ANTINOCICEPTIVE EFFECTS OF AN ANTI-CGRP ANTIBODY IN RAT MODELS OF COLON-BLADDER CROSS ORGAN SENSITIZATION

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Nonstandard Abbreviations: AWR: Abdominal withdrawal reflex; DAI: Disease activity index; IBS: Irritable bowel syndrome; BPS: Bladder pain syndrome; CRD: Colorectal distension; G: Conductance; CGRP: Calcitonin gene-related peptide; GI: Gastrointestinal; OVX: Ovariectomized; PS: protamine sulfate; TNBS: Trinitrobenzenesulfonic acid; TEER: Transepithelial electrical resistance; VMR: Visceromotor response

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ABSTRACT

Irritable bowel syndrome (IBS) and bladder pain syndrome/interstitial cystitis (BPS/IC) are comorbid visceral pain disorders seen commonly in women with unknown etiology, limited treatment options and can involve visceral organ cross-sensitization. Calcitonin gene-related peptide (CGRP) is a mediator of nociceptive processing and may serve as a target for therapy. In three rodent models, we employed a monoclonal anti-CGRP F(ab’)2 to investigate the hypothesis that visceral organ cross-sensitization is mediated by abnormal CGRP signaling. Visceral organ cross-sensitization was induced in adult female rats via transurethral infusion of protamine sulfate (PS) into the urinary bladder or infusion into the colon of trinitrobenzene sulfonic acid (TNBS). Colonic sensitivity was assessed via the visceromotor response (VMR) to colorectal distension (CRD). Bladder sensitivity was assessed as the frequency of abdominal withdrawal responses (AWR) to von Frey filaments applied to the suprapubic region. PS- or TNBS-induced changes in colonic and bladder permeability were investigated in vitro via quantification of transepithelial electrical resistance (TEER). Peripheral administration of an anti-CGRP F(ab’)2 inhibited PS-induced visceral pain behaviors and colon hyperpermeability. Similarly, TNBS-induced pain behaviors and colon and bladder hyperpermeability were attenuated by anti-CGRP F(ab’)2 treatment. PS into the bladder or TNBS into the colon significantly increased the VMR to CRD and AWR to suprapubic stimulation and decreased bladder and colon TEER. These findings suggest an important role of peripheral CGRP in visceral nociception and organ cross-sensitization and support the evaluation of CGRP as a therapeutic target for visceral pain in patients with IBS and/or BPS/IC.
Significance Statement: A monoclonal antibody against calcitonin gene-related peptide (CGRP) was found to reduce concomitant colonic and bladder hypersensitivity and hyperpermeability. The results of this study suggest that CGRP-targeting antibodies, in addition to migraine prevention, may provide a novel treatment strategy for multi-organ abdominopelvic pain following injury or inflammation.

Keywords: Irritable bowel syndrome, bladder pain syndrome, visceral pain, calcitonin gene-related peptide (CGRP), colon, urinary bladder, visceral hypersensitivity, visceral organ crosstalk, rat.
INTRODUCTION

Irritable bowel syndrome (IBS) is a common female predominant gut-brain disorder that affects up to 20% of adults in the United States (Saito et al., 2002; Longstreth et al., 2006). IBS features chronic abdominal pain and abnormal bowel habits presenting as IBS with diarrhea constipation, or a combination of both (Longstreth, 2005). IBS patients suffer from comorbid regional pain disorders, including bladder pain syndrome/interstitial cystitis (BPS/IC). BPS/IC is a chronic pelvic pain condition that affects 3-8 million women in the United States (Berry et al., 2011). Frequent urination, as well as increased urgency, and abdominal pain are the common symptoms of BPS/IC (Held et al., 1990; Patnaik et al., 2017). Of interest to the current study, up to 40% of patients diagnosed with BPS/IC also suffer from symptoms of IBS (Ustinova et al., 2006). Despite the prevalence and relatively high comorbidity of both BPS/IC and IBS, the mechanisms contributing to symptom overlap in patients are not well understood. Evidence suggests that abnormal visceral organ cross-communication may underlie the comorbidity of IBS and BPS/IC. In support, colonic irritation disrupts micturition patterns and increases urethral sphincter activity in a rat model (Pezzone et al., 2005). Moreover, inflammation of the large intestine induces abnormal bladder detrusor-muscle contractility and bladder hyperpermeability, whilst bladder irritation leads to colonic hypersensitivity and colonic hyperpermeability in rats (Bielefeldt et al., 2006; Winnard et al., 2006; Greenwood-Van Meerveld et al., 2015). The underlying mechanisms responsible for visceral organ cross-sensitization remain to be fully elucidated, evidence suggests that multiple factors may be involved, including the convergence of sensory neural pathways at various levels of the central nervous system, epithelial hyperpermeability, and visceral afferent sensitization (Lavelle et al., 2002; Malykhina et al., 2006; Christianson et al., 2007; Malykhina, 2007; Suckow and Caudle, 2009; Greenwood-Van
Meerveld et al., 2015). In summary, visceral pain disorders involve interconnected pathophysiological mechanism and suggest that a single therapeutic may provide relief of multifocal visceral pain associated with comorbid IBS and BPS/IC.

Calcitonin gene-related peptide (CGRP) is a 37 amino acid member of the calcitonin family of peptides and is produced in neurons throughout both peripheral and central nervous system. CGRP is a vasodilator, immune modulator, and is involved in nociception (Rosenfeld et al., 1983; Brain et al., 1985; Chen et al., 2010; Walsh et al., 2015). Anti-CGRP ligand antibodies and anti-CGRP receptor antibody are clinically approved therapeutics for the prevention of migraine (Do et al., 2019; Bhakta et al., 2021). CGRP is present within both intrinsic and extrinsic primary afferent neurons located within the enteric nervous system (ENS) and brain gut axis (Sundler et al., 1991; Domoto et al., 1992; Timmermans et al., 1992; Wolf et al., 2007; Makowska and Gonkowski, 2018). Evidence suggests that CGRP plays a key role in smooth muscle contractility, epithelial transport, neuroprotection and the transmission of sensory and nociceptive stimuli (Timmermans et al., 1992; Rytel and Calka, 2016; Makowska et al., 2017). CGRP-mediated visceral organ afferent sensitization is implicated in the development of abdominopelvic pain associated with colitis and cystitis (Qiao and Grider, 2007; Pan et al., 2010; Ceuleers et al., 2018; Utsumi et al., 2018). For example, inflammation of the colon significantly increases CGRP-immunoreactive nerve density in rat primary afferent pathways (Qiao and Grider, 2009). Furthermore, in inflammatory models featuring visceral hypersensitivity, a peptide CGRP receptor antagonist [human CGRP-(8-37)] attenuates visceral hypersensitivity (Plourde et al., 1997; Delafoy et al., 2006). However, the role CGRP-mediated mechanisms play in the initiation and maintenance of visceral organ cross-sensitization and their potential as therapeutic targets to treat IBS and BPS/IC have yet to be fully explored.
To advance our understanding of peripheral CGRP signaling in the context of overlapping visceral pain disorders, we evaluated the effect of a monoclonal anti-CGRP F(\(\text{ab'}\))\(_2\) on visceral nociception and permeability of the bladder and colon in three clinically relevant rodent models of visceral organ cross-sensitization. In adult female rats that protamine sulfate (PS)-induced disruption of urinary bladder results in hypersensitivity and hyperpermeability of both the bladder and undamaged colon. Conversely, we show that TNBS-induced colitis elicits hypersensitivity and hyperpermeability of the colon and undamaged bladder both during and following the resolution of acute colitis. In these models we show that anti-CGRP F(\(\text{ab'}\))\(_2\) treatment attenuates visceral pain behaviors and colon hyperpermeability. These findings provide insight into the mechanisms of visceral organ cross-sensitization underlying the poorly localized abdominopelvic pain observed in IBS and BPS and support investigation of CGRP as a therapeutic target for these indications.
METHODS

Preparation of F(ab’)₂ fragment antibody

Anti-CGRP F(ab’)₂ and isotype F(ab’)₂ control were used to explore the specific targeting of CGRP in the tested clinically relevant rodent models of visceral organ cross-sensitization. As described previously by Noor Mohammadi and colleagues fremanezumab (a humanized anti-CGRP mAb; Teva Pharmaceuticals, Redwood City, CA) and isotype control antibody (Teva Pharmaceuticals, Redwood City, CA) were digested with pepsin and affinity chromatography purification of F(ab’)₂ using Kappa-Select columns was performed. Purity and integrity of the F(ab’)₂ were analyzed by SDS-PAGE under reducing and non-reducing conditions. Similar binding affinity of anti-CGRP F(ab’)₂ to CGRP compared to full length anti-CGRP antibody was confirmed by surface plasmon resonance (Noor-Mohammadi et al., 2021). The antibody binds to both isoforms of rat CGRP with comparable affinity (data not shown).

Animals

A total of 300 ovariectomized female Sprague-Dawley rats (220–250 g) were purchased from Charles River Laboratories (Wilmington, Massachusetts). Rats were single housed (cage dimension: 140 square inches) within the University of Oklahoma Health Sciences Center (OUHSC), Department of Comparative Medicine's animal facility under controlled temperature (23°C ±3°C) and humidity (30-70%) with free access to food and water on a standard 12:12 hr light: dark cycle. All animals were acclimated to the facility and laboratory for a minimum of 2 weeks before experimentation. Female rats were used because of ease of urethral catheterization and ovariectomized to avoid any effects of hormonal cycling. All experimental procedures were
approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee (IACUC animal Protocol number: 17-014-CHFA).

Administration of Protamine Sulfate into the Bladder

Protamine Sulfate administration into the Bladder: This procedure was outlined in our previous publication (Noor-Mohammadi et al., 2021). Between 8:00 AM and 10:00 AM rats were anesthetized in the laboratory with isoflurane (2%) and a steady supply of oxygen for ~15 min. Body temperature was maintained with a homeothermic blanket, and PS solution was infused transurethrally as previously described (Lavelle et al., 2002). Briefly, the bladder was drained following catheterization using a 20-gauge intravenous catheter (Becton Dickinson infusion Therapy Systems Inc., Sandy UT, USA). Animals were monitored during the experimental procedure and animals with signs of blood in the urine were removed from the study (4 rats). Following the procedure the transurethral catheter was removed, and animals were returned to their home cages. Bladder and colonic hypersensitivity were assessed at 24-hrs and 5 days after the infusion of PS into the bladder (Greenwood-Van Meerveld et al., 2018; Grundy et al., 2020). In a separate cohort of rats, bladders and colons were harvested 24-hrs and 5 days following the infusion of PS for the in vitro permeability assessments. In previous unpublished experiments we found that infusion of saline into the bladder of OVX female rats caused a mild bladder irritation. Therefore, OVX female rats that were catheterized but not given saline served as control animals.

Administration of TNBS into the Colon
TNBS Administration in Colon: After an overnight fast with free access to water, rats were transported from the animal facility to the laboratory, where that were briefly anesthetized with isoflurane (3%) with a steady supply of oxygen. A solution of 50 mg/ml TNBS diluted in 25% ethanol and 25% saline was then given to the rats via an enema. Controls were infused with 100% saline. Bladder and colonic hypersensitivity were assessed 5 days (active colitis) and 30 days (post-inflammatory) after the infusion of TNBS into the colon. In a separate cohort of rats, bladders and colons were harvested 5 days (active colitis) and 30 days (post-inflammatory) following the infusion of TNBS for the in vitro permeability assessments. Intracolonic saline-treated OVX rats served as control animals.

Disease Activity Index (DAI)
The severity of TNBS induced colonic inflammation, was assessed by weighing the rats and grading the rats stools for 5 consecutive days after TNBS infusion. DAI was calculated as previously described (Greenwood-Van Meerveld and Tyler, 2006). The presence of blood in the feces was tested using an occult blood indicator test (Beckman Coulter, Fullerton, California).

Bladder Sensitivity
All bladder sensitivity reordering were performed between 10:00 AM and 12:00 PM in a separate behavioral room attached to the main laboratory. Rats were placed in individual Plexiglas chambers with a stainless-steel wire grid floor and were acclimated to the chambers for 30 min. Von Frey filaments applied to the suprapubic area of the animal was used to quantify bladder sensitivity (Laird et al., 2001; Rudick et al., 2007). The frequency of withdrawal responses to individual von Frey filaments with calibrated force of 0.16, 0.4, 1, 2,
4, 8, and 15 g was manually recorded. Each of the von Frey hairs was applied to the suprapubic region for 1-2 sec for a total of 10 applications/force with a 5-sec rest period between each application. Sharp retraction of the abdomen, immediate licking or grooming, or jumping was considered a positive response to the stimulus.

**Colonic Sensitivity**

Colonic sensitivity assessment was performed between 10:00 AM and 2:00 PM in a behavioral room attached to the main laboratory. The methodology used to measure colonic sensitivity has been previously described (Myers and Greenwood-Van Meerveld, 2007; Gosselin et al., 2010; Noor-Mohammadi et al., 2021). Rats were brought to the laboratory after an overnight fast and then briefly anesthetized with 5% isoflurane. A 5-cm colonic balloon was then inserted approximately 11-cm past the anus into the colon and secured to the base of the tail with tape and attached to a Distender series IIR barostat (G & J Electronics Inc., Toronto, Ontario, Canada). When the rats were freely ambulatory within in their clean home cage containing sani-chip bedding, a 10-min basal recording with the balloon inserted but not distended, then the colonic balloon catheter was distended to graded pressures (randomized 0-60 mmHg for 10min) of isobaric colorectal distension (CRD). Colonic sensitivity to CRD was quantified by counting the number of abdominal contractions in response to graded pressures of balloon distension (10-min inflation period then a 10-min rest period between distensions).

**Bladder and Colonic Permeability**
Rat bladders and colons were isolated (between 8:00 AM and 10:00 AM) and placed into ice cold Krebs buffer composed of 120 mM NaCl, 6 mM KCl, 1.2 mM MgCl₂, 1.2 mM H₂PO₄, 2.5 mM, CaCl₂, 14.4 mM NaHCO₃, and 11.5 mM glucose, aerated with 95 % O₂–5 % CO₂. The isolated tissue was sectioned longitudinally and mounted into biopsy perfusion chambers (bladder) or modified Ussing chambers (colon). In both in vitro preparations the colonic or bladder tissue was bathed in oxygenated Krebs solution at 37ºC for 30-45 min, before experimentation. Transepithelial electrical resistance (TEER) and conductance (G) were recorded as an indicator of tissue permeability. To calculate TEER and G, the potential difference (PD) and short circuit current (Isc) were recorded, and TEER/G was calculated using Ohm’s law: 

\[ I = \frac{PD}{R} \]

where R represents resistance.

**Drugs and Chemicals**

Anti-CGRP F(ab')₂, isotype F(ab')₂ control, and vehicle were supplied by TEVA Pharmaceuticals (Redwood City, CA) and stored at 4º C. In the PS induced bladder and colonic hypersensitive/hyperpermeability and active colitis models, rats received a single intraperitoneal (i.p.) administration of anti-CGRP F(ab')₂ (30 mg/kg), isotype F(ab')₂ control (30 mg/kg) or vehicle (saline) 24-hrs before the measurement of bladder and colonic sensitivity or permeability. However, in our model of post-TNBS hypersensitivity, rats received a single intraperitoneal (i.p.) administration of anti-CGRP F(ab')₂ (30 mg/kg), isotype F(ab')₂ control (30 mg/kg) or vehicle (saline) on days 25, 27, and 29 post TNBS or saline (i.e. 24-hrs prior to measuring bladder and colonic sensitivity or permeability). Fecal pellets were collected 6 hours after administration of the final dose on day 29 for assessment of the wet-to-dry ratio. The doses
for both Anti-CGRP F(ab’)2 and isotype F(ab’)2 control were employed based on previously published experiments (Noor-Mohammadi et al., 2021; Melo-Carrillo et al., 2017b).

**Statistical analysis and experimental rigor**

Prior to experimentation a power analysis was performed for each experiment to determine the number of animals needed to reach significance. All experiments were performed by one experimenter who was responsible for all components of the study, but he was blinded to treatments. To improve experimental rigor experiments were performed in a randomized manner (www.randomization.com). Statistical significance was determined using Two-Way Repeated Measure ANOVA followed by a Bonferroni post-test. Data for TEER calculations were generated via EVC4000 Voltage/Current Clamp instrumentation. PD and Isc were manually recorded into a laboratory notebook and later transferred into an Excel spreadsheet. One-Way ANOVA with Tukey’s post-hoc multi-comparison test were employed to determine statistical significance. All test values were expressed as mean ± standard error of the mean (SEM) and analyses were performed using GraphPad Prism (GraphPad Software 9XML, USA), and for all data a 95% confidence interval was used. A p<0.05 was considered statistically significant in all statistical tests.

**RESULTS**

**ANTI-CGRP F(ab’)2 ATTENUATES BLADDER AND COLONIC HYPERSENSITIVITY INDUCED BY INFUSION OF PS INTO THE BLADDER IN FEMALE RATS**

PS infusion is a well-established method to induce bladder hypersensitivity and permeability (Hurst et al., 2015). Therefore, for our first series of experiments we aimed to investigate
whether peripheral administration of anti-CGRP F(ab’)2 is capable of disrupting crosstalk between the bladder and colon to inhibit visceral hyperalgesia, 24-hrs and 5 days post PS infusion. Following a single i.p. administration of anti-CGRP F(ab’)2 (30 mg/kg), isotype F(ab’)2 control (30mg/kg) or vehicle bladder and colonic sensitivity were assessed in freely moving adult female rats. We demonstrated that infusion of PS into the bladder significantly increased bladder hypersensitivity as shown by the increased number of withdrawal responses at 1–15 g in both vehicle and isotype F(ab’)2 control groups when compared to anti-CGRP F(ab’)2. Pre-treatment with anti-CGRP F(ab’)2 significantly decreased withdrawal responses from low to high-threshold stimulation in comparison with vehicle and isotype F(ab’)2 control-treated rats, 24-hrs (Fig. 1A) (F [2, 31] = 14.20, P < 0.0001) and 5 days (Fig. 1B) (F [2, 22] = 10.31, P < 0.0001) post PS infusion into the bladder. Similarly, pre-treatment with anti-CGRP F(ab’)2, significantly decreased colonic hypersensitivity, illustrated by the significantly reduced number of abdominal contractions in anti-CGRP F(ab’)2-treated rats compared with vehicle and isotype F(ab’)2 control-treated rats at 24-hrs (F [2, 31] = 5.85, P < 0.0001) (Fig. 1C) and 5 days (F [23, 69] = 3.58, P < 0.0001) (Fig. 1D) post PS-infusion into the bladder.

ANTI-CGRP F(ab’)2 INHIBITS BLADDER AND COLONIC HYPERPERMEABILITY INDUCED BY PS-INFUSION IN THE BLADDER IN FEMALE RATS

In our next series of experiments, we considered whether anti-CGRP F(ab’)2 is capable of inhibiting visceral hypersensitivity via a reduction in mucosal hyperpermeability. As illustrated in Figure 2A, pre-treatment with anti-CGRP F(ab’)2 (30mg/kg, i.p.) had no significant effect on TEER illustrating no differences in bladder permeability at 24-hrs post PS-infusion into the bladder. However, anti-CGRP F(ab’)2 (30mg/kg, i.p.) significantly decreased bladder
permeability as shown by an increase in TEER 5 days post PS-infusion into the bladder when compared to isotype F(ab’)2 and vehicle treated groups (Fig. 2B) (F [2, 22] = 9.02, P < 0.01). To probe the effects of anti-CGRP F(ab’)2 on PS-induced colonic hyperpermeability, we isolated colon from rats pretreated with either vehicle, isotype F(ab’)2 control, or anti-CGRP F(ab’)2 24-hrs and 5 days post PS-infusion into the bladder. Compared with vehicle and isotype F(ab’)2 control-treated animals, anti-CGRP F(ab’)2 had a significant inhibitory effect on colonic hyperpermeability induced by infusion of PS into the bladder. Our finding revealed a significant increase in colonic TEER in tissue isolated from rats 24-hrs (Fig. 2C) (F [2, 49] = 19.79, P < 0.0001) and 5 days (Fig. 2D) (F [2, 48] = 18.78, P < 0.0001) after PS infusion in the bladder in anti-CGRP F(ab’)2 group compared with vehicle and isotype F(ab’)2 control-treated rats.

ANTI-CGRP F(ab’)2 INHIBITS BLADDER BUT NOT COLONIC HYPERSENSITIVITY DURING ACTIVE TNBS-INDUCED COLITIS IN FEMALE RATS

We observed a significant increase in disease activity index (DAI) following the TNBS enema due to the presence of blood in the feces and altered stool consistency (data not shown). The results indicated that 5 days post-TNBS infusion into the colon, bladder and colonic hypersensitivity was significantly increased in both vehicle and isotype F(ab’)2 control groups when compared to saline treated rats (Fig. 3A and B) (F [6, 91] = 76.74, P < 0.0001, Bladder) (F [23, 45]= 191.1, P < 0.0001, Colon). As illustrated in figure 3A, administration of anti-CGRP F(ab’)2 (30mg/kg, i.p.) was found to inhibit this increase in bladder sensitivity at 4-15 g of force (F [6, 182]= 164.6, P < 0.0001), but not to normal levels. However, pre-treatment with anti-CGRP F(ab’)2 had no effect on colonic hypersensitivity during active colitis in this rodent model (Fig. 3B).
ANTI-CGRP F(ab')2 REVERSES BLADDER AND COLONIC HYPERPERMEABILITY DURING ACTIVE TNBS-INDUCED COLITIS IN FEMALE RATS

We now aimed to study the effect of anti-CGRP F(ab')2 on bladder and colonic hyperpermeability during active colitis. Both bladder and colonic tissue was isolated 5 days post TNBS infusion into the colon. In response to TNBS-induced infusion in the colon, bladder permeability was increased as shown by a significant decrease in TEER in vehicle and isotype F(ab')2 control compared to saline-treated rats. Administration of anti-CGRP F(ab')2 (30mg/kg, i.p.) normalized bladder hyperpermeability (F [3, 36]= 17.44, P < 0.0001) (Fig. 4A). In the colon from TNBS-treated rats there was a significant decrease in TEER compared to saline treated controls and pre-treatment with anti-CGRP F(ab')2 largely reversed the TNBS-induced increase in colonic permeability when compared to vehicle and isotype F(ab')2 control (F [3, 75]= 19.30, P < 0.0001) but not to normal levels (Fig. 4B).

ANTI-CGRP F(ab')2 INHIBITS VISCERAL HYPERSENSITIVITY IN A RODENT MODEL OF POST-INFLAMMATORY TNBS-INDUCED COLITIS IN FEMALE RATS

Our next aim was to investigate whether anti-CGRP F(ab')2 treatment would affect the increased bladder and colonic sensitivity in a rodent model of post-inflammatory visceral hypersensitivity following recovery from a TNBS-induced colitis. Chronic post-inflammatory visceral hypersensitivity was assessed 30 days post infusion of TNBS into the colon. Bladder and colonic sensitivity were assessed in freely moving adult female rats following a single i.p. administration of anti-CGRP F(ab')2 (30 mg/kg), isotype F(ab')2 control (30mg/kg) or vehicle on days 25, 27, and 29 post TNBS infusion into the colon. Rats that received intra-colonic infusion of saline
served as saline control group. We demonstrated that an intracolonic enema of TNBS significantly increased bladder sensitivity as illustrated by the higher number of abdominal withdrawal responses at 4–15 g in both vehicle and isotype F(ab’)2 control groups compared to the saline enema control group (Fig. 5A) (F [6, 196] = 182.3, P <0.0001). Pre-treatment with anti-CGRP F(ab’)2 (30mg/kg, i.p.) significantly decreased withdrawal response to high-threshold stimulation (8 and 15 g) compared with vehicle treated rats (F [3, 196] = 10.74, P <0.0001) (Fig. 5A). As shown in figure 5B, vehicle-treated animals previously exposed to TNBS, exhibited significantly higher number of abdominal contractions at distension pressures of 40 mmHg (P <0.0001) and 60 mmHg (P<0.0001) when compared to saline control, confirming 30 days post infusion of TNBS into the colon induces colonic hypersensitivity (F [3, 112] = 466.4, P <0.0001). Post-hoc analysis revealed that animals treated with anti-CGRP F(ab’)2 demonstrated significantly fewer abdominal contractions in response to CRD at balloon distension pressures of 40 mmHg (P <0.0001) and 60 mmHg (P < 0.001) compared to vehicle-treated animals and isotype F(ab’)2 control (F [3, 112] = 59.17, P <0.0001).

ANTI-CGRP F(ab’)2 REVERSES BLADDER AND COLONIC HYPERPERMEABILITY IN A RODENT MODEL OF POST-INFLAMMATORY TNBS-INDUCED COLITIS IN FEMALE RATS

Thirty days post infusion of TNBS into the colon there was a significant decrease in TEER in the vehicle- and isotype F(ab’)2 control- treated animals when compared to the saline control group (F [3, 36] = 14.35, P <0.0001) (Fig. 6A) demonstrating of a significant elevation of bladder permeability. Pre-treatment with of anti-CGRP F(ab’)2 (30mg/kg, i.p.) reversed the bladder hyperpermeability 30 days post TNBS-infusion into the colon (F [3, 74] = 12.98, P <0.0001) (Fig.
6A). In response to TNBS infusion in the colon, the colonic TEER was significantly decreased in vehicle and isotype F(ab’)2 control groups compared to saline control rats (F [3, 74] = 12.98, P <0.0001) (Fig. 6B). Administration of anti-CGRP F(ab’)2 (30mg/kg, i.p.) reversed the effect on colonic permeability 30 days post TNBS-infusion into the colon (F [3, 57] = 341.3, P <0.0001) (Fig. 6B).

ANTI-CGRP F(ab’)2 OR ISOTYPE F(ab’)2 CONTROL HAD NO SIGNIFICANT EFFECT ON THE AVERAGE FECAL PELLET WET/DRY RATIO

We next investigated whether chronic treatment of anti-CGRP F(ab’)2 or isotype F(ab’)2 control affected fecal wet to dry ratio on day 29 post-TNBS infusion into the colon. The fecal wet-to-dry ratio was assessed in fecal pellet collected from freely moving adult female rats following a single i.p. administration of anti-CGRP F(ab’)2 (30 mg/kg), isotype F(ab’)2 control (30mg/kg) or vehicle on days 25, 27, and 29 post TNBS/saline infusion into the colon. 1 hour post final administration of compounds or vehicle, fecal pellets produced were weighed and recorded at 30-minute intervals for 1 hour. The results indicated that there was no significant difference in the average wet to dry ratio between vehicle and saline control groups. Anti-CGRP F(ab’)2 or Isotype F(ab’)2 control had no effect on average fecal wet to dry ratio on day 29 post-TNBS infusion into the colon (Fig. 7).

DISCUSSION

The experimental finding in the current study provide compelling evidence that peripheral CGRP–mediated mechanisms play a role in visceral organ cross-sensitization. In a rodent model we confirmed that PS infusion into the bladder induced acute hypersensitivity of
not only the bladder but also in the undamaged colon. Conversely, infusion of TNBS into the colon induced a transient colitis that resulted in chronic post-inflammatory hypersensitivity of both the colon and undamaged urinary bladder. These tissue hypersensitivity responses after PS infusion into the bladder or TNBS infusion into the colon were inhibited by an anti-CGRP F(ab’)2. Specifically, anti-CGRP F(ab’)2 attenuated bladder and colonic hyperalgesia. In an attempt to understand the mechanism(s) underlying the antinociceptive effect of CGRP inhibition, we investigated whether a reduction of visceral hypersensitivity by an anti-CGRP F(ab’)2 may result from inhibition of urothelial and mucosal hyperpermeability, which in turn decreases activation of visceral afferents by sensitizing agents in the lumen or urinary bladder and results in a reduction in the transmission of nociceptive information to the spinal cord. In the current study we showed that bladder and colonic permeability were increased following PS infusion into the bladder. Additionally, in another cohort of animals, infusion of TNBS into the colon induced both colonic and bladder hyperpermeability that persisted beyond the spontaneous resolution of the acute TNBS-induced colitis (Johnson and Greenwood-Van Meerveld, 2017). In further support of our hypothesis, we demonstrated that anti-CGRP F(ab’)2 significantly inhibited both PS and TNBS-induced colonic and urinary bladder hyperpermeability, respectively. These findings suggest that blockade of CGRP signaling reduces visceral pain behaviors emanating from the bladder and colon through visceral organ crosstalk mechanisms that involve effects on visceral organ permeability. These findings are relevance since they offer further insight into novel therapies for patients with comorbid post-inflammatory IBS and BPS/IC.

Although the comorbidity between IBS and BPS/IC is well documented, the etiology of these disorders is incompletely understood and treatment options for patients with IBS and
BPS/IC diagnosed with debilitating and painful symptoms are limited. Previously, we employed rodent models of colonic hypersensitivity induced by either chronic stress in adulthood or following early life stress. In these experimental models, inhibition of CGRP with an anti-CGRP F(ab’)_2 elicited antinociceptive effects via a mechanism involving inhibition of nociceptive signaling at the level of the thoracolumbar spinal cord (Noor-Mohammadi et al., 2021). In the current study, we advanced our earlier studies and investigated whether targeting CGRP is capable of decreasing colonic and bladder hypersensitivity induced by a challenge to another visceral organ. We showed that infusion of PS into the bladder increased both colonic and bladder sensitivity without altering the histologic appearance of the colon and inducing only minimal damage to the bladder urothelium. Conversely, infusion of TNBS into the colon increased colonic and bladder sensitivity in the absence of any overt damage to the bladder. Our findings show that CGRP inhibition attenuates both colonic and bladder hypersensitivity in the PS-induced and post-inflammatory visceral organ crosstalk models. However, CGRP blockade had no effect on colonic hypersensitivity in the active phase of TNBS-induced colitis model, reversing hypersensitivity in only the undamaged urinary bladder and pointing to the possibility that differing mechanism(s) may drive visceral nociception in the presence or absence of inflammation. Although CGRP may modulate immune responses (Holzmann, 2013; Holzer and Farzi, 2014), the differences in efficacy of CGRP inhibition observed in the TNBS-inflamed colon versus the undamaged urinary bladder, taken together with our previous findings in models of stress-induced visceral pain (Noor-Mohammadi et al., 2021), suggest the analgesic effect of anti-CGRP F(ab’)_2 is not the result of decreased local inflammation. Rather, the analgesic effect of anti-CGRP F(ab’)_2 may be through reduction of central
sensitization via blockade of peripheral CGRP, which is a key mediator in transmitting nociceptive information from sensitized visceral afferents to the CNS (Noor-Mohammadi et al., 2021). This hypothesis is supported by studies in rodent models of migraine demonstrating that fremanezumab inhibits the activation of peripheral Aδ fibers that converge on and activate central high threshold neurons, thus disrupting transmission of nociceptive signals to the CNS (Melo-Carrillo et al., 2017a; Melo-Carrillo et al., 2017b). Additionally, quantification of CGRP-immunoreactive myenteric neurons in mouse colon has shown that 20% of intrinsic myenteric neurons contain CGRP (Hibberd et al., 2022). In addition, there are three major morphological types of CGRP-immunoreactive nerve endings in the bladder wall (Sharma et al., 2020). Based on these observations, we hypothesize that CGRP-mediated attenuation of visceral hypersensitivity may be a result of inhibition of CGRP-reactive nociceptors within the gut and a subsequent reduction in second order neuron activation, leading to decreased sensitivity of peripheral and central nociceptive pathways. This inhibition of nociceptive signaling between the periphery and CNS further could then attenuate neurogenic disruption of epithelial permeability within the gut to further decrease visceral afferent sensitization and signaling (Cottrell, 2019). Furthermore, CGRP is implicated in models of visceral pain and is the most abundant peptide in capsaicin sensitive afferent fibers within the gastrointestinal tract of primary afferent neurons (Sternini et al., 1987). CGRP may also interact with other neuropeptides, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) at the level of the gut, DRG and spinal cord to induce colonic hypersensitivity suggesting that CGRP may act as a neuromodulator to induce visceral hypersensitivity (Delafoy et al., 2006). Specifically, the cascade involves BDNF,
which needs NGF, which in turn needs CGRP to induce colonic hypersensitivity (Delafoy et al., 2006).

Evidence suggests that the overlapping symptomology of IBS and BPS/IC may be linked to visceral organ cross-sensitization, during which heightened epithelial permeability of one visceral organ drives increases in the sensitivity of another visceral organ. Abnormal intestinal barrier function and secretory activity have also been implicated in symptomatology in IBS (Camilleri et al., 2012; Bischoff et al., 2014). Furthermore, clinical and preclinical evidence points to the theory that increased colonic permeability leads to sensitization of sensory afferents, increased spinal neuronal excitability, and central sensitization (Zhou and Verne, 2011; Camilleri et al., 2012; Hurst et al., 2015). To explore the role that visceral organ cross-communication plays in the development of visceral pain and the impact CGRP inhibition has on these processes, permeability of the colon and urinary bladder was assessed by electrophysiology in modified Ussing chambers. In agreement with previous studies, we observed that infusion of PS into the bladder or TNBS into the colon significantly decreased TEER in both the infused and adjacent undamaged organ, demonstrated by enhanced urothelial and mucosal permeability. Moreover, in the post-inflammatory cohort, we found that hyperpermeability persists in both the bladder and colon despite the resolution of TNBS-induced colitis (Johnson and Greenwood-Van Meerveld, 2017). This apparent disconnect between inflammation, visceral organ hyperpermeability and hypersensitivity provides evidence for the concept that these pathophysiological changes may be initiated by peripheral inflammation and/or damage but are likely maintained via neurogenic mechanisms related to central sensitization. Administration of anti-CGRP F(ab’)2 normalized permeability in both the colon and bladder of PS and post-inflammatory animals. In contrast, CGRP inhibition
during active colitis normalized bladder permeability but only partially reversed colonic hyperpermeability. The inability of anti-CGRP F(\(ab')_2\) to completely normalize colonic permeability or hypersensitivity during an overt inflammatory phase, further supports the hypothesis that CGRP-mediated attenuation of enhanced visceral nociception plays a role in the restoration of epithelial and urothelial permeability. Furthermore, these differential responses also point to the involvement of mechanism(s) related to the activation of CGRP-insensitive inflammatory pathways in the colon, but not the bladder, during active colitis that continue to drive colonic hyperpermeability and nociception despite inhibition of peripheral CGRP signaling. Alternatively, these experimental findings could be related to the differential expression of CGRP under inflammatory conditions within the colon. Previous studies have demonstrated a significant reduction in neuronal expression of CGRP in the colon during active colitis, potentially due to mass neurotransmitter release or loss of CGRP-containing fibers within the mucosa during the initial stages of inflammation (Miampamba and Sharkey, 1998; Li et al., 2013). In agreement, CGRP-containing afferents in the colon may serve a protective function during inflammation and mitigate mucosal damage in colitis (Evangelista and Tramontana, 1993; Reinshagen et al., 1998; Thompson et al., 2008; de Jong et al., 2015). Further exploration of CGRP-related mechanisms in rodent models of colon-bladder cross-sensitization are required to clearly elucidate the role CGRP plays in the development and maintenance of visceral pain and to further evaluate it as a potentially useful therapeutic target in these indications.

To conclude, although the etiology(ies) of comorbid IBS and BPS/IC remain unclear, a considerable amount of evidence points to the involvement of visceral organ cross-sensitization mechanisms. Previous investigations have implicated CGRP signaling pathways
in this process, making it a potentially valuable target for the treatment of multifocal visceral pain disorders. In the current study, using three clinically relevant rodent models of visceral organ cross-sensitization, we show that administration of an anti-CGRP F(ab’)_2 reverses persistent colonic and urinary bladder hyperpermeability and hypersensitivity following an acute insult to either organ. These data highlight the involvement of CGRP-mediated mechanisms of visceral organ cross-sensitization in the development of comorbid IBS and BPS/IC and support additional evaluations of CGRP signaling as a therapeutic target for these indications.
**Authorship Contributions**

*Participated in research design:* Noor-Mohammadi, Mackenzie, Stratton, Shnider, and Greenwood-Van Meerveld.

*Conducted experiments:* Noor-Mohammadi, Ligon, Mackenzie and Greenwood-Van Meerveld.

*Contributed new reagents or analytic tools:* Noor-Mohammadi, Ligon, Mackenzie, Stratton, Shnider, and Greenwood-Van Meerveld.

*Performed data analysis:* Noor-Mohammadi, Ligon, Mackenzie and Greenwood-Van Meerveld.

*Wrote or contributed to the writing of the manuscript:* Noor-Mohammadi, Ligon, Mackenzie, Stratton, Shnider, and Greenwood-Van Meerveld.
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Footnotes

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Disclosures

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FIGURE LEGENDS

Figure 1. Effect of protamine sulfate (PS) infusion into the bladder, on bladder and colonic hypersensitivity. (A) 24 hrs and (B) 5 days post PS infusion into the bladder, there was a significant increase in the number of withdrawal responses to suprapubic stimulation in vehicle (24 hrs: n=7, 5 days: n=7) and isotype F(ab’)2 control (24 hrs: n=10, 5 days: n=6) rats. Administration of anti-CGRP F(ab’)2 at 30 mg/kg, i.p., (24 hrs: n=8, 5 days: n=6) 24 hrs prior to bladder hypersensitivity assessment, significantly decreased the average withdrawal response frequencies to suprapubic stimulation. (C) 24 hrs and (D) 5 days post PS infusion into the bladder, an increase in colonic sensitivity was shown as an exaggerated visceromotor response to CRD in vehicle (24 hrs: n=7, 5 days: n=7) and isotype F(ab’)2 control (24 hrs: n=10, 5 days: n=6) treated rats and pretreatment with anti-CGRP F(ab’)2 at 30 mg/kg, i.p., (24 hrs: n=8, 5 days: n=6) reversed the effect of PS-induced colonic hypersensitivity, as shown in the significant reduction of the visceromotor response to CRD. Data are expressed as the mean (S.E.M.). Statistical significance was determined using two-way ANOVA followed by a Bonferroni post-test. *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001 compared to PS + Vehicle, +p <0.05, ++p <0.01, +++p <0.001, +++++p <0.0001 compared to PS + isotype control F(ab’)2.

Figure 2. Effect of protamine sulfate (PS) infusion into the bladder, on bladder and colonic permeability. (A) 24 hrs post PS infusion into the bladder, there was no significant difference in
the permeability of the bladder as demonstrated by comparable Transepithelial Electrical Resistance (TEER) values cross all three groups (n=9-10). (B) Anti-CGRP F(ab’)2 (30mg/kg, i.p.) significantly decreased bladder permeability as shown by an increase in TEER 5 days post PS-infusion into the bladder when compared to isotype F(ab’)2 and vehicle treated groups (n=8-9). (C) 24 hrs and (D) 5 days post PS was infused into the bladder, anti-CGRP F(ab’)2 at 30 mg/kg, i.p., (24 hrs: n=10/18, 5 days: n=8/18) induced an increase in TEER when compared to vehicle (24 hrs: n=10/18, 5 days: n=8/15) and isotype F(ab’)2 control (24 hrs: n=9/16, 5 days: n=9/18), indicating a reduction in colonic permeability. Data are expressed as the mean (S.E.M.). Statistical significance was determined using one-way ANOVA followed by a Bonferroni post-test. **p <0.01, ***p <0.001, ****p <0.0001 compared to PS + Vehicle, ++p <0.01, ++++p <0.0001 compared to PS + Isotype F(ab’)2 Control.

**Figure 3.** Effect of intracolonic administration of Trinitrobenzenesulfonic acid (TNBS) on bladder and colonic hypersensitivity 5 days post infusion. (A) Hypersensitivity was assessed in undamaged bladder 5 days after intracolonic TNBS infusion. There was a significant increase in the number of withdrawal responses to suprapubic stimulation in vehicle (n=10) and isotype F(ab’)2 control (n=10) rats when compared to saline controls (n=5). Administration of anti-CGRP F(ab’)2 at 30 mg/kg, i.p., (n=10) 24 hrs prior to bladder hypersensitivity assessment significantly decreased the average withdrawal response frequencies to suprapubic stimulation compared with vehicle-treated animals. (B) 5 days post-intracolonic TNBS infusion, an increase in colonic sensitivity was shown as an exaggerated visceromotor response to CRD in vehicle (n=10) and isotype F(ab’)2 control (n=10) when compared to saline control (n=5) treated rats. Pre-treatment with anti-CGRP F(ab’)2 at 30 mg/kg, i.p., (n=10) had no significant effect on
TNBS-induced colonic hypersensitivity. Data are expressed as the mean (S.E.M.). Statistical significance was determined using two-way ANOVA followed by a Bonferroni post-test. **p <0.01, ****p <0.0001 compared to TNBS + VEH, †p <0.05, ‡‡p <0.001, ‡‡‡‡p <0.0001 compared to TNBS + Isotype F(ab’)2 Control, #p <0.05, ##p <0.01, ####p <0.0001 compared to Saline + VEH.

**Figure 4.** Effect of intracolonic administration of Trinitrobenzenesulfonic acid (TNBS) bladder and colonic permeability 5 days post infusion. (A) Permeability was assessed in undamaged bladder 5 days after intracolonic TNBS infusion. There was a significant increase in the permeability of the bladder shown as the decrease in Transepithelial Electrical Resistance (TEER) in vehicle (n=10) and isotype F(ab’)2 control (n=10) compared with saline controls (n=10). Pre-treatment with anti-CGRP F(ab’)2 at 30 mg/kg, i.p., (n=10) significantly reversed the effect of TNBS-induced bladder hyperpermeability, as assessed via an increase in TEER to the levels seen in Sham controls. (B) 5 days post TNBS infusion into the colon, an increase in colonic permeability was observed as indicated by a decreased in TEER in vehicle (n=10/19) and isotype F(ab’)2 control (n=10/20) compared with saline controls (n=10/20) and pretreatment with anti-CGRP F(ab’)2 at 30 mg/kg, i.p., (n=10/20) reversed the TNBS-induced changes in colonic permeability as assessed via an increase in TEER. (n=X/X denotes the number of animals/number of chambers). Data are expressed as the mean (S.E.M.). Statistical significance was determined using one-way ANOVA followed by a Bonferroni post-test. ***p <0.001, ****p <0.0001 compared to TNBS + VEH, ‡‡‡‡p <0.0001 compared to TNBS + Isotype F(ab’)2 Control, ##p <0.01 compared to Saline + VEH. “ns” denotes not significant.
**Figure 5.** Effect of intracolonic administration of Trinitrobenzenesulfonic acid (TNBS) bladder and colonic hypersensitivity 30 days post infusion. (A) Hypersensitivity was assessed in undamaged bladder 30 days after intracolonic TNBS infusion. There was a significant increase in the number of withdrawal responses to suprapubic stimulation in vehicle (n=7) and isotype F(ab’)2 control (n=7) rats when compared to saline control (n=10). Administration of anti-CGRP F(ab’)2 at 30 mg/kg, i.p., (n=8) on days 25, 27, and 29 post intracolonic TNBS infusion, significantly decreased the average withdrawal response frequencies to suprapubic stimulation at 4-15 g of force compared with vehicle control animals. (B) 30 days post-intracolonic infusion of TNBS, an increase in colonic sensitivity was shown as an exaggerated visceromotor response to CRD in vehicle (n=7) and isotype F(ab’)2 control (n=7) treated rats when compared to saline control (n=10). Pre-treatment with anti-CGRP F(ab’)2 at 30 mg/kg, i.p. (n=8) reversed the effect of TNBS-induced colonic hypersensitivity, as shown in the significant reduction of the visceromotor response to CRD when compared to vehicle and isotype F(ab’)2 control groups. Data are expressed as the mean (S.E.M.). Statistical significance was determined using two-way ANOVA followed by a Bonferroni post-test. *p < 0.05, ***p < 0.001, ****p < 0.0001 compared to TNBS + VEH, +p < 0.05, ++p < 0.01, +++p < 0.001, ++++p < 0.0001 compared to TNBS + Isotype F(ab’)2 Control, #p < 0.05, ###p < 0.001 compared to Saline + VEH. “ns” denotes not significant.

**Figure 6.** Effect of intracolonic administration of Trinitrobenzenesulfonic acid (TNBS) on bladder and colonic permeability, 30 days post infusion. (A) Permeability was assessed in undamaged bladder 30 days after intracolonic TNBS infusion. There was a significant increase in the permeability of the bladder shown as the decrease in Transepithelial Electrical Resistance (TEER) in vehicle (n=10) and isotype F(ab’)2 control (n=10) compared with saline controls.
Administration of anti-CGRP F(ab’)2 at 30 mg/kg, i.p., (n=10) on days 25, 27, and 29 post intracolonic TNBS infusion, significantly reversed the effect of TNBS-induced bladder hyperpermeability, as assessed via an increase in TEER to Sham control levels. (B) 30 days post TNBS infusion into the colon, an increase in colonic permeability was observed as indicated by a decreased in TEER in vehicle (n=10/19) and isotype F(ab’)2 control (n=10/20) compared with saline controls (n=10/20). Pre-treatment with anti-CGRP F(ab’)2 at 30 mg/kg, i.p., (n=10/20) reversed the TNBS-induced changes in colonic permeability as assessed via an increase in TEER. (n=X/X denotes the number of animals/number of chambers). Data are expressed as the mean (S.E.M.). Statistical significance was determined using one-way ANOVA followed by a Bonferroni post-test. **p <0.01, ***p <0.001, ****p <0.0001 compared to TNBS + VEH, ++p <0.01, +++p <0.001, ++++p <0.0001 compared to TNBS + Isotype F(ab’)2 Control. “ns” denotes not significant.

**Figure 7.** Effect of anti-CGRP F(ab’)2 on average fecal wet to dry ratio on day 29 post-TNBS infusion into the colon. Fecal pellet wet to dry ratio was assessed in all groups, 29 days post intracolonic administration of TNBS. There was no significant difference in the average wet to dry ratio between vehicle and saline control groups. Anti-CGRP F(ab’)2 or Isotype F(ab’)2 control had no effect on average fecal wet to dry ratio on day 29 post-TNBS infusion into the colon.
Figure 2

(24-hr Post-PS Infusion)

Bladder

Vehicle
Isotype F(ab')2
Control
Anti-CGRP F(ab')2

(10)
(9)

(8)
(9)

Colon

Vehicle
Isotype F(ab')2
Control
Anti-CGRP F(ab')2

(10/18)
(9/16)

(8/15)
(8/18)
Figure 4

A) Bladder

- VEH
- Isotype F(ab')2
- Anti-CGRP F(ab')2
- Saline + VEH

B) Colon

- VEH
- Isotype F(ab')2
- Anti-CGRP F(ab')2
- Saline + VEH

TEER (Ω.cm²)

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Figure 5

A) Average Number of Withdrawal Reflexes

- TNBS + VEH
- TNBS + Isotype F(ab')2 Control
- TNBS + Anti-CGRP F(ab')2
- Saline + VEH

Force (g)

B) Average Number of Abdominal Contractions

- TNBS + VEH
- TNBS + Isotype F(ab')2 Control
- TNBS + Anti-CGRP F(ab')2
- Saline + VEH

Distension Pressure (mmHg)

Bladder

Colon
Figure 7

Wet/Dry Ratio

0.0  0.5  1.0  1.5  2.0

VEH

Isotype F(ab')2

Control

Anti-CGRP

F(ab')2

Saline + VEH

+ TNBS

7

7

4

10

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