. T., Szewczuk, M. R., & Wallace, J. L. (1989). Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology, 96*(3), 795-803.

Motavallian-Naeini, A., Andalib, S., Rabbani, M., Mahzouni, P., Afsharipour, M., & Minaiyan, M. (2012). Validation and optimization of experimental colitis induction in rats using 2, 4, 6-trinitrobenzene sulfonic acid. *Research in pharmaceutical sciences, 7*(3), 159.

Randhawa, P. K., Singh, K., Singh, N., & Jaggi, A. S. (2014). A review on chemical-induced inflammatory bowel disease models in rodents. *Korean J Physiol Pharmacol, 18*(4), 279-288. doi: 10.4196/kjpp.2014.18.4.279

Rogler, G., & Andus, T. (1998). Cytokines in inflammatory bowel disease. *World J Surg, 22*(4),
382-389.

Romac, J M-J and Liddle, RA (2012). Transient Receptor Potential Receptor Vanilloid 1 (TRPV1).

Sherrington, C. S., & Laslett, E. E. (1903). Observations on some spinal reflexes and the interconnection of spinal segments. *The Journal of Physiology, 29*(1), 58-96. doi: 10.1113/jphysiol.1903.sp000946

Takami, Y., Mantyh, C. R., Pappas, T. N., Takahashi, T., Koda, K., & Miyazaki, M. (2009). Extrinsic surgical denervation ameliorates TNBS-induced colitis in rats. *Hepatogastroenterology, 56*(91-92), 682-686.

te Velde, A. A., Verstege, M. I., & Hommes, D. W. (2006). Critical appraisal of the current practice in murine TNBS-induced colitis. *Inflammatory Bowel Diseases, 12*(10), 995-999. doi: 10.1097/01.mib.0000227817.54969.5e

Varga, G., Erces, D., Fazekas, B., Fulop, M., Kovacs, T., Kaszaki, J., . . . Boros, M. (2010). N-Methyl-D-aspartate receptor antagonism decreases motility and inflammatory activation in the early phase of acute experimental colitis in the rat. *Neurogastroenterol Motil, 22*(2), 217-225, e268. doi: 10.1111/j.1365-2982.2009.01390.x

Woolf, C. J., & Ma, Q. (2007). Nociceptors--noxious stimulus detectors. *Neuron, 55*(3), 353-364. doi: 10.1016/j.neuron.2007.07.016

VITA

Rebekah Olaoluwa John

Candidate for the Degree of

Master of Science

Thesis: MITIGATION OF TNBS-INDUCED COLITIS WITH THE GLUTAMINASE INHIBITOR, 6-DIAZO-5-OXO-L-NORLEUCINE

Major Field: Biomedical Sciences

Biographical:

Education:

Completed the requirements for the Master of Science in your major at Oklahoma State University, Stillwater, Oklahoma in December, 2015.

Completed the requirements for the Bachelor of Science in Biology at the University of Oklahoma, Norman, Oklahoma in 2013

Experience: Studied in laboratory of Dr. Safiejko-Mrozcka, 2012-2013
Studied in laboratory of Dr. Miller, 2014-Present
Employed in animal facility at OSU Center for Health Sciences, 2013-2014

Professional Memberships: Member of Student National Medical Association, American College of Physicians, Oklahoma Osteopathic Association