Comparative Medicine
Copyright 2020
by the American Association for Laboratory Animal Science

Vol 70, No 1
February 2020
Pages 25–34

Original Research

Role of the δ-Opioid Receptor in 2 Murine Models of Colitis

Tia R Bobo,[1,†*] Leo R Fitzpatrick,[2,‡] Tiffany L Whitcomb,[1] Timothy K Cooper,[1,3,§] Sorana Raiciulescu,[4] and Jill P Smith[5§§]

Crohn disease and ulcerative colitis, collectively referred to as inflammatory bowel disease (IBD), are chronic inflammatory disorders of the gastrointestinal tract. Currently, the etiology of IBD is unknown, and immunosuppressive therapies have become the standard of care to reduce the inflammation; however, these agents only induce remission 50% of the time in patients and can have serious side effects. Recently, endogenous opioids and opioid receptors have been shown to play a role in the mediation of inflammation. In addition, opioid receptor blockade with a nonselective antagonist, naltrexone, has been shown to reduce colitis in both murine models and human subjects. The goal of the current study was to determine if the antiinflammatory effects of naltrexone are mediated through the delta (δ) opioid receptor. Male C57BL/6NCrl (6 to 8 wk.; *n* = 110) and female BALB/cAnNCrl (6–8 wk.; *n* = 91) mice were studied using 2 animal models of chemically induced colitis: dextran sodium sulfate (DSS) and 2, 4, 6-trinitrobenzenesulfonic acid (TNBS). The selective δ-receptor antagonists naltrindole and 7-benzylidenenaltrexone were administered to examine the role of the δ-opioid receptor in colonic inflammation. The quantitative measurement of colitis activity, colon weight and length, Hct, WBC count, and gross and microscopic aberrations were analyzed. Administration of naltrexone in the DSS colitis model significantly improved overall disease activity indices on day 5 of therapy. The use of δ-antagonists and naltrexone had limited to no effect on TNBS colitis. Similar findings were obtained by using the DSS colitis model. Based on the current findings, the authors conclude that naltrexone therapy has limited effect on the improvement of colitis in 2 murine models; however, the δ-opioid receptor was not responsible for mediating the effects.

Abbreviations: CD, Crohn disease; DAI, disease activity index; DSS, dextran sodium sulfate; HDN, high-dose naltrexone; LDN, low-dose naltrexone; TNBS, 2, 4, 6-trinitrobenzenesulfonic acid

DOI: 10.30802/AALAS-CM-19-000024

Inflammatory bowel disease (IBD) has become a global disease due to the steady increase of developing countries whose societies mimic westernized living. In the West alone, more than 1 million residents in the United States and 2.5 million in Europe are estimated to have IBD, and associated substantial costs for health care.[13] In addition, the CDC reported that an estimated 1.3% of United States adults (3 million) were diagnosed with IBD in 2015 as compared with 0.9% (2 million) in 1999.

IBD is a chronic intestinal inflammatory disorder that encompasses Crohn disease (CD) and ulcerative colitis and can lead to irreversible impairment of gastrointestinal function and structure.[4] Although the etiology of IBD is unknown, research suggests that environmental, genetic, immune, nonimmune, and microbial factors play a role in disease onset.[2,15] Although there are many similarities between the aforementioned diseases, there are also marked differences in the immunologic, pathologic, and clinical features. Lesions in CD patients can occur throughout the entire gastrointestinal tract but are most commonly located in the terminal ileum, cecum, perineum, and colon, with transmural granulomatous inflammation, fissuring ulceration, and fibrosis.[4] Patients can experience a wide range of disease presentations from diarrhea, abdominal pain, bowel obstruction, and abscess development to fistulous tracts. Conversely, ulcerative colitis tends to affect the superficial mucosal layers with infiltration of mononuclear and polymorphonuclear leukocytes and lesions are restricted to the colon. In addition, ulcerations and crypt abscesses are present microscopically. Patients with chronic ulcerative colitis or CD have an increased chance of developing colorectal cancer, and people with small intestinal CD are at increased risk of small bowel adenocarcinoma and lymphoma.[33]

Animal models of IBD are well characterized and include chemical induction, adoptive transfer, and spontaneous colitis and the use of genetically engineered or transgenic animals.[26] In fact, 66 different IBD animal models have been described in the literature.[18] Chemically induced colitis models are typically used to replicate histopathologic and morphologic changes in the intestine. In addition, these models are economical, accessible, and time-saving.[18] Examples of chemical induction models involve 2, 4, 6-trinitrobenzenesulfonic acid (TNBS),[20] dextran sodium sulfate (DSS),[22] oxazolone,[37] acetic acid,[26] NSAID,[3] carrageenan,[26] and peptidoglycan-polysaccharide.[26] In the current study, we used TNBS, a haptenating agent that elicits a

Received: 21 Feb 2019. Revision requested: 28 Mar 2019. Accepted: 26 Jul 2019.
Departments of [1]Comparative Medicine, [2]Pharmacology, [3]Pathology, and [5]Medicine, Penn State College of Medicine, Hershey, Pennsylvania; Department of [4]Preventive Medicine and Biostatistics, Uniformed Services University, Bethesda, Maryland
Current affiliation: †Office of Animal Research, The George Washington University, Washington DC; ‡Department of Pharmaceutical & Biomedical Sciences, California Northstate University, Elk Grove, California; §Charles River Laboratories, Contractor Supporting National Institute of Allergy and Infectious Disease, Frederick, Maryland; §§Department of Medicine, Georgetown University, Washington, DC
**Corresponding author. Email: bobotia@yahoo.com*

cell-mediated immune response (thereby closely mimicking CD). This model has been proven effective in inducing colitis in mice, rats, rabbits, and swine. In mice, the efficacy of TNBS is highly dependent on the strain. For example, SJL/J, C3H/HeJ, and BALB/c are susceptible, whereas C57BL/6 and DBA2/J are highly resistant.[18,28] We also used DSS, a polyanionic salt that causes hyperosmotic damage to the epithelial cells and thereby morphologically and symptomatically resembles ulcerative colitis. Immunodeficient mice lacking T and B cells, C3H/HeJ strains (as compared with other strains such as C57BL/6), and male mice have been noted to have increased susceptibility to colitis induction.[18] In the current study, we chose to use female BALB/c mice for the TNBS model because of their increased susceptibility and less aggressive nature. We used male C57BL/6N mice for the DSS model in light of their susceptibility, cost, and vendor availability.

Current therapies for IBD include compounds that reduce the inflammatory response such as 5-aminosalicylate, corticosteroids, immunomodulators (for example, azathioprine, methotrexate, cyclosporine), and biologics (for example, antiTNFα agents, vedolizumab, ustekinumab).[1,2] Although new biologic drugs may induce remission in as many as 50% of patients, concerns exist that long-term immunosuppression may increase the risk of infections and malignancy. Furthermore, despite the efficacy of these biologic regimens, half of patients with IBD do not achieve endoscopic remission; therefore, there still remains a need for safe, well tolerated therapeutics with rapid onset and ability to induce remission. One pathway that has been shown to modulate gastrointestinal immune responses includes endogenous opioids (enkephalins, endorphins, and dynorphins) and opioid receptors.[16,30,39] Three classic and distinct opioid receptors (δ, κ, and μ) have been characterized, which exhibit variable densities in different tissues throughout the body and are distinguished by selectivity and affinity for specific opioid drugs.[14,17] A nonclassic opioid receptor, opioid growth factor receptor, is nuclear bound.[6]

Naltrexone, a nonselective opioid receptor antagonist, is typically used in the treatment of opioid and alcohol addiction.[5,32] Naltrexone has the highest affinity for μ-opioid receptors[32] and to a lesser extent, δ-opioid receptors.[5] When administered as a low-dose formulation in clinical use, naltrexone has shown benefits for chronic inflammatory conditions including multiple sclerosis, fibromyalgia, cancer, complex regional pain syndrome, and Hailey–Hailey disease.[32] In addition naltrexone has been shown to significantly decrease inflammatory scores in the DSS animal model of colitis[16] and in humans with CD.[30] Furthermore, naltrexone therapy lowered plasma inflammation markers and promoted mucosal healing in adult patients with active CD.[29]

Given that δ-opioid receptors are the predominant receptor subtype associated with inflammatory cells,[34] we hypothesized in the current study that the mechanism by which naltrexone reduced colonic inflammation in IBD is mediated through blockade at the δ-receptor. To test this hypothesis, 2 δ-receptor selective antagonists—naltrindole, a δ-1 receptor antagonism and 7-benzylidenenaltrexone, a δ2 receptor antagonist, were administered to animals with chemically induced IBD. The primary outcome of this investigation was to assess improvement of intestinal inflammation by histology. Secondary outcomes included reduction in intestinal inflammation as assessed by myeloperoxidase activity in colonic tissue, disease activity index (DAI) scores (DSS model only), hematologic analyses, and gross examination of the large intestine. The results from this study should help elucidate whether naltrexone reverses intestinal inflammation through the δ-opioid receptor or by another mechanism, as well as determining drugs that selectively interact with the δ-opioid receptor for the treatment of both forms of IBD. Furthermore, these studies examined the role of opioid agents in a TNBS-induced murine model of colitis.

Materials and Methods

Animals. Male C57BL/6NCrl (15 to 20 g; age 6 to 8 wk.; *n* = 110) and female BALB/cAnNCrl (15 to 20 g; 6 to 8 wk.; *n* = 91) mice were obtained from Charles River Laboratories (Wilmington, MA). The male mice were individually housed in polycarbonate cages on a ventilated rack with corncob bedding (7092, Harlan Teklad, Madison, WI), without restriction access to irradiated rodent chow (2918, Harlan Teklad), and municipal water. Female mice were socially housed (5 mice/cage) under static conditions with microfilter lids. The facility was maintained at a room temperature of 20 ± 2 °C, relative humidity 50% ± 20% and a 12:12-h light: dark cycle. Environmental enrichment was provided in the form of nesting material or colored plastic tubes. Mice were given a 1-wk acclimation period prior to experimental manipulations. According to vendor health reports, mice were free of mouse hepatitis virus, mouse minute virus, mouse parvovirus, mouse rotavirus, Theiler murine encephalomyelitis virus, pneumonia virus of mice, Sendai virus, lymphocytic choriomeningitis virus, murine norovirus, ectromelia virus, Hantaan virus, mouse adenovirus types 1 and 2, mouse cytomegalovirus, respiratory enteric virus 3, K virus, lactate dehydrogenase elevating virus, polyoma virus, thymic virus, β-hemolytic *Streptococcus* spp., *Bordetella bronchiseptica*, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium kutscheri*, *Klebsiella oxytoca*, *Klebsiella pneumonia*, *Mycoplasma* spp., *Pasteurella pneumotropica*, other *Pasteurella* spp., *Pseudomonas aeruginosa*, *Salmonella* spp., *Staphylococcus aureus*, *Streptococcus pneumonia*, cilia-associated respiratory bacillus, *Helicobacter hepaticus*, *H. bilis*, *Pneumocystis murina*, endo-and ectoparasites, enteric protozoa, and *Encephalitozoon cuniculi*. All experiments were conducted in accordance with institutional guidelines, the *Guide for the Care and Use of Laboratory Animals*[10] and approved by Penn State College of Medicine IACUC.

Chemicals. DSS (molecular weight, 36,000 to 50,000) was obtained from MP Biomedicals (Solon, OH). TNBS was obtained from Sigma-Aldrich (St Louis, MO). Key reagents for the myeloperoxidase assay (3, 3′, 5, 5′ tetramethylbenzidine, hexadecyltrimethylammonium bromide; N, N dimethylformamide, and hydrogen peroxide) were obtained from Sigma-Aldrich. Naltrexone, naltrindole, and 7-benzylidenenaltrexone were purchased from Sigma-Aldrich (St Louis, MO). Lyophilized drugs were dissolved in filtered tap water and sterilized using a 0.2 μm PES Bottle Top Filtration system from VWR (Radnor, PA). Sterile stock solutions were reconstituted to the appropriate concentrations in filtered tap water. TNBS was dissolved in ethanol (50% w/vol).

DSS-induced murine model of colitis. Male C57BL/6NCrl mice were randomized to receive either filtered drinking water (*n* = 30) or 2% DSS drinking water (*n* = 30) without restriction for 7 d (Figure 1). Mice were individually housed to measure daily water consumption and ensure adequate consumption of DSS. The acute model of DSS induced colitis was modified from a previous study.[22] Mice were divided into treatment and control groups respectively: low dose (0.1 mg/kg) naltrexone (LDN), high dose (1.0 mg/kg) naltrexone (HDN), and sterile saline with dose volumes of 5 mL/kg subcutaneously. Dosing and route was determined based on a previous published study.[16] A second cohort of mice was given filtered drinking water

Treatment group	No colitis control (untreated water)	Colitis control (water treated with 2% DSS)	Colitis (water treated with 2% DSS)
Saline	20	20	not applicable
Naltrexone (0.1 mg/kg)	10	not applicable	20
Naltrexone (1.0 mg/kg)	10	not applicable	10
7-Benzylidenenaltrexone (0.1 mg/kg)	not applicable	not applicable	10
Naltrindole (0.1 mg/kg)	not applicable	not applicable	10

Figure 1. Study design for the DSS-induced murine model of colitis.

Score	Weight loss (%)	Stool consistency	Occult blood or gross bleeding
0	None	Normal	Negative
1	1–5	Loose stool	Negative
2	5–10	Loose stool	Hemoccult test positive
3	10–15	Diarrhea	Hemoccult test positive
4	>15	Diarrhea	Gross bleeding

Figure 2. Criteria for scoring disease activity index, as adopted from reference 21. The disease activity index (plotted in Figure 8) was calculated by combining scores for weight loss (from baseline), stool consistency, and occult or gross bleeding.

	No. of mice		
Treatment group	No colitis control (PBS enema)	Vehicle control (EtOH enema)	Colitis control (TNBS enema)
Saline	10	10	16
Naltrexone (0.1 mg/kg)	10	not applicable	15
Naltrexone (1.0 mg/kg)	10	not applicable	10
7-Benzylidenenaltrexone (0.1 mg/kg)	not applicable	not applicable	5
Naltrindole (0.1 mg/kg)	not applicable	not applicable	5

Figure 3. Study design for the TNBS-induced murine model of colitis.

(n = 10) or 2% DSS drinking water (n = 40) without restriction for 7 d to assess the response to δ-receptor antagonist administration. Mice were divided into 4 treatment groups with dose volumes of 5 mL/kg SC: LDN, naltrindole (0.1 mg/kg), 7-benzylidenenaltrexone (0.1 mg/kg), and sterile saline. Dosing for δ antagonists were extrapolated from a prior published study.[16] Injections began on the first day of DSS consumption and were administered once daily until study endpoint. A DAI score was calculated daily for each mouse according to the criteria outlined in reference [21] by using percentage of weight change, presence of fecal occult blood, and stool consistency (Figure 2). The final score was the sum of the assessed parameters divided by 3. A fecal blood kit (Hemoccult, Beckman-Coulter, Fullerton, CA) was used to determine presence of occult blood. On day 7, mice were euthanized by using CO_2 inhalation followed by terminal blood collection through cardiocentesis. Samples were stored in K_2EDTA-treated microtainer tubes (Becton Dickinson, Franklin Lakes, NJ) and submitted for CBC analyses (CBC-Diff Veterinary Hematology System, Heska, Loveland, CO).

TNBS-induced murine model of colitis. Female BALB/cAnNCrl mice were randomized by weight into groups receiving a one-time intracolonic enema of TNBS (n = 30), PBS (n = 25), or 50% ethanol (n = 5). A modified version of the acute TNBS model was used.[8,28] In brief, mice were fasted overnight; the next day, they received a single 40-µL dose per rectum of TNBS (20 mg/kg in 50% ethanol/PBS) under isoflurane anesthesia (3% to 4%) for 5 min. The enema was administered 4 cm into the colon by-using a 100 µL Hamilton syringe (Fisher Scientific, Waltham, MA) attached to a 20-gauge, 1.5-in. gavage needle (Fisher). Mice were allowed access to food and water without restriction on recovery from anesthesia. Mice were divided into control and treatment groups and received injections of LDN, HDN, or saline (5 mL/kg SC) at1 h prior to enema administration and once daily thereafter. A second cohort of mice each received an enema of TNBS (n = 21), PBS (n = 5), or 50% ethanol (n = 5). Mice were given either LDN, naltrindole (0.1 mg/kg SC), 7-benzylidenenaltrexone (0.1 mg/kg SC), or sterile saline once daily until study endpoint (Figure 3). Mice were euthanized through CO_2 inhalation on day 6 or 7. Blood collection after euthanasia and CBC analyses were performed.

Macroscopic evaluation of colon. At study endpoint, mice were euthanized, the colon was transected from the rectum, and tissues were rinsed in 1× PBS. Proximal and distal sections were evaluated subjectively for macroscopic changes by using a handheld magnifier in the TNBS-induced colitis model only.[27] Because DSS-induced colitis follows a different pathophysiologic sequence than TNBS-induced colitis, macroscopic scoring was not warranted for DSS-induced colitis. The scoring system is shown in Figure 4 and evaluated multiple features, including ulceration, adhesion, evidence of diarrhea, and colonic thickness. The total score was calculated by adding scores for ulceration, adhesion, and diarrhea and then multiplying by thickness.[27] This scoring system has been validated in rat and mouse models of colitis.[8,27]

Histopathologic evaluation of colon. At necropsy, the entire colon was excised from the cecocolic junction to the anus and the colon length was measured as in indirect marker of inflammation. Only 5 cm of measured colon was weighed, and a 2.5-cm longitudinal section was Swiss-rolled according the technique in reference 19. The remaining 2.5-cm section was placed in liquid nitrogen and stored in a –80 °C freezer for myeloperoxidase assay. Tissue specimens were fixed in 10% neutral buffered formalin, paraffin-embedded, and sectioned for staining with hematoxylin and eosin. Colon sections were evaluated microscopically and scored blindly by a board-certified veterinary pathologist. The overall index was calculated according to an established scoring system (Figure 5).[12] The 4 individual inflammatory parameters assessed were severity of inflammation, ulceration, percentage of area involved, and hyperplasia and dysplasia.

	Score
Ulceration	
Normal appearance	0
Focal hyperemia, no ulcers	1
Ulceration without hyperemia or bowel wall thickening	2
Ulceration with inflammation at one site	3
Ulceration or inflammation at 2 or more sites	4
Sites of major damage, extending >1 cm along length of colon	5
Score increased by 1 for each additional centimeter of involved colon beyond 2 cm	6–10
Adhesion	
None	0
Minor (colon can be separated from other tissue with little effort)	1
Major	2
Diarrhea	
No	0
Yes	1
Thickness	not applicable
Maximal bowel wall thickness (in mm)	

Figure 4. Macroscopic colonic evaluation of TNBS-induced murine colitis by using scores adopted from reference 28. The total score (plotted in Figure 7) was calculated by adding scores for ulceration, adhesion, and diarrhea and then multiplying by thickness.

	Score
Severity of inflammation	
Normal mucosa	0
Mild (either focal or widely separated multifocal inflammation limited to basal 1/3 of mucosa with loss of crypts)	1
Moderate (either multifocal or locally extensive, with or without fibrosis in a maximum of 2/3 of crypts)	2
Severe (mucosal ulcers with monocytes and polymorphonuclear leukocytes infiltrated into the mucosa, submucosa, muscularis propria, or subserosa or multiple affected layers)	3
Ulceration	
Absent	0
Present	1
Area of Inflammation	
0%	0
1% to 25%	1
26% to 50%	2
51% to 75%	3
76% to 100%	4
Hyperplasia or dysplasia	
Normal	0
Mild (epithelial cells lined normally, but crypts 2 to 4 times thicker than normal crypts)	1
Low-grade (epithelial thickness increased 2 to 4 times normal, hyperchromatic cells, few goblet cells, and scattered crypts developing an arborizing pattern)	2
High-grade (epithelial thickness increased >4 times normal, hyperchromasia, few to no goblet cells, high mitotic index in crypts with arborizing pattern, and extension of crypts to muscularis mucosa or submucosa)	3

Figure 5. Microscopic evaluation of DSS- and TNBS-induced colitis according to scores adopted from reference 12. The inflammation index was the sum of 4 individual inflammatory parameters: inflammation severity, ulceration, inflammation area involved, and hyperplasia and dysplasia. Lesions associated with each score are shown in Figures 6 and 7.

Myeloperoxidase assay. To assess granulocytic infiltration and to quantify myeloperoxidase activity, a 2.5-cm segment of colon was processed as previously described.[9] Briefly, tissues were homogenized in hexadecyltrimethylammonium bromide buffer, and the homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C. The pellet was retained for myeloperoxidase measurement using the tetramethylbenzidine substrate method. Absorbance was measured at 655 nm on a spectrophotometer. All measurements were performed in triplicate. One unit of myeloperoxidase activity was defined as the amount that caused a 1.0-unit change in absorbance per minute at 37 °C. Myeloperoxidase activity was expressed as units per gram of tissue.

Statistical analysis. Statistical analyses of colon length and weight, myeloperoxidase activity, Hct, WBC, macroscopic damage, histology score, ulcer score, and DAI were analyzed by using the Kruskall–Wallis test, with Dunn correction for multiple comparisons (Prism 7, GraphPad Software, La Jolla, CA). A P value of less than 0.05 was considered to be statistically significant. Data are reported as medians with lower and upper quintiles.

Results

Blood test results, colonic measurements, and assessments for inflammation. In the DSS colitis model, the CBC values, specifically WBC and Hct, varied across all groups and thus was potentially indicative of anemia, dehydration, or hemoconcentration (Figure 6). The Hct values between the no-colitis and DSS colitis control groups differed significantly ($P < 0.001$).

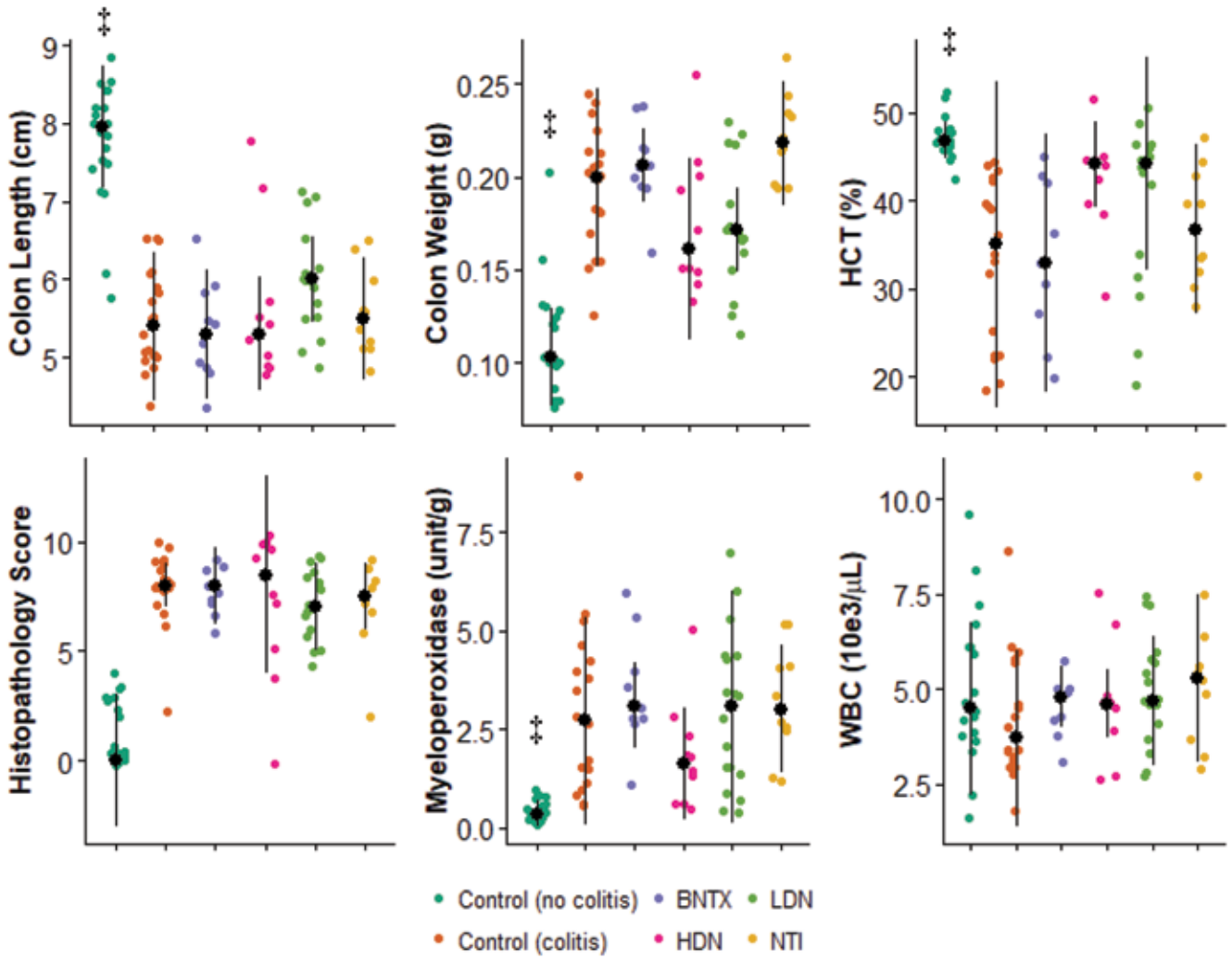

Figure 6. Measured parameters of colitis induction by model and treatment group. Data are presented as median ± IQR. Significant (‡, $P < 0.001$) difference between values for no colitis compared with DSS colitis controls. There are no significant differences between colitis control groups and opioid-treated groups. The reference range for Hct is 42% to 44% and for WBC is 5.1 to 11.3 × 10^3/μL. Except for CBC analyses, group size was: controls, n = 20; low-dose naltrexone (LDN), n = 17; and high-dose naltrexone (HDN), naltrindole (NTI), and 7-benzylidenenaltrexone (BNTX), n = 10 each; group size for CBC analyses was: controls, n = 18; LDN, n = 17; HDN, n = 9; NTI, n = 9; and BNTX, n = 10.

Colonic length was decreased in the DSS colitis control group compared with the no-colitis control group ($P < 0.001$). The treated mice had similar colon lengths to those of the DSS colitis controls, but lengths were subjectively shorter than those of the no-colitis controls. In contrast, colon weight in the DSS colitis group was twice that of the no-colitis control groups ($P < 0.001$). Colon weight was not significantly reduced in the low- and high-dose naltrexone groups. According to the myeloperoxidase assay, marked elevations of neutrophilic inflammation within colonic tissue segments were expressed despite opioid treatment with naltrexone, 7-benzylidenenaltrexone, or naltrindole. Significance ($P < 0.001$) was reached between the no-colitis and DSS colitis control groups, indicative of successful induction of colitis.

Results for the TNBS colitis model are presented in Figure 7. Due to small sample size (as a result from poor sample quality), HDN, naltrindole, and 7-benzylidenenaltrexone groups were excluded from the CBC analysis. Regardless, the difference in Hct between the TNBS (no colitis) control compared with the LDN group reached significance ($P = 0.01$), although all groups had evidence of hemoconcentration. Along the same lines, colon weights did not differ among the opioid receptor antagonist-treated and untreated mice. Treatment with drug had no effect on colon length as compared with no colitis and colitis control or vehicle groups. In the myeloperoxidase assay, neutrophilic inflammation levels were nonsignificantly induced, as evidenced in the controls, vehicle, and opioid antagonist groups. Macroscopic damage was significant ($P = 0.03$) between the no colitis and colitis control groups. There was no clear pattern among the groups that was indicative of opioid receptor drug reduced colonic damage. Moreover, an increase in macroscopic damage did not correlate with an increase in ulcer score (one component of the scoring method), which was atypical for this type of colitis model. For example, HDN and naltrindole groups did not have gross ulcers present despite an increase in macroscopic scoring.

DAI in mice with DSS-induced colitis. At day 3, mice in each respective DSS-treated group began to show symptoms of colitis as evidenced by change in weight, fecal output and consistency. Regardless of opioid treatment type, DAI scores continued in an upward trend until study endpoint (Figure 8). An increase in scores correlated with an advanced stage of colitis, such as hunched posture, weight loss, dehydration and occult to gross blood in feces. At day 4, DSS colitis control compared with no

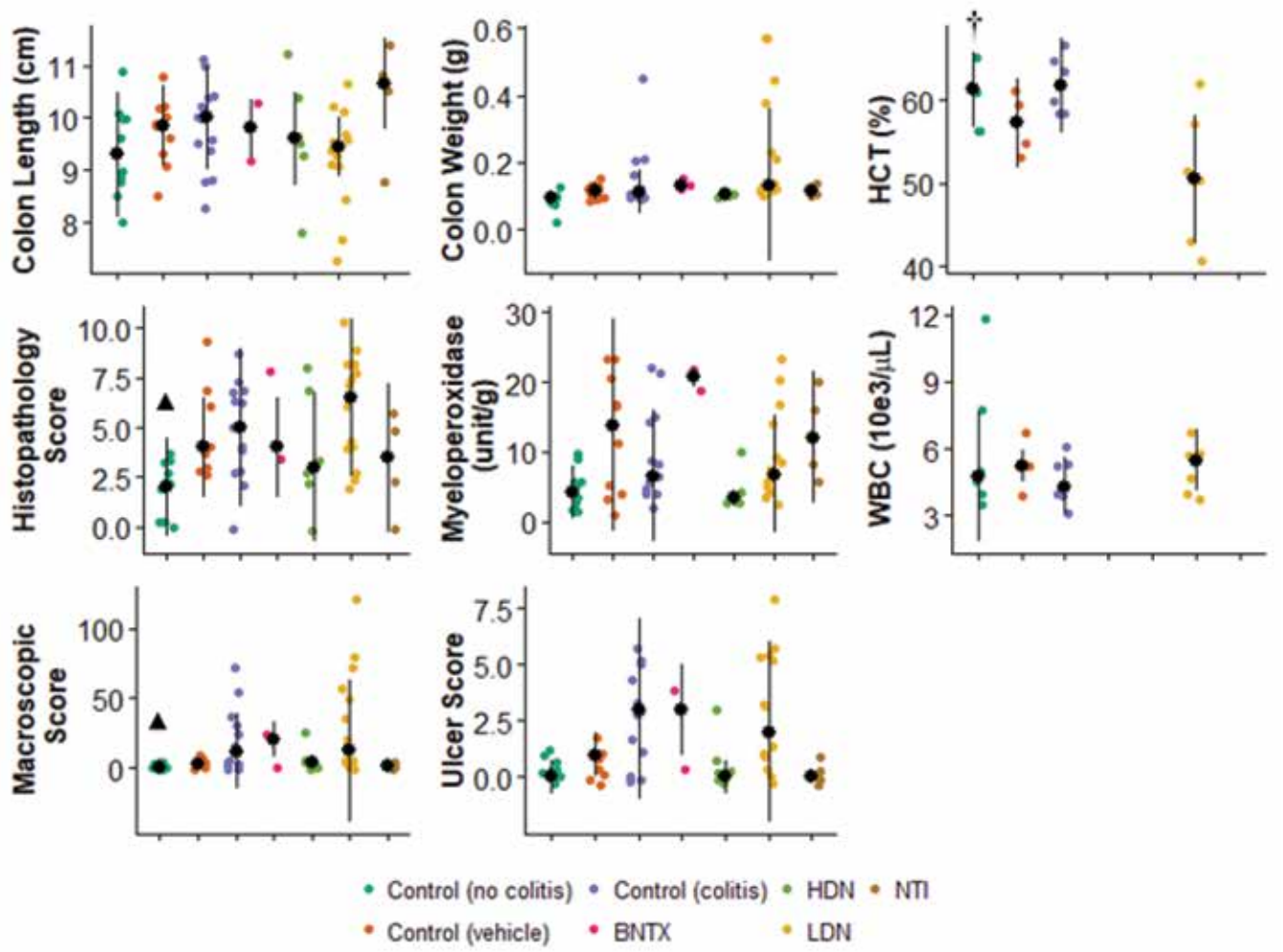

Figure 7. Measured parameters of colitis induction according to model and treatment group. Data are presented as median ± interquartile range. †, $P = 0.01$, control (no colitis) compared with HDN; ▲, $P = 0.03$, control (no colitis) compared with control (colitis). There are no significant differences between colitis control groups, vehicle, and opioid treated groups. Reference range for HCT and WBC are 42% to 44% and 5.1-11.26 $\times 10^3$/μL, respectively. Except for CBC analyses, group size was: no colitis control, $n = 10$; colitis control, $n = 13$; vehicle, $n = 10$; LDN, $n = 14$; HDN, $n = 6$; naltrindole, $n = 4$; 7-benzylidenenaltrexone, $n = 3$. Group size for CBC analyses was: control (no colitis) $n = 6$; control (vehicle), $n = 4$; control (colitis), $n = 7$; and LDN, $n = 7$.

colitis control groups differed ($P = 0.004$), whereas treatment with the opioid antagonist had no effect. DAI scores continued to increase by day 5 in the DSS colitis control group compared with that of no-colitis group ($P = 0.0001$). In addition, treatment with HDN significantly ($P = 0.04$) reduced DAI scores compared with colitis controls. In addition, day 6 showed marked ($P = 0.0001$) differences between the colitis and no-colitis control groups. On days 5 and 6, treatment with the nonselective and selective δ-antagonists did not significantly reduce overall scores as compared with the colitis control.

Histologic analysis of inflammation. Histologic sections from representative DSS-treated and no-colitis control mice are shown in Figure 9. Microscopically, the colon in no-colitis control mice had a normal appearance. In contrast, the DSS-mice that received saline or opioid treatment showed evidence of marked colitis, with loss of crypts, infiltration of inflammatory cells, and thickening of the muscularis mucosa, submucosal edema and ulcerations. There was a stark contrast in histology scores (Figure 6) between treated and untreated mice; however, all opioid treatment groups showed limited to no drug effect. Quantitatively, control mice (no colitis) had a score of 0 (indicating no disease), whereas the colitis control group showed severe, active disease (as confirmed by histopathology). When comparing opioid treated and colitis control mice, there was limited to no drug effect and histologic analysis showed similar pathologic changes between all groups. Results are not reported for negative-control groups, because no difference was noted in the between-group analyses.

Microscopic changes in the TNBS colitis model are shown in Figure 9. Sections whereby proximal colon was assessed were largely unaffected in this model, as expected. Microscopically, the colon had a normal appearance in the control (no colitis) mice. Multifocal areas of mononuclear and polymorphonuclear inflammation expanding the lamina propria were present in the vehicle (image not shown) and control groups. Opioid antagonist groups had mild to moderate mononuclear and polymorphonuclear inflammatory infiltrates within the lamina propria and submucosal layer, focal to focally extensive mucosal ulcerations, and on rare occasion, submucosal edema. Histopathologic scores are reported in Figure 7, and TNBS-induced colitis control mice significantly ($P = 0.03$) differed from the no-colitis control group. Scores in the HDN, naltrindole, and 7-benzylidenenaltrexone groups were not significantly different from the TNBS colitis control group. As for the DSS

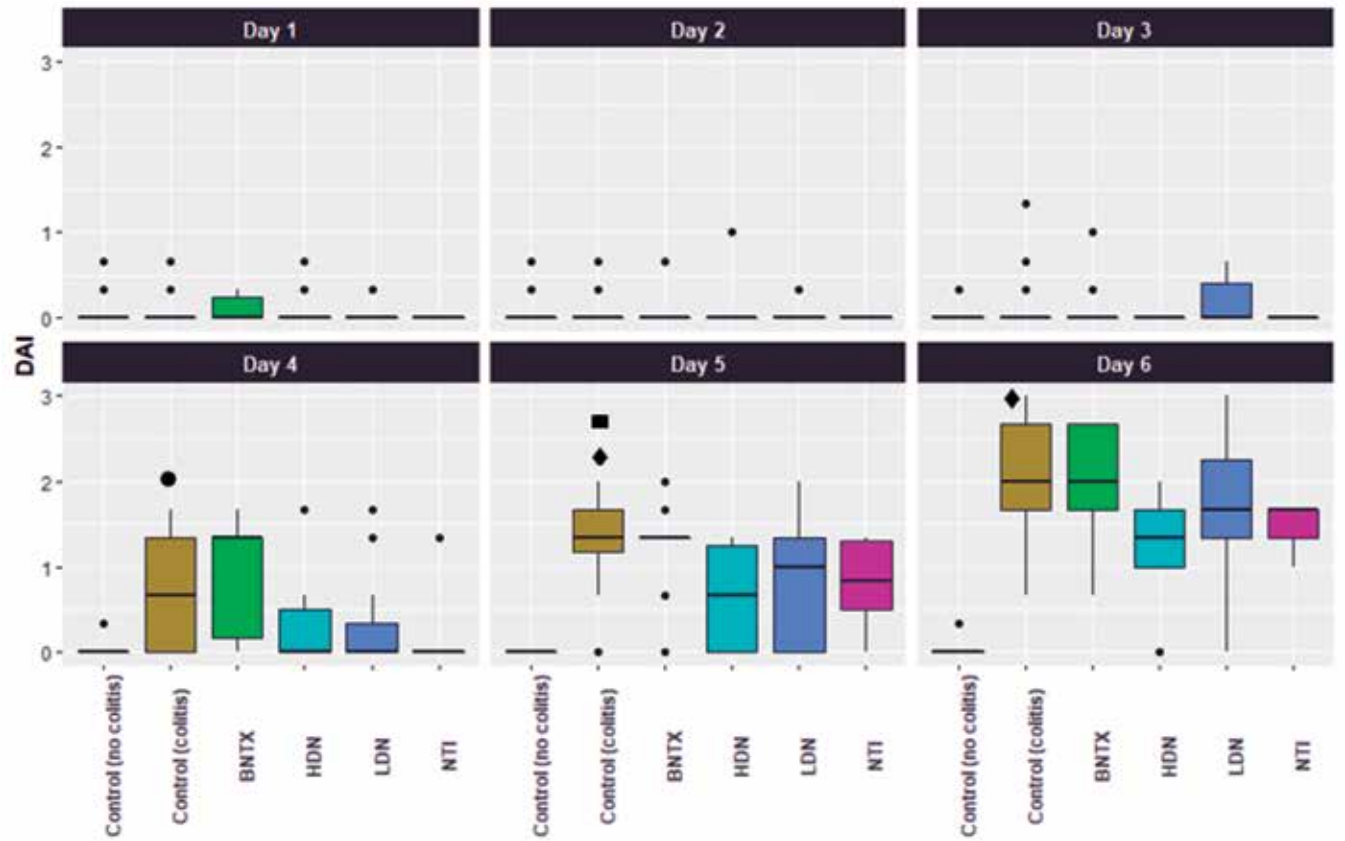

Figure 8. Disease activity index scores over time in DSS-colitis mice; median values are shown for each group. Scores differ significantly between DSS colitis control mice compared with no-colitis control (•, $P = 0.004$; ♦, $P = 0.0001$) and between the HDN group compared with DSS colitis controls (■, $P = 0.04$).

study, results for negative control groups in the TNBS model are not reported because no differences were noted in the between-group analyses.

Mortality. The final numbers of mice in the control and opioid receptor antagonist-treated groups varied as a result of early euthanasia at the approved humane endpoint (body weight loss of 15% or more or gross rectal bleeding) or (rarely) due to anesthetic complications. In the DSS colitis model, 15% mortality occurred in the LDN group as a result of reaching humane endpoint criteria. In the TNBS colitis study, mortality rates were 17%, 7%, 20%, and 40% for the colitis control, LDN, naltrindole, and 7-benzylidenenaltrexone groups, respectively.

Discussion

In the present study, we were able to successfully induce colonic inflammation in 2 established murine models of experimental colitis, as demonstrated with histology by the presence of acute ulcerations, submucosal edema, and polymorphonuclear as well as mononuclear cell inflammatory infiltrates. Further confirmatory evidence of the adequacy of these models was in the finding of the elevation in myeloperoxidase activity, increased DAI (DSS only), reduced colon length (DSS only), and increased colon weight (DSS only) from baseline control values. Overall, with the dosages used in this study, there was limited reversal of inflammation with the nonselective opioid receptor antagonist naltrexone but not with the agents that specifically antagonized the δ-receptors. In the DSS model, both doses of naltrexone appeared to have some effect at decreasing inflammation, whereas in the TNBS model, the higher dose was more effective (although neither difference was significant). These findings are on trend with that of a previous report regarding naltrexone therapy in DSS-treated mice with moderately induced colitis.[16] At present, none of the studies in the literature evaluate the therapeutic effects of naltrexone in TNBS-induced colitis.

In the current study, we hypothesized that reduction in colonic inflammation might occur through blockade of the δ-receptor, given that δ-opioid receptors are the predominant receptor subtype associated with inflammatory cells.[34] Our current study failed to demonstrate significant effects in either mouse model by using a δ-receptor opioid antagonist. It is possible that the administered dosages of these compounds were insufficient to achieve therapeutic effects, or perhaps the use of these specific δ-receptor antagonists does not play a role in gastrointestinal inflammation induced through DSS or TNBS.

The effects of naltrexone varied between the 2 colitis models. In the DSS model, LDN improved colon length and maintained Hct values within the normal reference range. In contrast, HDN had improved colon weight, reduced myeloperoxidase activity, low histology, macroscopic and ulcer scores in the TNBS colitis mice. Because the etiology and pathogenesis of CD and ulcerative colitis differ, these findings may therefore result in the differing therapeutic effects of naltrexone as evidenced in this study. Therefore, it is not unreasonable to expect that the higher dose of naltrexone reduced inflammation in this model more than did the lower dose. The unusual feature is that the higher dose of naltrexone was not more potent in the DSS model. Others have shown that, in the DSS model, lower rather than higher doses of naltrexone are more effective.[16] One explanation for this phenomenon is that the μ-receptor is blocked at the higher dose and results in increased inflammation. Alternatively, one group

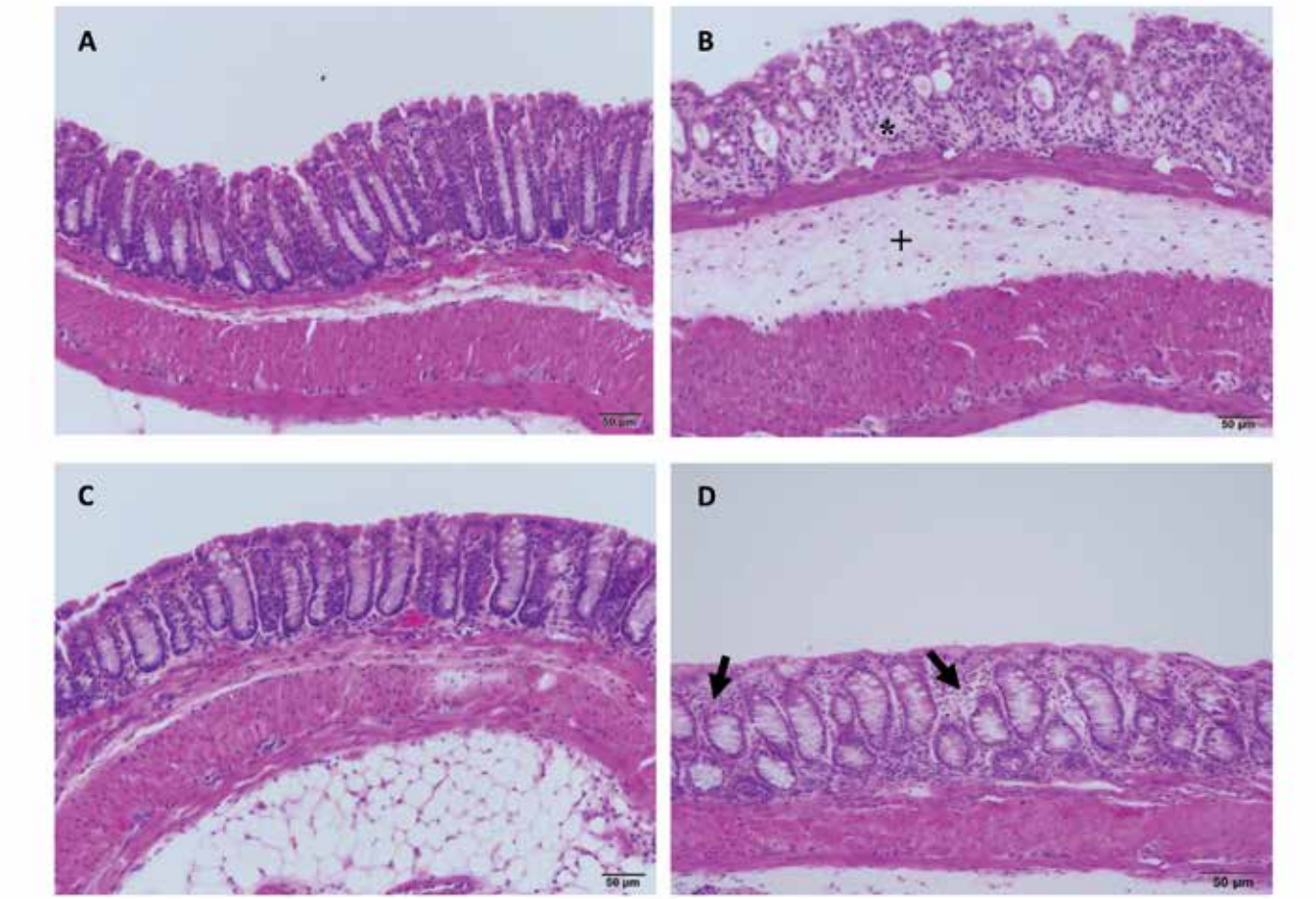

Figure 9. Histologic evaluation of colon. (A) Section of normal colon. (B) Representative section from control mouse with DSS-induced colitis. Note the loss of mucosal epithelium and crypts and the expansion of the lamina propria due to mononuclear inflammatory infiltrates (*). Diffuse submucosal edema (+) is present also. (C) Section of normal colon. (D) Representative section from control mouse with TNBS-induced colitis. Multifocal areas of PMN and mononuclear inflammation expand the lamina propria (signified by arrows). Images not shown for opioid receptor antagonist treated groups. Hematoxylin and eosin stain; bar: 50 µm (A through D).

of researchers proposed that the reason is related to intermittent blockade of opioid growth factor receptor, which promotes production of endogenous enkephalins and endorphins, both of which agents act on the opioid receptors to downregulate inflammation.[39] If too high a dosage of naltrexone is used, then enkephalins and endorphins cannot interface with and act on the opioid receptors.

The exact mechanism of action of naltrexone on inflammation and its associated effects in IBD are unknown, and several mechanisms have been proposed in the literature. Studies investigating this interaction have focused on immune cell cytokine production whereby elevations in TNFα, IL6, and IL12 were reduced by naltrexone administration in a rat and mouse model of colitis.[16,31] Through direct effects on bowel motility and opioid receptor- modulated immunologic effects, antagonism of the gastrointestinal tract opioid receptors may be therapeutic to inflamed mucosa. In addition, interference with endogenous opioids, such as endorphins and enkephalins, has been postulated.[25] Lastly, toll-like receptors play an integral role in the initiation of immune responses to infections and inappropriate activity and/or recognition of self-ligands have been associated with inflammatory conditions and autoimmunity.[7] Blockade of TLR4 can inhibit release of proinflammatory cytokines, substance P, nitric oxide, and excitatory amino acids, leading to downregulation of chemokine receptor expression and adhesion molecules.[38] One group reported that naltrexone inhibited IL6 and TNFα produced by peripheral blood mononuclear cells after stimulation with known ligands for TLR7, TLR8, and TLR9 but not TLR4.[5] In recent studies, the opioid inactive +-isomers of naltrexone inhibited LPS-induced TLR4 signaling, a bacterial-induced inflammatory pathway contributing to IBD.[35,36]

There were several limitations to our study. This study involved blood collection by cardiocentesis. Due to dehydration or blood loss in feces, hematologic samples were often difficult to obtain or were not of diagnostic quality. In addition, intrarectal administration of TNBS caused varying degrees of colitis in control and treated mice; therefore it is possible that the haptenating agent did not remain in the colon long enough to induce inflammation and was expelled from the anus of the mice during recovery from anesthesia.

In conclusion, we were able to demonstrate that nonselective opioid receptor blockade with naltrexone exerted limited preventive and therapeutic intestinal antiinflammatory effects in 2 murine models of inflammatory bowel disease. The mechanism for this antiinflammatory effect did not appear to be mediated by the δ opioid receptor. One of the key features of intestinal inflammation is upregulation of opioid receptors in the gastrointestinal tissues, both at the mRNA and protein level.[11,23,24] Future studies might include assessment of proinflammatory

cytokines, dose optimization for naltrexone, dose optimization for selective δ-opioid receptor antagonists, use of different selective antagonists, and the role of opioid receptor blockade in chronic animal models of IBD.

Acknowledgments

Special thanks to the Department of Comparative Medicine diagnostic laboratory staff and Dr Hannah Atkins for providing histology and hematological services, husbandry staff for providing animal care, Mr Jeffery Small for technical assistance and departmental chair, Dr Ronald P Wilson, for his support on this project.

Funding Acknowledgments

This work was supported by the Department of Comparative Medicine endowment funds.

References

1. **Bandzar S, Gupta S, Platt MO.** 2013. Crohn's disease: a review of treatment options and current research. Cell Immunol **286:**45–52. https://doi.org/10.1016/j.cellimm.2013.11.003.
2. **Baumgart DC, Sandborn WJ.** 2012. Crohn's disease. Lancet **380:**1590–1605. https://doi.org/10.1016/S0140-6736(12)60026-9.
3. **Berg DJ, Zhang J, Weinstock JV, Ismail HF, Earle KA, Alila H, Pamukcu R, Moore S, Lynch RG.** 2002. Rapid development of colitis in NSAID-treated IL-10-deficient mice. Gastroenterology **123:**1527–1542. https://doi.org/10.1053/gast.2002.1231527.
4. **Bouma G, Strober W.** 2003. The immunological and genetic basis of inflammatory bowel disease. Nat Rev Immunol **3:**521–533. https://doi.org/10.1038/nri1132.
5. **Cant R, Dalgleish AG, Allen RL.** 2017. Naltrexone inhibits IL-6 and TNFα production in human immune cell subsets following stimulation with ligands for intracellular toll-like receptors. Front Immunol **8:**1–8.
6. **Donahue RN, McLaughlin PJ, Zagon IS.** 2011. Low-dose naltrexone targets the opioid growth factor-opioid growth factor receptor pathway to inhibit cell proliferation: mechanistic evidence from a tissue culture model. Exp Biol Med (Maywood) **236:**1036–1050. https://doi.org/10.1258/ebm.2011.011121.
7. **Fischer M, Ehlers M.** 2008. Toll-like receptors in autoimmunity. Ann N Y Acad Sci **1143:**21–34. https://doi.org/10.1196/annals.1443.012.
8. **Fitzpatrick LR, Deml L, Hofmann C, Small JS, Groeppel M, Hamm S, Lemstra S, Leban J, Ammendola A.** 2010. 4SC-101, a novel immunosuppressive drug, inhibits IL-17 and attenuates colitis in two murine models of inflammatory bowel disease. Inflamm Bowel Dis **16:**1763–1777. https://doi.org/10.1002/ibd.21264.
9. **Fitzpatrick LR, Wang J, Le T.** 2000. In vitro and in vivo effects of gliotoxin, a fungal metabolite: efficacy against dextran sodium sulfate-induced colitis in rats. Dig Dis Sci **45:**2327–2336. https://doi.org/10.1023/A:1005630723111.
10. **Institute for Laboratory Animal Research.** 1996. Guide for the care and use of laboratory animals. Washington (DC): National Academies Press.
11. **Jiménez N, Puig MM, Pol O.** 2006. Antiexudative effects of opioids and expression of k- and δ-opioid receptors during intestinal inflammation in mice: involvement of nitric oxide. J Pharmacol Exp Ther **316:**261–270. https://doi.org/10.1124/jpet.105.091991.
12. **Ju J, Hao X, Lee MJ, Lambert JD, Lu G, Xiao H, Newmark HL, Yang CS.** 2009. A γ-tocopherol-rich mixture of tocopherols inhibits colon inflammation and carcinogenesis in azoxymethane and dextran sulfate sodium-treated mice. Cancer Prev Res (Phila) **2:**143–152. https://doi.org/10.1158/1940-6207.CAPR-08-0099.
13. **Kaplan GG.** 2015. The global burden of IBD: from 2015 to 2025.Nat Rev Gastroenterol Hepatol **12:**720–727. https://doi.org/10.1038/nrgastro.2015.150.
14. **Koneru A, Satyanarayana S, Rizwan S.** 2009. Endogenous opioids: their physiological role and receptors. Global Journal of Pharmacology **3:**149–153.
15. **Leone V, Chang EB, Devkota S.** 2013. Diet, microbes, and host genetics: the perfect storm in inflammatory bowel diseases. J Gastroenterol **48:**315–321. https://doi.org/10.1007/s00535-013-0777-2.
16. **Matters GL, Harms JF, McGovern C, Fitzpatrick L, Parikh A, Nilo N, Smith JP.** 2008. The opioid antagonist naltrexone improves murine inflammatory bowel disease. J Immunotoxicol **5:**179–187. https://doi.org/10.1080/15476910802131469.
17. **McDonald J, Lambert DG.** 2005. Opioid receptors. Continuing education in anaesthesia, critical care & pain **5:**22–25. https://doi.org/10.1093/bjaceaccp/mki004.
18. **Mizoguchi A.** 2012. Animal models of inflammatory bowel disease. Prog Mol Biol Transl Sci **105:**263–320. https://doi.org/10.1016/B978-0-12-394596-9.00009-3.
19. **Moolenbeek C, Ruitenberg EJ.** 1981. The "Swiss roll": a simple technique for histological studies of the rodent intestine. Lab Anim **15:**57–59. https://doi.org/10.1258/002367781780958577.
20. **Morris GP, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL.** 1989. Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology **96:**795–803. https://doi.org/10.1016/S0016-5085(89)80079-4.
21. **Murthy SN, Cooper HS, Shim H, Shah RS, Ibrahim SA, Sedergran DJ.** 1993. Treatment of dextran sulfate sodium-induced murine colitis by intracolonic cyclosporin. Dig Dis Sci **38:**1722–1734. https://doi.org/10.1007/BF01303184.
22. **Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R.** 1990. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology **98:**694–702. https://doi.org/10.1016/0016-5085(90)90290-H.
23. **Pol O, Alameda F, Puig MM.** 2001. Inflammation enhances μ-opioid receptor transcription and expression in mice intestine. Mol Pharmacol **60:**894–899. https://doi.org/10.1124/mol.60.5.894.
24. **Pol O, Palacio JR, Puig MM.** 2003. The expression of δ- and k-opioid receptor is enhanced during intestinal inflammation in mice. J Pharmacol Exp Ther **306:**455–462. https://doi.org/10.1124/jpet.103.049346.
25. **Raknes G, Simonsen P, Småbrekke L.** 2018. The effect of low-dose naltrexone on medication in inflammatory bowel disease: a quasi experimental before-and-after prescription database study. J Crohns Colitis **12:**677–686. https://doi.org/10.1093/ecco-jcc/jjy008.
26. **Randhawa PK, Singh K, Singh N, Jaggi AS.** 2014. A review on chemical-induced inflammatory bowel disease models in rodents. Korean J Physiol Pharmacol **18:**279–288. https://doi.org/10.4196/kjpp.2014.18.4.279.
27. **Reuter BK, Asfaha S, Buret A, Sharkey KA, Wallace JL.** 1996. Exacerbation of inflammation-associated colonic injury in rat through inhibition of cyclooxygenase-2. J Clin Invest **98:**2076–2085. https://doi.org/10.1172/JCI119013.
28. **Scheiffele F, Fuss IJ.** 2002. Induction of TNBS colitis in mice. Curr Protoc Immunol **49:**15.19.1–15.19.14. https://doi.org/10.1002/0471142735.im1519s49
29. **Smith JP, Bingaman SI, Ruggiero F, Mauger DT, Mukherjee A, McGovern CO, Zagon IS.** 2011. Therapy with the opioid antagonist naltrexone promotes mucosal healing in active Crohn's disease: a randomized placebo-controlled trial. Dig Dis Sci **56:**2088–2097. https://doi.org/10.1007/s10620-011-1653-7.
30. **Smith JP, Stock H, Bingaman S, Mauger D, Rogosnitzky M, Zagon IS.** 2007. Low-dose naltrexone therapy improves active Crohn's disease. Am J Gastroenterol **102:**820–828. https://doi.org/10.1111/j.1572-0241.2007.01045.x.
31. **Tawfik DI, Osman AS, Tolba HM, Khattab A, Abdel-Salam LO, Kamel MM.** 2016. Evaluation of therapeutic effect of low dose naltrexone in experimentally-induced Crohn's disease in rats. Neuropeptides **59:**39–45. https://doi.org/10.1016/j.npep.2016.06.003.
32. **Toljan K, Vrooman B.** 2018. Low-Dose Naltrexone (LDN)—Review of Therapeutic Utilization. Med Sci (Basel) **6:**1–18.
33. **Triantafillidis JK, Nasioulas G, Kosmidis PA.** 2009. Colorectal cancer and inflammatory bowel disease: epidemiology, risk factors, mechanisms of carcinogenesis and prevention strategies. Anticancer Res **29:**2727–2737.

34. **van Rijn RM, Defriel JN, Whistler JL.** 2013. Pharmacological traits of delta opioid receptors: pitfalls or opportunities? Psychopharmacology (Berl) **228:**1–18. https://doi.org/10.1007/s00213-013-3129-2.
35. **Wang X, Zhang Y, Peng Y, Hutchinson MR, Rice KC, Yin H, Watkins LR.** 2016. Pharmacological characterization of the opioid inactive isomers (+)-naltrexone and (+)-naloxone as antagonists of toll-like receptor 4. Br J Pharmacol **173:**856–869. https://doi.org/10.1111/bph.13394.
36. **Watkins LR, Wang X, Mustafa S, Hutchinson MR.** 2014. In vivo veritas: (+)-Naltrexone's actions define translational importance: A letter in response to Skolnick et al. 'Translational potential of naloxone and naltrexone as TLR4 antagonists'. Trends Pharmacol Sci **35:**432–433. https://doi.org/10.1016/j.tips.2014.07.002.
37. **Wirtz S, Neufert C, Weigmann B, Neurath MF.** 2007. Chemically induced mouse models of intestinal inflammation. Nat Protoc **2:**541–546. https://doi.org/10.1038/nprot.2007.41.
38. **Younger J, Parkitny L, McLain D.** 2014. The use of low-dose naltrexone (LDN) as a novel anti-inflammatory treatment for chronic pain. Clin Rheumatol **33:**451–459. https://doi.org/10.1007/s10067-014-2517-2.
39. **Zagon IS, McLaughlin PJ.** 2011. Targeting opioid signaling in Crohn's disease: new therapeutic pathways. Expert Rev Gastroenterol Hepatol **5:**555–558. https://doi.org/10.1586/egh.11.62.