1521-0103/379/3/270–279$35.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

https://doi.org/10.1124/jpet.121.000731
J Pharmacol Exp Ther 379:270–279, December 2021

The Journal of PHARMACOLOGY And Experimental Therapeutics

A Monoclonal Anti–Calcitonin Gene-Related Peptide Antibody Decreases Stress-Induced Colonic Hypersensitivity

Ehsan Noor-Mohammadi, Casey Owen Ligon, Kimberly Mackenzie, Jennifer Stratton, Sara Shnider, and Beverley Greenwood-Van Meerveld

Department of Physiology, University of Oklahoma Health Science Center, Oklahoma City, Oklahoma (E.N.-M., C.O.L., B.G.-V.M); and Teva Pharmaceutical Industries, Ltd., Redwood City, California (K.M., J.S., S.S.)

Received May 14, 2021; accepted September 24, 2021

ABSTRACT

Irritable bowel syndrome (IBS) is a brain-gut disorder characterized by abdominal pain and altered bowel habits. Although the etiology of IBS remains unclear, stress in adulthood or in early life has been shown to be a significant factor in the development of IBS symptomatology. Evidence suggests that aberrant calcitonin gene-related peptide (CGRP) signaling may be involved in afferent sensitization and visceral organ hypersensitivity. Here, we used a monoclonal anti-CGRP divalent antigen-binding fragment [F(ab′)$_2$] antibody to test the hypothesis that inhibition of peripheral CGRP signaling reverses colonic hypersensitivity induced by either chronic adult stress or early life stress. A cohort of adult male rats was exposed to repeated water avoidance stress. Additionally, a second cohort consisting of female rats was exposed to a female-specific neonatal odor-attachment learning paradigm of unpredictable early life stress. Colonic sensitivity was then assessed in adult animals via behavioral responses to colorectal distension (CRD). To analyze spinal nociceptive signaling in response to CRD, dorsal horn extracellular signal–regulated kinase (ERK) 1/2 phosphorylation was measured via immunohistochemistry. Repeated psychologic stress in adulthood or unpredictable stress in early life induced colonic hypersensitivity and enhanced evoked ERK1/2 phosphorylation in the spinal cord after CRD in rats. These phenotypes were reversed by administration of a monoclonal anti-CGRP F(ab′)$_2$ fragment antibody. Stress-induced changes in visceral sensitivity and spinal nociceptive signaling were reversed by inhibition of peripheral CGRP signaling, which suggests a prominent role for CGRP in central sensitization and the development of stress-induced visceral hypersensitivity.

SIGNIFICANCE STATEMENT

Targeting peripheral calcitonin gene-related peptide (CGRP) with a monoclonal anti-CGRP divalent antigen-binding fragment antibody reduced central sensitization and attenuated colonic hypersensitivity induced by either chronic adult stress or early life stress. CGRP-targeting antibodies are approved for migraine prevention, and the results of this study suggest that targeting CGRP may provide a novel treatment strategy for irritable bowel syndrome–related, stress-induced visceral pain.

Introduction

Irritable bowel syndrome (IBS) is a common brain-gut disorder estimated to affect 10%–20% of the North American population. IBS features a constellation of clinical symptoms, including abdominal pain and discomfort, bloating, and abnormal bowel habits (Saito et al., 2002; Longstreth, 2005, 2006). Currently, the etiology of this disorder remains unclear, although evidence suggests that stress is a trigger for the development of IBS symptomatology. Clinical studies have demonstrated that patients with IBS frequently report high levels of lifetime stress, which correlates with worsening of symptoms (Prusator et al., 2016). Early life stress (ELS) has also been implicated as a potential risk factor for IBS, with a substantial percentage of diagnosed patients reporting instances of childhood trauma or abuse (Drossman, 1997; Nickel et al., 2011; van Tilburg, 2011; Bradford et al., 2012; Tran et al., 2013). These findings are supported by several preclinical studies in animal models linking both adult stress and ELS to IBS symptomatology (Coutinho et al., 2002; Bradesi et al., 2005; Myers and Greenwood-Van Meerveld, 2012; Tran et al., 2012; Chaloner and Greenwood-Van Meerveld, 2013; Ackerman et al., 2015; Prusator and Greenwood-Van Meerveld, 2015; Mohammadi et al., 2016; Wang et al., 2017). Because of the extraordinarily complex etiology of IBS, there is currently a paucity of effective therapies for the treatment of this chronic pain disorder. Given the negative impact of IBS on quality of life and its financial burden on society, there is an obvious need for additional therapeutic options for relief of IBS-associated abdominal pain (Drossman et al., 2002; Pare et al., 2006; Davis et al., 2014).

Calcitonin gene-related peptide (CGRP) is a 37-amino-acid member of the calcitonin family of peptides produced in both

This work was supported by Teva Pharmaceutical Industries, Ltd., Redwood City, CA.
K.M., J.S., and S.S. are full-time employees of Teva Pharmaceutical Industries, Ltd.
dx.doi.org/10.1124/jpet.121.000731.

ABBREVIATIONS: CRD, colorectal distension; CGRP, calcitonin gene-related peptide; ELS, early life stress; ERK, extracellular signal–regulated kinase; F(ab′)$_2$, divalent antigen-binding fragment; Fc, fragment crystallizable; GI, gastrointestinal; IBS, irritable bowel syndrome; IR, immunoreactive; L, lumbar; pERK, phosphorylated ERK; PND, postnatal day; T, thoracic; VEH, vehicle; VMR, visceromotor response; WAS, water avoidance stress.

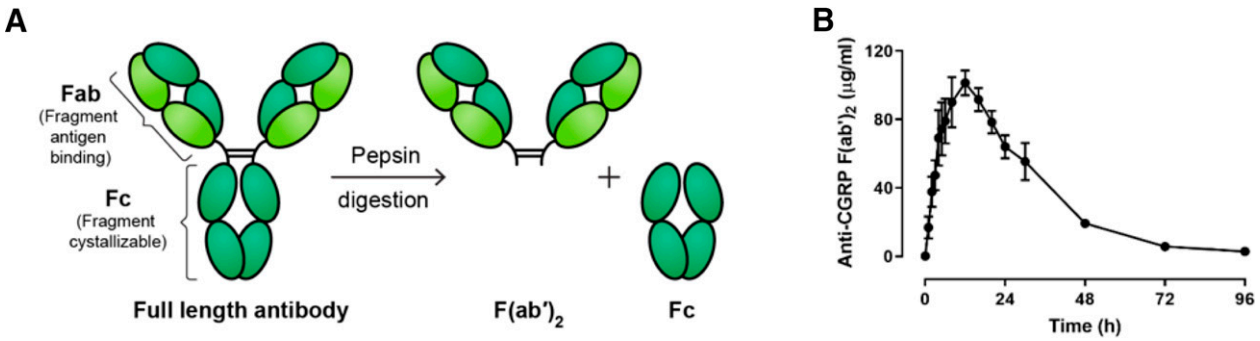

Fig. 1. Generation and pharmacokinetic characterization of human anti-CGRP F(ab′)$_2$. (A) Schematic representation of full-length antibody digestion with pepsin. (B) Pharmacokinetic profile of human anti-CGRP F(ab′)$_2$ in rats (n = 5 per group). Serum concentrations of the anti-CGRP F(ab′)$_2$ fragments were measured by ELISA after a single intraperitoneal administration of 30 mg/kg. Data are expressed as the mean (S.E.M.).

peripheral and central neurons, which functions as a potent vasodilator and plays a role in the transmission of nociception (Rosenfeld et al., 1983; Brain et al., 1985; Chen et al., 2010). In the gastrointestinal (GI) tract specifically, CGRP is widely distributed throughout the enteric nervous system from the esophagus to the rectum in both intrinsic and extrinsic primary afferent neurons of various mammalian species, although it should be noted that CGRP expression levels vary significantly depending on the species and the subregion of the GI tract (Sundler et al., 1991; Domoto et al., 1992; Timmermans et al., 1992; Wolf et al., 2007; Makowska and Gonkowski, 2018). Furthermore, CGRP is known as an important factor in the transmission of sensory and pain stimuli and numerous other aspects of GI physiology, including motility, secretion, and neuroprotection (Timmermans et al., 1992; Rytel and Calka, 2016; Makowska et al., 2017). Current evidence suggests that CGRP-mediated visceral organ afferent sensitization may contribute to abdominal and pelvic pain associated with inflammatory conditions, such as colitis and cystitis (Qiao and Grider, 2007; Pan et al., 2010; Ceuleers et al., 2018; Utsumi et al., 2018). For instance, in a rodent model of colitis, CGRP-immunoreactive nerve density is significantly increased in rat primary afferent pathways (Qiao and Grider, 2009). Additionally, in inflammatory models that exhibit visceral hypersensitivity, administration of a CGRP receptor antagonist [human CGRP-(8-37)] has demonstrated reduction in visceral hypersensitivity (Plourde et al., 1997; Delafoy et al., 2006). However, it remains unknown whether a peripheral CGRP-mediated mechanism is involved in stress-induced visceral pain in models lacking overt GI inflammation as observed in patients with IBS. Thus, to gain a better understanding of the involvement of peripheral CGRP in visceral pain associated with stress, we examined the effect of an anti-CGRP divalent antigen-binding fragment [F(ab′)$_2$] antibody on enhanced visceral nociception in two clinically relevant rodent models of stress-induced visceral pain. In one model, colonic hypersensitivity was induced in adult male rats via exposure to a repeated psychologic stressor (Bradesi et al., 2005; Tran et al., 2013). In a second model, these same phenotypes were induced in adult female rats after exposure to an unpredictable odor-attachment learning paradigm during the neonatal period (Chaloner and Greenwood-Van Meerveld, 2013; Prusator and Greenwood-Van Meerveld, 2015). In both models, peripheral CGRP blockade attenuated stress-induced pain behaviors and inhibited evoked spinal extracellular signal–related kinase (ERK) 1/2 phosphorylation [phosphorylated ERK (pERK)], which suggests that CGRP plays a significant role in stress-induced visceral nociception and central sensitization.

Methods

Preparation of F(ab′)$_2$ Fragment Antibody

F(ab′)$_2$ fragment antibody was used as a tool to investigate the selective targeting of peripheral CGRP without the possible immunomodulatory effects of the fragment crystallizable (Fc) region of the full-length antibody in the tested animal models. Briefly, fremanezumab (fully humanized anti-CGRP monoclonal antibody; Teva Pharmaceuticals, Redwood City, CA) and isotype control antibody (Teva Pharmaceuticals) 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 nonreducing conditions. Similar binding affinity of anti-CGRP F(ab′)$_2$ to CGRP compared with full-length anti-CGRP was confirmed by surface plasmon resonance.

Pharmacokinetics of Anti-CGRP F(ab')$_2$

Human anti-CGRP F(ab′)$_2$ (30mg/kg) was administered once via intraperitoneal route to male Sprague-Dawley rats purchased from Charles River Laboratories (Wilmington, MA). Serial blood samples were collected into serum tubes and stored at room temperature for at least 30 minutes to allow for clotting. Samples were processed to serum by centrifugation (3500 rpm for 10 minutes at 4°C) within 1 hour of collection, and serum was stored at −80°C until analysis. The concentration of anti-CGRP F(ab′)$_2$ in the serum was quantified using a Human Kappa ELISA Kit (Abcam, Cambridge, MA).

Chronic Adult Stress Induced by Water Avoidance Stress

Animals. Experiments were performed on male Fischer 344 rats (250–350 g) purchased from Charles River Laboratories (Wilmington, MA). Rats were housed two per cage with free access to food and water at 21–23°C and 12-hour light/dark cycle within the University of Oklahoma Health Sciences Center Department of Comparative Medicine animal facility in Oklahoma City, OK. All animals were acclimated to facility housing for a minimum of 1 week before experimentation. To further minimize experimental stress, the rats were brought to the laboratory for an additional week to acclimate to the laboratory

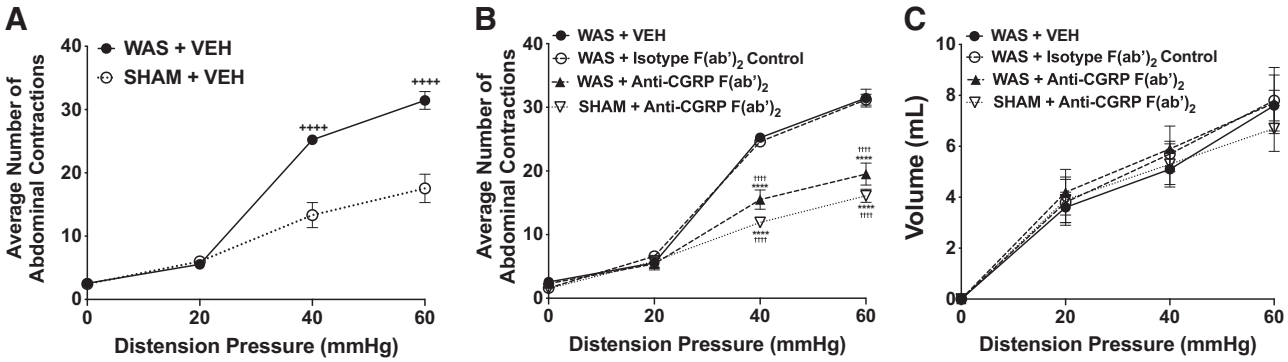

Fig. 2. Effect of repeated WAS on visceral sensitivity. (A) Number of abdominal contractions exhibited by adult male rats receiving CRD after 10 days of WAS was significantly ($P < 0.0001$) higher than those in animals exposed to SHAM ($n = 10$ per group). (B) Rats with colonic hypersensitivity after exposure to WAS showed decreased VMR upon administration of anti-CGRP F(ab′)$_2$ at 30 mg/kg, i.p., 24 hours prior to the CRD assessment compared with VEH and isotype F(ab′)$_2$ control–dosed rats ($P < 0.0001$) ($n = 10$ per group). (C) Administration of anti-CGRP F(ab′)$_2$ does not alter colonic compliance. Pressure-volume curves, as a measure of colonic compliance, were similar in all groups. Data are expressed as the mean (S.E.M.). Statistical significance was determined using two-way ANOVA followed by a Bonferroni post-test. $^{++++}P < 0.0001$ compared with SHAM + VEH, $^{****}P < 0.0001$ compared with WAS + VEH, and $^{\dagger\dagger\dagger\dagger}P < 0.0001$ compared with WAS + isotype F(ab′)$_2$ control.

environment and animal handlers. All experimental procedures were approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee (Protocol 20-001-FH).

Water Avoidance Stress Paradigm. Rats in the water avoidance stress (WAS) group were placed on a square platform (8 × 8 × 8 cm) mounted in the center of a white semitransparent plastic container (50 × 35 × 33 cm) filled with fresh, room-temperature water to 1 cm below the surface of the platform. Animals in the SHAM stress group were placed in containers without water. All animals were left undisturbed for 60 minutes, and the number of fecal pellets produced during the WAS or SHAM stress was recorded to evaluate stress-induced autonomic outflow (unpublished data). The WAS or SHAM procedure was repeated for 10 consecutive days.

Odor-Attachment Learning Model of ELS

Animals. Timed-pregnant Long-Evans rats were purchased from Charles Rivers Laboratories (Wilmington, MA) to arrive on embryonic day 9. The dams were housed on aspen wood shavings at 23°C on a 12-hour light/dark cycle (7:00 AM to 7:00 PM). Food (5053 Irradiated PicoLab Rodent Diet; LabDiet, St. Louis, MO) and water were available ad libitum. One day after parturition, designated postnatal day (PND) 1, litters were sexed, crossfostered, and culled to a maximum of 12 female pups, with a minimum of 8 pups, per litter. From PND 8–12, female pups were exposed to an odor-shock conditioning paradigm as previously described (Chaloner and Greenwood-Van Meerveld, 2013). In this study, pups were classically conditioned by an unpaired (unpredictable) odor-shock presentation or by an odor-only presentation (control). The exposure to an unpredictable stressor during the neonatal period induces colonic hypersensitivity in adult female rats only, whereas other ELS models, such as maternal separation or limited nesting material, seemingly produce hypersensitivity exclusively in adult male animals.

ELS Paradigm. An ELS daily conditioning paradigm consisted of 11 trials of a 30-second peppermint odor presentation with a 4-minute interval. Peppermint odor was administered using peppermint oil (Thermo Fisher Scientific, Waltham, MA) vaporized at a 1:10 odor concentration in a flow dilution olfactometer (Med Associates, Georgia, VT) set to a delivery rate of 2 l/min. Unpredictable odor-shock pups received a 0.5-mA shock (Coulbourn Instruments, Whitehall, PA) to the base of the tail 2 minutes after odor presentation. Odor-only pups were exposed to a 30-second peppermint odor presentation with no subsequent shock. The behavioral activation of the pups was recorded during each conditioning trial. A scale of 0–5 was used to score behavioral activation, in which 0 represented no movement and 5 represented movement of all four limbs and the head. Preodor activation scores were compared with odor activation scores as an indication of a learned response. Y-maze testing was used on PND 13 to assess the development of a learned odor preference. Pups underwent five trials wherein they were placed in one arm of the maze and given 1 minute to choose between an arm containing fresh aspen shavings and an arm containing peppermint odor. Failure to make a choice would be scored as "no choice," and pups that accumulated three "no choice" scores would be removed from the study. In the current study, no animals were removed because of the failure to make an odor choice. Animals were weaned on PND 22 and housed two per cage according to ELS treatment. All experimental procedures were approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee (Protocol 20-001-FH).

Colonic Sensitivity

Colonic sensitivity in adult animals (~PND 90) was assessed as a measurement of the visceromotor response (VMR) to colorectal distension (CRD) as described previously (Johnson et al., 2015; Prusator and Greenwood-Van Meerveld, 2016a). After overnight fasting, animals were anesthetized with 2%–5% isoflurane, and a 5-cm latex balloon catheter was inserted up to 11 cm beyond the anal verge and secured to the base of the tail with surgical tape. After a 30-minute recovery period, the catheter was connected to a Distender Series IIR barostat (G & J Electronics Inc., North York, Canada) for controlled, isobaric, colonic distensions at randomized pressures of 0, 20, 40, and 60 mmHg. Each colonic distension period lasted 10 minutes each with a 10-minute recovery period between each distension. Colonic compliance was assessed in each rat by using the

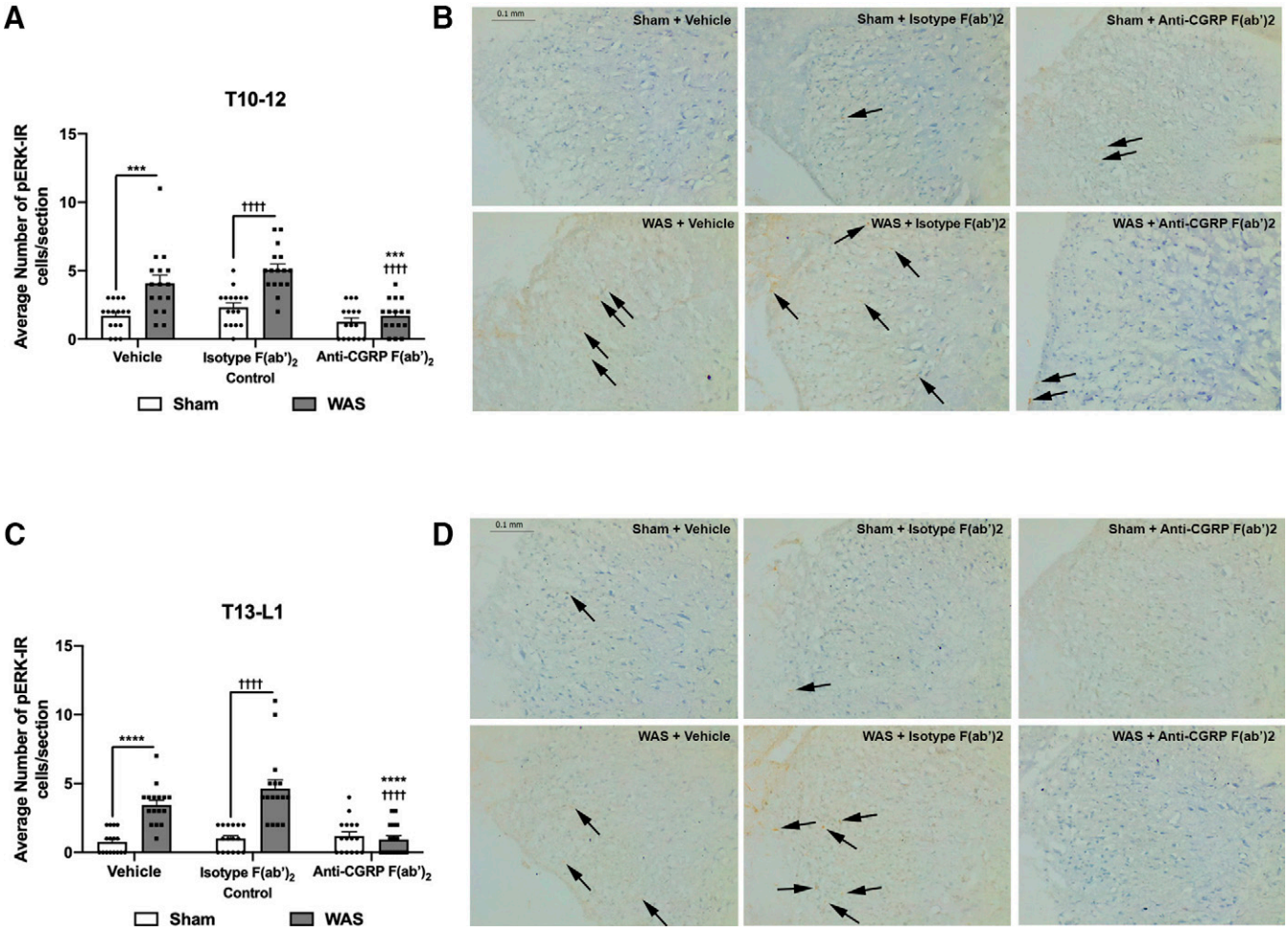

Fig. 3. Effect of repeated WAS on nociceptive signaling in the dorsal horn of the spinal cord. After repeated exposure to WAS, there was a significant increase ($P < 0.001$) in neuronal activation in the dorsal horn of the spinal cord in response to noxious CRD when compared with that in SHAM ($n = 10$ per group). (A–D) Administration of anti-CGRP F(ab′)$_2$ at 30 mg/kg, i.p., 24 hours prior to CRD assessment significantly reduced the number of pERK-IR–activated neurons (black arrows) in the T10–T12 (A and B) and T13–L1 (C and D) spinal cord compared with that in VEH and isotype F(ab′)$_2$ control–dosed rats ($P < 0.0001$) ($n = 10$ per group). Data are expressed as the mean (S.E.M.). Statistical significance was determined using two-way ANOVA followed by a Bonferroni post-test. ***$P < 0.001$ and ****$P < 0.0001$ compared with WAS + VEH; $^{††††}P < 0.0001$ compared WAS + isotype F(ab′)$_2$ control.

pressure-volume data generated by the Distender Series IIR barostat. All experiments occurred between 10:00 AM and 2:00 PM.

pERK Immunohistochemistry. Immediately after the final CRD, the rat was anesthetized with isoflurane (5%) and underwent transcardial perfusion with ice-cold PBS, which was followed by ice-cold 4% paraformaldehyde in PBS. After transcardial perfusion, the thoracolumbar [thoracic (T) 10 to lumbar (L) 1] regions of the spinal cord were removed and postfixed for 18 hours at 4°C in 4% paraformaldehyde in PBS. After fixation, the spinal cord was cryoprotected in 30% sucrose in PBS overnight at 4°C and then block-frozen in optimal cutting temperature compound. Frozen sections (10 μm) were cut using a cryostat and placed on Fisherbrand Superfrost Plus Microscope Slides (Fisher Scientific, Pittsburg, PA) for future immunohistochemical analysis. Frozen sections were air-dried for 20 minutes, postfixed in 4% paraformaldehyde for 10 minutes, and then washed three times with PBS. The sections were incubated with Biocare's Rodent Block R (Catalog# RBR962H, Biocare Medical, Pacheco, CA) for 20 minutes at room temperature to block nonspecific binding of antibodies. After blocking, the sections were washed three times with 0.2% Triton-X-PBS and then incubated overnight at 4°C with anti–phospho- p44/42 mitogen-activated protein kinase (ERK1/2) (Thr202/Tyr204) antibody (pERK; 1:400, 4370; Cell Signaling Technology, Danvers, MA) diluted in Biocare Da Vinci Green Diluent (Cat# PD900H, Biocare Medical, Pacheco, CA). Sections were then washed three times with 0.2% Triton-X-PBS prior to incubation for 1 hour at room temperature with secondary donkey anti-rabbit IgG HRP antibody diluted in Biocare Da Vinci Green Diluent (donkey anti-rabbit IgG; 1:500, Cat# 711-035-152, Jackson ImmunoResearch Laboratories Inc. West Grove, PA). Sections were washed three times with PBS, incubated with Betazoid DAB chromogen kit (Cat# BDB2004H, Biocare Medical, Pacheco, CA) at room temperature for 5 minutes, washed with PBS, and rinsed in deionized water. Sections were counterstained with hematoxylin. Two sections per region of the spinal cord (T10–T12; T13–L1) per animal were randomly selected, and pERK-immunoreactive cells in the dorsal horn of each section were identified and

counted using a Zeiss Axiovert epifluorescence microscope (Zeiss, Jena, Germany).

Drug Administration

Anti-CGRP F(ab′)$_2$, isotype F(ab′)$_2$ control, and vehicle were supplied by Teva Pharmaceuticals and stored at 4°C. To reduce possible immunomodulatory effects of the Fc region of the full-length antibody in the tested animal models, F(ab')$_2$ fragment antibody prepared through pepsin digestion of the full-length antibody was used. F(ab′)$_2$ fragment antibodies have two F(ab) portions linked together by disulfide bonds and have a molecular mass of ∼110 kDa (Fig. 1A). Rats received a single intraperitoneal administration of anti-CGRP F(ab′)$_2$ (30 mg/kg), isotype F(ab′)$_2$ control (30 mg/kg), or vehicle (saline) 24 hours prior to measuring colonic sensitivity.

Statistical Analysis and Experimental Rigor

All data are presented as mean ± S.E.M. A power analysis was performed to determine the minimum number of animals required for the study. A single experimenter followed all animals during the study and was anonymized to treatments. All experiments were conducted in a randomized order. Colonic sensitivity was recorded as the average number of abdominal contractions and analyzed with a two-way ANOVA followed by a Bonferroni multicomparison post-test. Spinal cord signaling was quantified as the average number of pERK-immunoreactive (IR) neurons observed in the dorsal horn region of each section of the spinal cord. Statistical significance was determined using two-way ANOVA, which was followed by a Bonferroni post-test. A two-way repeated-measures ANOVA was used to analyze the mean behavioral activation during conditioning with odor presentation and trial number as factors. An unpaired t test was used to analyze odor preference, and a one-way ANOVA was used to analyze weaning weights (n = 10–11 per group).

Results

Pharmacokinetics of Anti-CGRP F(ab′)$_2$

To reduce possible immunomodulatory effects of the Fc region of the full-length antibody in the tested animal models, F(ab′)$_2$ fragment antibody was used as an experimental tool to determine the specific effect of peripheral CGRP inhibition. Fremanezumab (anti-CGRP antibody) and isotype control were digested with pepsin, and the resulting F(ab′)$_2$ fragments containing two antigen-binding–fragment portions normally linked together by disulfide bonds at the hinge were purified (Fig. 1A). Human anti-CGRP F(ab′)$_2$ (30 mg/kg, i.p.) was administered to rats, and F(ab′)$_2$ concentrations were measured in serial serum samples by ELISA (Fig. 1B).

Anti-CGRP F(ab′)$_2$ Inhibits Colonic Hypersensitivity Induced by WAS without an Effect on Colonic Compliance

For our first series of experiments, we employed a rodent model of repeated WAS to investigate whether peripheral CGRP blockade with anti-CGRP F(ab′)$_2$ could inhibit stress-induced visceral nociceptive behaviors. We demonstrated that 24 hours after the final stress procedure induced by water avoidance, there was a significant increase in a VMR to CRD quantified as the number of abdominal contractions during the distention period when compared with SHAM-treated animals ($F_{[3,\ 64]}$ = 108.3, $P < 0.0001$) (Fig. 2A). In a second cohort of rats pretreated with anti-CGRP F(ab′)$_2$ (30mg/kg, i.p.), there were a significantly reduced number of abdominal contractions induced by WAS when compared with vehicle or isotype F(ab′)$_2$ control groups ($F_{[9,\ 140]}$ = 18.88, $P < 0.0001$) (Fig. 2B). Compliance of the colonic musculature was assessed in each rat via the pressure-volume relationship; in rats previously exposed to WAS, colonic compliance was similar to that in rats exposed to SHAM treatment, with a nonsignificant effect of drug/vehicle (VEH) treatment on colonic compliance ($F_{[2,65]}$ = 0.09; P = 0.41.) (Fig. 2C). Furthermore, no significant change in CRD-evoked VMR responses and colonic compliance was observed in SHAM animals treated with anti-CGRP F(ab′)$_2$, isotype F(ab′)$_2$ control, and vehicle (unpublished data).

Anti-CGRP F(ab′)$_2$ Inhibits Thoracolumbar Spinal Cord Signaling Induced by CRD in Rats Previously Exposed to WAS

To investigate the effect of anti-CGRP F(ab′)$_2$ on spinal cord dorsal horn neuronal signaling in response to CRD, we analyzed neuronal responses via pERK immunohistochemistry in the adult male rats previously exposed to WAS or SHAM stress controls. Our findings revealed that there was a significant increase in pERK-IR neurons in the thoracolumbar segment of the spinal cord, which innervates the colon via the lumbar splanchnic nerve (Brierley et al., 2018), immediately after noxious CRD in rats exposed to WAS compared with SHAM ($F_{[2,\ 90]}$ = 18.91, $P < 0.0001$) and anti-CGRP F(ab′)$_2$ pretreatment significantly reduced the number of pERK-IR neurons compared with vehicle and isotype F(ab′)$_2$ control–treated rats, as illustrated in Fig. 3, A–D ($F_{[2,\ 90]} = 11.75, P < 0.0001$).

Rodent Model of ELS: Behavioral Activation, Odor Preference, and Weaning Weights

Female pups underwent neonatal conditioning from PND 8–12. The conditioning paradigm involved an assessment of behavioral activation scores prior to and during presentation of the conditioned peppermint odor (Davis et al., 2014). The pups in the odor-only control and unpredictable odor-shock conditioning groups showed no differences in behavioral activation upon exposure to the odor, which indicates that no association was made between the odor and the aversive stimuli (Fig. 4, A and B). Y-maze testing revealed no significant effect of ELS treatment on odor preference in both odor-only animals and unpredictable ELS animals, which confirms a lack of a learned odor preference in both groups (Fig. 4C). Upon weaning, pups were weighed to ensure that the neonatal conditioning had no effect on body weight. No significant differences between weaning weights were observed between odor-only controls and pups subjected to unpredictable ELS, which indicates the animals exposed to unpredictable ELS showed no association between odor-shock and did not develop an odor preference (Fig. 4D).

Anti-CGRP F(ab′)$_2$ Inhibits Colonic Hypersensitivity in Adult Animals after Neonatal Unpredictable ELS without an Effect on Colonic Compliance

We next investigated whether anti-CGRP F(ab′)$_2$ treatment would affect the increased VMR to CRD in adult female rats previously exposed to unpredictable ELS. Colonic sensitivity was assessed in freely moving adult female rats after a single

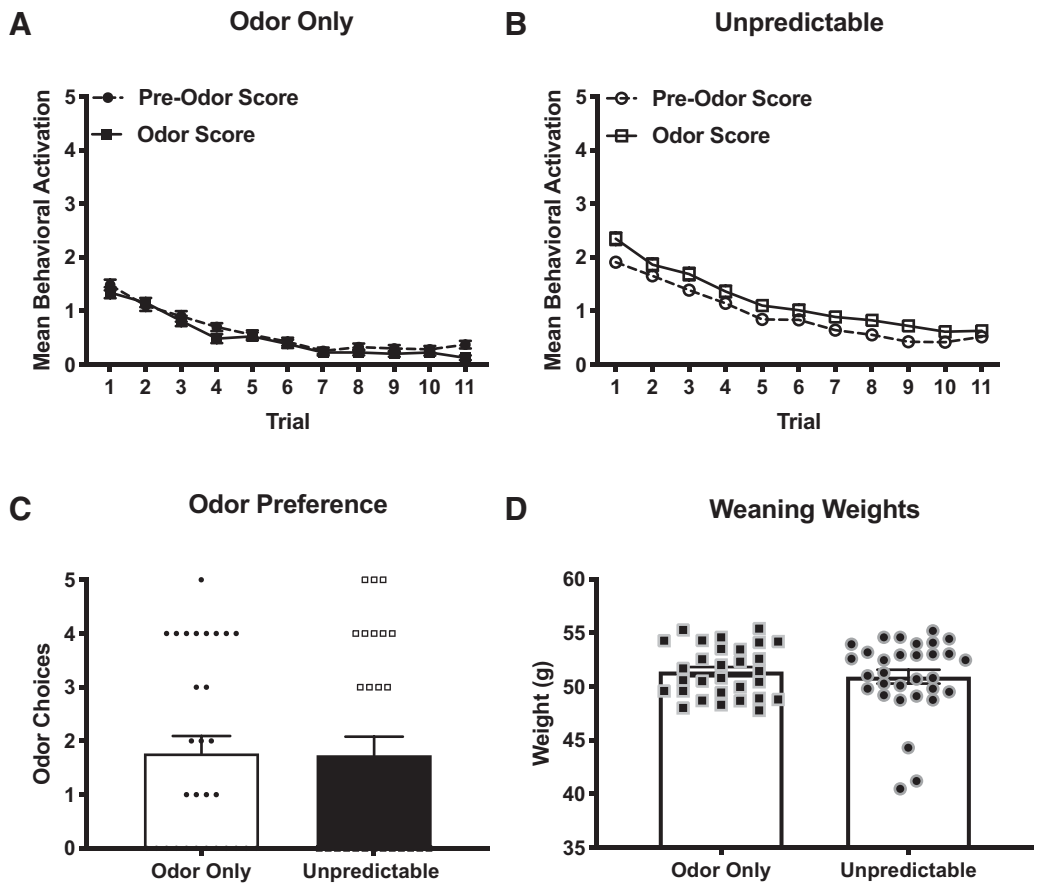

Fig. 4. Neonatal learning acquisition in response to classic conditioning. Neonatal behavioral activation was recorded during each trial from PND 8–12 during odor presentation for pups experiencing (A) odor only (n = 10) and (B) unpredictable odor-shock (n = 11) and compared with their prestimulus score on that same day. Animals experiencing odor-only control or unpredictable odor-shock exhibited no increases in behavioral activation during odor presentation. (C) Odor preference analysis using the Y-maze between the odor-only controls and unpredictable odor shock showed an absence of odor preference. (D) An unpaired t test was used to analyze odor preference, and a one-way ANOVA was used to analyze weaning weights. Data are expressed as the mean (S.E.M.).

administration of anti-CGRP F(ab′)$_2$ (30 mg/kg, i.p.), isotype F(ab′)$_2$ control (intraperitoneal), or vehicle. Analysis of the behavioral response to distension of the colon of vehicle-treated animals revealed a significant main effect of distension pressure ($F_{[3, 57]} = 341.3$, $P < 0.0001$) and ELS ($F_{[1, 19]} = 108.3$, $P < 0.0001$) with a significant interaction between the two factors ($F_{[3, 57]} = 60.71$, $P < 0.0001$). As shown in Fig. 5A, vehicle-treated animals previously exposed to unpredictable ELS exhibited a significantly higher number of abdominal contractions at distension pressures of 40 ($P < 0.0001$) and 60 mmHg ($P < 0.0001$) when compared with odor-only controls, which confirms that unpredictable ELS induces colonic hypersensitivity. In animals previously exposed to unpredictable ELS, a main effect of distension pressure ($F_{[2.1, 94.8]} = 437.6$, $P < 0.0001$) and drug treatment ($F_{[4, 46]} = 26.87$, $P < 0.0001$) was observed. Post hoc analysis revealed that animals treated with anti-CGRP F(ab′)$_2$ demonstrated significantly fewer abdominal contractions in response to CRD at distension pressures of 40 ($P < 0.001$) and 60 mmHg ($P < 0.05$) compared with vehicle-treated animals and those in the isotype F(ab′)$_2$ control, and there was no effect on animals in odor-only controls (Fig. 5B). The colonic compliance as assessed via pressure-volume relationships in rats previously exposed to unpredictable ELS were similar to that in rats exposed to odor-only controls with a nonsignificant effect of drug/VEH treatment ($F_{[1,76]} = 1.22$; $P = 0.27$.) (Fig. 5C). Furthermore, no significant change in CRD-evoked VMR responses and colonic compliance was observed in SHAM animals treated with anti-CGRP F(ab′)$_2$, isotype F(ab′)$_2$ control, and vehicle (unpublished data).

Anti-CGRP F(ab′)$_2$ Inhibits Thoracolumbar Spinal Cord Signaling Induced by CRD in Rats Previously Exposed to Neonatal Unpredictable ELS

Our next series of experiments were designed to probe the effect the anti-CGRP F(ab′)$_2$ on CRD-induced spinal cord pERK expression in adult female rats previously exposed to an unpredictable ELS. Our findings revealed that there was a significant increase in pERK-IR neurons in the thoracolumbar segment of the spinal cord immediately after noxious CRD in rats exposed to an unpredictable ELS compared with those in the odor-only control ($F_{[2, 90]} = 18.91$, $P < 0.0001$). In rats treated with the anti-CGRP F(ab′)$_2$, there was a significant reduction ($F_{[2, 90]} = 11.75$, $P < 0.0001$) in the number of pERK-IR neurons compared with those in vehicle and isotype F(ab′)$_2$ control–treated rats as illustrated in Fig. 6, A–D.

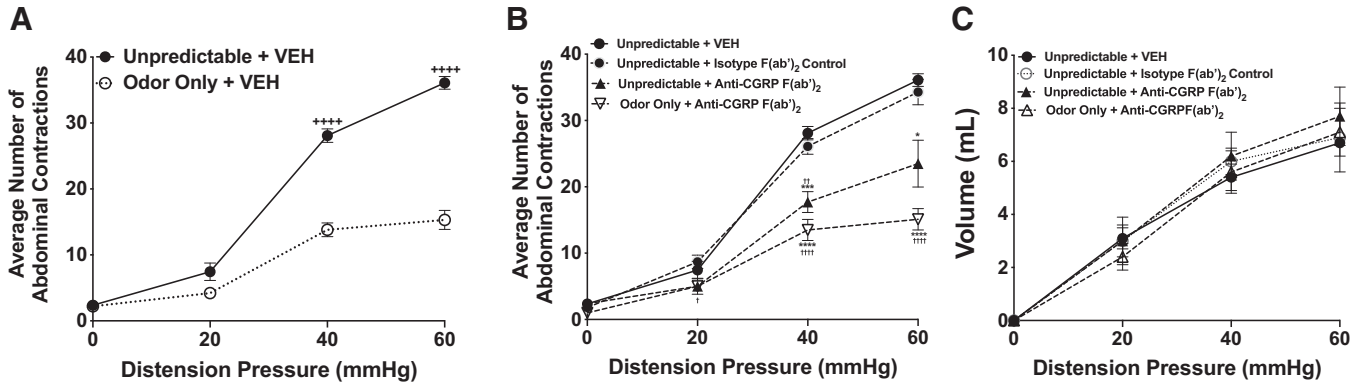

Fig. 5. Effect of unpredictable ELS on visceral sensitivity. After unpredictable ELS, there was a significant increase ($P < 0.001$) in visceral sensitivity ($n = 10$). (A) VEH-treated rats previously exposed to unpredictable ELS exhibited an increased number of abdominal contractions compared with those in VEH-treated odor-only controls ($P < 0.0001$) in response to CRD. (B) In animals exposed to neonatal unpredictable ELS, treatment with anti-CGRP F(ab′)$_2$ (30 mg/kg, i.p.) in adulthood significantly reduced the number of abdominal contractions in response to CRD when compared with that in isotype F(ab′)$_2$ and vehicle control–treated animals ($n = 10$–11 per group). (C) Administration of anti-CGRP F(ab′)$_2$ does not alter colonic compliance. Pressure-volume curves, as a measure of colonic compliance, were similar in all groups. Data are expressed as the mean (S.E.M.). Statistical significance was determined using two-way ANOVA followed by a Bonferroni post-test. $^{++++}P < 0.0001$ compared with SHAM + VEH; $^{*}P < 0.05$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ compared with ELS + VEH; and $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger\dagger}P < 0.0001$ compared ELS + isotype F(ab′)$_2$ control.

Discussion

The aim of this study was to evaluate whether antibody-mediated peripheral blockade of CGRP prevented stress-induced visceral pain in adulthood. Using clinically relevant rodent models of repeated WAS and unpredictable ELS previously reported to induce visceral hypersensitivity (Camp and Rudy, 1988; Cámara-Lemarroy et al., 2016), we provide evidence that peripheral CGRP blockade attenuates colonic hypersensitivity and inhibits neuronal activation in the dorsal horn of the thoracolumbar spinal cord in response to noxious colonic distension as assessed via quantification of pERK-IR neurons. These findings suggest that peripheral CGRP plays a key role in the development of stress-induced colonic hypersensitivity.

Previous studies have reported that prolonged psychologic stress is associated with a myriad of chronic pain conditions, including disorders that feature visceral pain, such as IBS (Lampe et al., 2003; Blanchard et al., 2008; Johnson and Greenwood-Van Meerveld, 2014). Stress exposure is believed to induce a "top-down" sensitization of the central nervous system resulting from maladaptive changes in signaling pathways that modulate nociception. These signaling pathways interact with a multitude of neurotransmitters, including glucocorticoids, corticotropin-releasing factor, serotonin, glutamate, GABA, and endocannabinoids, to promote enhanced pain perception (Mora et al., 2012; Timmermans et al., 2013; Grace et al., 2014; Johnson and Greenwood-Van Meerveld, 2014). However, the role CGRP plays in stress-induced chronic pain is less clear, particularly in visceral pain. In this study, we sought to determine the involvement of CGRP signaling in the development of stress-induced colonic hypersensitivity, a key feature observed in patients with abdominal pain. To accomplish this, we used repeated, daily exposure to WAS, a well validated chronic-stress paradigm that has been shown to alter stimulus-evoked neuronal activation and nociceptive signaling pathways and induces long-lasting visceral hypersensitivity in adult rats (Eutamene et al., 2010; Myers and Greenwood-Van Meerveld, 2012; Nash et al., 2012; Tran et al., 2013). It should be noted that WAS is a homotypic stressor, and therefore, rats exposed to repeated WAS may potentially habituate to the stressor. However, measurements of fecal pellet output during stress exposure suggest that the WAS paradigm induces sustained stress axis activation.

CGRP has been implicated in the pathogenesis of several chronic pain conditions, including arthritis, IBS with diarrhea, and fibromyalgia (Walsh et al., 2015; Liang et al., 2016; Iyengar et al., 2017; Schou et al., 2017). Recent approval of anti-CGRP ligand antibodies and anti-CGRP receptor monoclonal antibodies for migraine prevention has increased interest in CGRP-targeted–pathway treatment strategies for chronic pain. Taking advantage of the use of an anti-CGRP ligand binding F(ab')$_2$ fragment antibody, we report that peripheral CGRP plays a key role in stress-induced visceral pain. In agreement with previous studies, we found that repeated WAS exposure induced colonic hypersensitivity in adult male rats. Anti-CGRP F(ab')$_2$ treatment 24 hours prior to CRD assessment completely inhibited colonic sensitivity compared with vehicle treatment. Moreover, building on earlier work performed in rodent models of visceral hypersensitivity demonstrating increased spinal ERK phosphorylation in response to noxious CRD (Castro et al., 2013; Mohammadi et al., 2018), we observed a significant increase in pERK immunoreactivity in the thoracolumbar dorsal horn of the spinal cord in rats exposed to a repeated WAS compared with those in SHAM controls. Next, we investigated whether blockade of peripheral CGRP signaling resulted in inhibition of peripheral sensory signaling to the spinal cord to reduce central sensitization and thus visceral hypersensitivity. In rats exposed to WAS, administration of anti-CGRP F(ab')$_2$ decreased CRD-induced pERK expression in the thoracolumbar spinal cord, specifically in the superficial lamina of the dorsal horn, which is the major site of afferent terminations responding to nociceptive input from the colon. These findings suggest that repeated stress may modulate neuronal activation in response to stimuli via a CGRP-related mechanism to increase transmission of nociceptive signals to the spinal cord, resulting in visceral hypersensitivity.

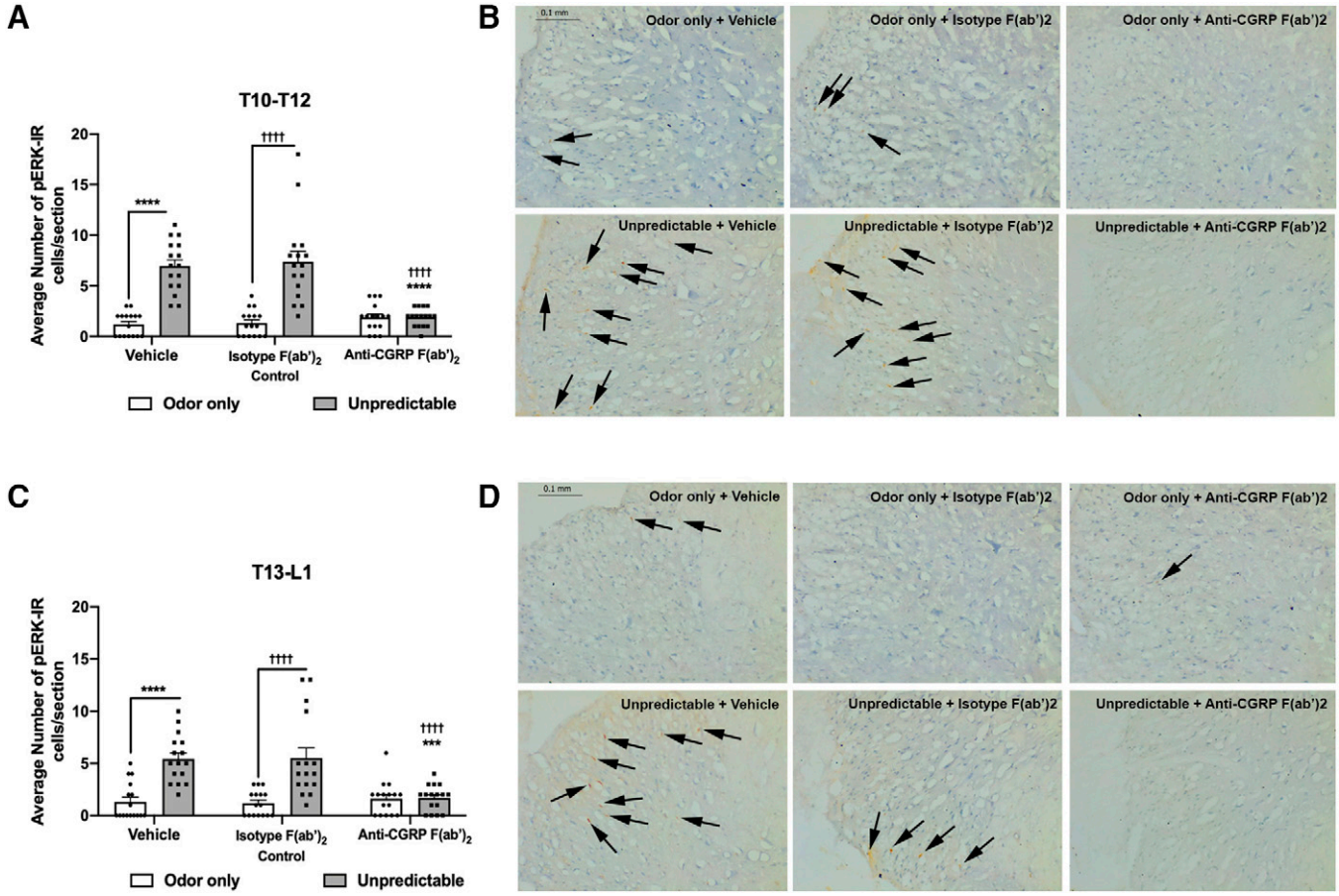

Fig. 6. Effect of unpredictable ELS on nociceptive signaling in the dorsal horn of the spinal cord. After unpredictable ELS, there was a significant increase ($P < 0.0001$) in nociceptive signaling in the dorsal horn of the spinal cord in response to noxious CRD when compared with that in SHAM ($n = 10$ per group). (A–D) Administration of anti-CGRP F(ab′)$_2$ at 30 mg/kg, i.p., significantly reduced the number of pERK-IR neurons (black arrows) in the T10–T12 (A and B) and T12–L1 (C and D) spinal cord compared with VEH and isotype F(ab′)$_2$ control–dosed rats ($P < 0.0001$) ($n = 10$ per group). Data are expressed as the mean (S.E.M.). Statistical significance was determined using two-way ANOVA followed by a Bonferroni post-test. ***$P < 0.001$ and ****$P < 0.0001$ compared with ELS + VEH; $^{\dagger\dagger\dagger\dagger}P < 0.0001$ compared ELS + isotype F(ab′)$_2$ control.

ELS is also an important risk factor for chronic pain later in life, especially in females (Talley, 1991; Bradford et al., 2012). Specifically, childhood adversity increases the likelihood of developing adult GI disorders, such as IBS (Whitehead et al., 1990; Talley et al., 1991). However, although the link between ELS and functional pain disorders, such as IBS, is apparent, the mechanisms by which ELS promotes chronic visceral pain remain unclear. Thus, a second goal of this study was to investigate the role that CGRP signaling plays in ELS-induced visceral hypersensitivity. Exposure to an unpredictable stressor during the neonatal period induced colonic hypersensitivity in adult female rats only (Prusator and Greenwood-Van Meerveld, 2016b). The ELS model used in this study utilizes Pavlovian conditioning and relies upon conditioned responses to an unpaired odor-shock stimulus to mimic what is considered an attachment to an abusive caregiver (Camp and Rudy, 1988; Sullivan et al., 2000). Using this paradigm, we have previously shown that when compared with a paired odor-shock or odor-only stimulus, unpaired (i.e., unpredictable) neonatal conditioning induces visceral hypersensitivity in adult female rats (Tyler et al., 2007; Chaloner and Greenwood-Van Meerveld, 2013).

In the next experiments, we used the same anti-CGRP F(ab′)$_2$ to evaluate the effect of peripheral CGRP inhibition on ELS-induced visceral hypersensitivity and stimulus-evoked neuronal activation in the spinal cord. Similarly to the results observed in the WAS model, CGRP inhibition reduced the VMR to CRD to a level resembling nonstressed controls. In agreement with our findings in the WAS model, after CRD, we observed a significant increase in pERK-IR neurons in the thoracolumbar dorsal horn of the spinal cord of vehicle-treated adult animals previously exposed to unpredictable ELS when compared with odor-only controls. Moreover, peripheral anti-CGRP F(ab′)$_2$ led to a robust inhibition of CRD-induced pERK expression in this model. Our results suggest that peripheral CGRP may play a significant role in central sensitization and resultant visceral hypersensitivity not only in models of repeated stress in adulthood but also in models of early life adversity.

Although our findings demonstrate a role of CGRP in stress-induced visceral hypersensitivity, little is currently known about the precise mechanisms by which stress, both in adulthood and in early life, modulates CGRP. One potential mechanism may involve CGRP's role in transmission of nociceptive information. Immunohistochemical studies show an abundance of CGRP-containing fibers in the lower thoracic and the lumbar level of the dorsal horn of the spinal cord. Ablation of these inputs results in a loss of CGRP-positive neurons in the dorsal horn, consistent

with the role of CGRP as a primary afferent neurotransmitter communicating with second-order neurons in the dorsal horn (Iyengar et al., 2017). CGRP-mediated neurotransmission occurs through the cAMP-dependent phospho-ERK signaling cascade that modulates neuronal excitability and neurotransmitter release. This potentially explains the decrease in CRD-evoked spinal phospho-ERK immunoreactivity in response to anti-CGRP $F(ab')_2$ treatment observed in the current study. Thus, it is possible that peripheral CGRP inhibition directly attenuates the transmission of nociceptive signals from the gut to the central nervous system.

Alternatively, CGRP's role in immune function and inflammation may underlie the analgesic effects of CGRP blockade. Two studies using partial wrap restraint stress and maternal-separation models of visceral hypersensitivity demonstrated an increased number of mast cells in close proximity to CGRP-IR fibers in the gut (Barreau et al., 2008; Traini et al., 2016). Furthermore, CGRP is thought to have an antimicrobial impact on several bacteria strains that are part of a healthy gut microbiome, which suggests stress-altered CGRP signaling may have effects on the microbiota-gut-brain axis (Holzer and Farzi, 2014). Thus, it is conceivable that both adult stress and ELS increase CGRP expression resulting in abnormal immune cell activation and dysbiosis that drives sensitization of colonic afferents. CGRP inhibition may therefore prevent activation of immune mediators to attenuate sensitization of these afferents and reverse colonic hypersensitivity.

Given the clinical efficacy of CGRP pathway therapeutics for migraine, there are some possible similarities between migraine and IBS. For example, both conditions are chronic prevalent disorders predominantly affecting women with visceral and thermal cutaneous hypersensitization (Chang and Lu, 2013). Additionally, stress is linked to IBS symptomatology and is a predisposing factor and trigger in patients with migraines (Sauro and Becker, 2009). Also, studies have revealed comorbidities between migraine/tension headache and IBS; however, the mechanisms that underlie this association are unclear (Arzani et al., 2020). Our finding that peripheral CGRP blockade reduces stress-induced visceral hypersensitivity suggests that anti-CGRP ligand antibodies may not only offer treatment for migraine but also concurrent treatment of comorbid IBS or other visceral pain conditions.

In conclusion, although IBS is a multifaceted disorder, evidence suggests that stress, both in early life and in adulthood, is a significant contributing factor for the development and exacerbation of the disorder. Here we used two clinically relevant rodent models in which a repeated stressor in adulthood or early life induces hallmark features of IBS, including colonic hypersensitivity and abnormal neuronal activation. We report that administration of a $F(ab')_2$ fragment antibody targeting CGRP inhibits stress-induced colonic hypersensitivity and stimulus-evoked spinal ERK1/2 phosphorylation, which emphasizes the role of CGRP in the regulation of persistent visceral pain seen in adult rats exposed to repeated stress in adulthood or ELS.

Authorship Contributions

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

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

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

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

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

References

Ackerman AL, Jellison FC, Lee UJ, Bradesi S, and Rodriguez LV (2015) Glt1 glutamate receptor mediates the establishment and perpetuation of chronic visceral pain in an animal model of stress-induced bladder hyperalgesia. *Am J Physiol Renal Physiol* **310**: F628–F636.

Arzani M, Jahromi SR, Ghorbani Z, Vahabizad F, Martelletti P, Ghaemi A, Sacco S, and Togha M; School of Advanced Studies of the European Headache Federation (EHF-SAS) (2020) Gut-brain axis and migraine headache: a comprehensive review. *J Headache Pain* **21**:15.

Barreau F, Salvador-Cartier C, Houdeau E, Bueno L, and Fioramonti J (2008) Long-term alterations of colonic nerve-mast cell interactions induced by neonatal maternal deprivation in rats. *Gut* **57**:582–590.

Blanchard EB, Lackner JM, Jaccard J, Rowell D, Carosella AM, Powell C, Sanders K, Krasner S, and Kuhn E (2008) The role of stress in symptom exacerbation among IBS patients. *J Psychosom Res* **64**:119–128.

Bradesi S, Kokkotou E, Simeonidis S, Patierno S, Ennes HS, Mittal Y, McRoberts JA, Ohning G, McLean P, Marvizon JC, et al. (2006) The role of neurokinin 1 receptors in the maintenance of visceral hyperalgesia induced by repeated stress in rats. *Gastroenterology* **130**:1729–1742.

Bradesi S, Schwetz I, Ennes HS, Lamy CM, Ohning G, Fanselow M, Pothoulakis C, McRoberts JA, and Mayer EA (2005) Repeated exposure to water avoidance stress in rats: a new model for sustained visceral hyperalgesia. *Am J Physiol Gastrointest Liver Physiol* **289**:G42–G53.

Bradford K, Shih W, Videlock EJ, Presson AP, Naliboff BD, Mayer EA, and Chang L (2012) Association between early adverse life events and irritable bowel syndrome. *Clin Gastroenterol Hepatol* **10**:385–390e31-3.

Brain SD, Williams TJ, Tippins JR, Morris HR, and MacIntyre I (1985) Calcitonin gene-related peptide is a potent vasodilator. *Nature* **313**:54–56.

Brierley SM, Hibberd TJ, and Spencer NJ (2018) Spinal afferent innervation of the colon and rectum. *Front Cell Neurosci* **12**:467.

Cámara-Lemarroy CR, Rodriguez-Gutierrez R, Monreal-Robles R, and Marfil-Rivera A (2016) Gastrointestinal disorders associated with migraine: a comprehensive review. *World J Gastroenterol* **22**:8149–8160.

Camp LL and Rudy JW (1988) Changes in the categorization of appetitive and aversive events during postnatal development of the rat. *Dev Psychobiol* **21**:25–42.

Castro J, Harrington AM, Hughes PA, Martin CM, Ge P, Shea CM, Jin H, Jacobson S, Hannig G, Mann E, Cohen MB, MacDougall JE, Lavins BJ, Kurtz CB, Silos-Santiago I, Johnston JM, Currie MG, Blackshaw LA, and Brierley SM (2013) Linaclotide inhibits colonic nociceptors and relieves abdominal pain via guanylate cyclase-C and extracellular cyclic guanosine 3′,5′-monophosphate. *Gastroenterology* **145**:1334–46.e1-11.

Ceuleers H, Hanning N, Heirbaut J, Van Remoortel S, Joossens J, Van Der Veken P, Francque SM, De Bruyn M, Lambeir AM, De Man JG, et al. (2018) Newly developed serine protease inhibitors decrease visceral hypersensitivity in a post-inflammatory rat model for irritable bowel syndrome. *Br J Pharmacol* **175**:3516–3533.

Chaloner A and Greenwood-Van Meerveld B (2013) Sexually dimorphic effects of unpredictable early life adversity on visceral pain behavior in a rodent model. *J Pain* **14**:270–280.

Chang FY and Lu CL (2013) Irritable bowel syndrome and migraine: bystanders or partners? *J Neurogastroenterol Motil* **19**:301–311.

Chen LJ, Zhang FG, Li J, Song HX, Zhou LB, Yao BC, Li F, and Li WC (2010) Expression of calcitonin gene-related peptide in anterior and posterior horns of the spinal cord after brachial plexus injury. *J Clin Neurosci* **17**:87–91.

Coutinho SV, Plotsky PM, Sablad M, Miller JC, Zhou H, Bayati AI, McRoberts JA, and Mayer EA (2002) Neonatal maternal separation alters stress-induced responses to viscerosomatic nociceptive stimuli in rat. *Am J Physiol Gastrointest Liver Physiol* **282**:G307–G316.

Davis NF, Brady CM, and Creagh T (2014) Interstitial cystitis/painful bladder syndrome: epidemiology, pathophysiology and evidence-based treatment options. *Eur J Obstet Gynecol Reprod Biol* **175**:30–37.

Delafoy L, Gelot A, Ardid D, Eschalier A, Bertrand C, Doherty AM, and Diop L (2006) Interactive involvement of brain derived neurotrophic factor, nerve growth factor, and calcitonin gene related peptide in colonic hypersensitivity in the rat. *Gut* **55**:940–945.

Domoto T, Yang H, Bishop AE, Polak JM, and Oki M (1992) Distribution and origin of extrinsic nerve fibers containing calcitonin gene-related peptide, substance P and galanin in the rat upper rectum. *Neurosci Res* **15**:64–73.

Drossman DA (1997) Irritable bowel syndrome and sexual/physical abuse history. *Eur J Gastroenterol Hepatol* **9**:327–330.

Drossman DA, Camilleri M, Mayer EA, and Whitehead WE (2002) AGA technical review on irritable bowel syndrome. *Gastroenterology* **123**:2108–2131.

Eutamene H, Bradesi S, Larauche M, Theodorou V, Beaufrand C, Ohning G, Fioramonti J, Cohen M, Bryant AP, Kurtz C, et al. (2010) Guanylate cyclase C-mediated antinociceptive effects of linaclotide in rodent models of visceral pain. *Neurogastroenterol Motil* **22**:312–e84.

Grace PM, Hutchinson MR, Maier SF, and Watkins LR (2014) Pathological pain and the neuroimmune interface. *Nat Rev Immunol* **14**:217–231.

Holzer P and Farzi A (2014) Neuropeptides and the microbiota-gut-brain axis. *Adv Exp Med Biol* **817**:195–219.

Iyengar S, Ossipov MH, and Johnson KW (2017) The role of calcitonin gene-related peptide in peripheral and central pain mechanisms including migraine. *Pain* **158**:543–559.

Johnson AC and Greenwood-Van Meerveld B (2014) Stress-induced pain: a target for the development of novel therapeutics. *J Pharmacol Exp Ther* **351**:327–335.

Johnson AC, Tran L, and Greenwood-Van Meerveld B (2015) Knockdown of corticotropin-releasing factor in the central amygdala reverses persistent viscerosomatic hyperalgesia. *Transl Psychiatry* **5**:e517.
Lampe A, Doering S, Rumpold G, Sölder E, Krismer M, Kantner-Rumplmair W, Schubert C, and Söllner W (2003) Chronic pain syndromes and their relation to childhood abuse and stressful life events. *J Psychosom Res* **54**:361–367.
Liang WJ, Zhang G, Luo HS, Liang LX, Huang D, and Zhang FC (2016) Tryptase and protease-activated receptor 2 expression levels in irritable bowel syndrome. *Gut Liver* **10**:382–390.
Longstreth GF (2005) Definition and classification of irritable bowel syndrome: current consensus and controversies. *Gastroenterol Clin North Am* **34**:173–187.
Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, and Spiller RC (2006) Functional bowel disorders. *Gastroenterology* **130**:1480–1491.
Makowska K and Gonkowski S (2018) The influence of inflammation and nerve damage on the neurochemical characterization of calcitonin gene-related peptide-like immunoreactive (CGRP-LI) neurons in the enteric nervous system of the porcine descending colon. *Int J Mol Sci* **19**:548.
Makowska K, Obremski K, Zielonka L, and Gonkowski S (2017) The influence of low doses of zearalenone and T-2 toxin on calcitonin gene related peptide-like immunoreactive (CGRP-LI) neurons in the ENS of the porcine descending colon. *Toxins (Basel)* **9**:98.
Mohammadi E, Prusator DK, Healing E, Hurst R, Towner RA, Wisniewski AB, and Greenwood-Van Meerveld B (2016) Sexually dimorphic effects of early life stress in rat pups on urinary bladder detrusor muscle contractility in adulthood. *Biol Sex Differ* **7**:8.
Mohammadi EN, Ligon CO, Silos-Santiago A, Ge P, Kurtz C, Higgins C, Hannig G, and Greenwood-Van Meerveld B (2018) Linaclotide attenuates visceral organ crosstalk: role of guanylate cyclase-c activation in reversing bladder-colon cross-sensitization. *J Pharmacol Exp Ther* **366**:274–281.
Mora F, Segovia G, Del Arco A, de Blas M, and Garrido P (2012) Stress, neurotransmitters, corticosterone and body-brain integration. *Brain Res* **1476**:71–85.
Myers B and Greenwood-Van Meerveld B (2012) Differential involvement of amygdala corticosteroid receptors in visceral hyperalgesia following acute or repeated stress. *Am J Physiol Gastrointest Liver Physiol* **302**:G260–G266.
Nash MS, McIntyre P, Groarke A, Lilley E, Culshaw A, Hallett A, Panesar M, Fox A, and Bevan S (2012) 7-tert-Butyl-6-(4-chloro-phenyl)-2-thioxo-2,3-dihydro-1H-pyrido[2,3-d]pyrimidin-4-one, a classic polymodal inhibitor of transient receptor potential vanilloid type 1 with a reduced liability for hyperthermia, is analgesic and ameliorates visceral hypersensitivity. *J Pharmacol Exp Ther* **342**:389–398.
Nickel JC, Tripp DA, Pontari M, Moldwin R, Mayer R, Carr LK, Doggweiler R, Yang CC, Mishra N, and Nordling J (2011) Childhood sexual trauma in women with interstitial cystitis/bladder pain syndrome: a case control study. *Can Urol Assoc J* **5**:410–415.
Pan XQ, Gonzalez JA, Chang S, Chacko S, Wein AJ, and Malykhina AP (2010) Experimental colitis triggers the release of substance P and calcitonin gene-related peptide in the urinary bladder via TRPV1 signaling pathways. *Exp Neurol* **225**:262–273.
Paré P, Gray J, Lam S, Balshaw R, Khorasheh S, Barbeau M, Kelly S, and McBurney CR (2006) Health-related quality of life, work productivity, and health care resource utilization of subjects with irritable bowel syndrome: baseline results from LOGIC (Longitudinal Outcomes Study of Gastrointestinal Symptoms in Canada), a naturalistic study. *Clin Ther* **28**:1726–1735, discussion 1710–1711.
Plourde V, St-Pierre S, and Quirion R (1997) Calcitonin gene-related peptide in visceroosensitive response to colorectal distension in rats. *Am J Physiol* **273**:G191–G196.
Prusator DK, Andrews A, and Greenwood-Van Meerveld B (2016) Neurobiology of early life stress and visceral pain: translational relevance from animal models to patient care. *Neurogastroenterol Motil* **28**:1290–1305.
Prusator DK and Greenwood-Van Meerveld B (2015) Gender specific effects of neonatal limited nesting on viscerosomatic sensitivity and anxiety-like behavior in adult rats. *Neurogastroenterol Motil* **27**:72–81.
Prusator DK and Greenwood-Van Meerveld B (2016a) Sex differences in stress-induced visceral hypersensitivity following early life adversity: a two hit model. *Neurogastroenterol Motil* **28**:1876–1889.
Prusator DK and Greenwood-Van Meerveld B (2016b) Sex-related differences in pain behaviors following three early life stress paradigms. *Biol Sex Differ* **7**:29.
Qiao LY and Grider JR (2007) Up-regulation of calcitonin gene-related peptide and receptor tyrosine kinase TrkB in rat bladder afferent neurons following TNBS colitis. *Exp Neurol* **204**:667–679.
Qiao LY and Grider JR (2009) Colitis induces calcitonin gene-related peptide expression and Akt activation in rat primary afferent pathways. *Exp Neurol* **219**:93–103.
Rosenfeld MG, Mermod JJ, Amara SG, Swanson LW, Sawchenko PE, Rivier J, Vale WW, and Evans RM (1983) Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature* **304**:129–135.
Rytel L and Calka J (2016) Acetylsalicylic acid-induced changes in the chemical coding of extrinsic sensory neurons supplying the prepyloric area of the porcine stomach. *Neurosci Lett* **617**:218–224.
Saito YA, Schoenfeld P, and Locke GR III (2002) The epidemiology of irritable bowel syndrome in North America: a systematic review. *Am J Gastroenterol* **97**:1910–1915.
Sauro KM and Becker WJ (2009) The stress and migraine interaction. *Headache* **49**:1378–1386.
Schou WS, Ashina S, Amin FM, Goadsby PJ, and Ashina M (2017) Calcitonin gene-related peptide and pain: a systematic review. *J Headache Pain* **18**:34.
Sullivan RM, Stackenwalt G, Nasr F, Lemon C, and Wilson DA (2000) Association of an odor with activation of olfactory bulb noradrenergic beta-receptors or locus coeruleus stimulation is sufficient to produce learned approach responses to that odor in neonatal rats. *Behav Neurosci* **114**:957–962.
Sundler F, Ekblad E, and Håkanson R (1991) Occurrence and distribution of substance P- and CGRP-containing nerve fibers in gastric mucosa: species differences. *Adv Exp Med Biol* **298**:29–37.
Talley NJ (1991) Diagnosing an irritable bowel: does sex matter? *Gastroenterology* **100**:834–837.
Talley NJ, Zinsmeister AR, Van Dyke C, and Melton 3rd LJ (1991) Epidemiology of colonic symptoms and the irritable bowel syndrome. *Gastroenterology* **101**:927–934.
Timmermans JP, Scheuermann DW, Barbiers M, Adriaensen D, Stach W, Van Hee R, and De Groodt-Lasseel MH (1992) Calcitonin gene-related peptide-like immunoreactivity in the human small intestine. *Acta Anat (Basel)* **143**:48–53.
Timmermans W, Xiong H, Hoogenraad CC, and Krugers HJ (2013) Stress and excitatory synapses: from health to disease. *Neuroscience* **248**:626–636.
Traini C, Evangelista S, Girod V, Faussone-Pellegrini MS, and Vannucchi MG (2016) Changes of excitatory and inhibitory neurotransmitters in the colon of rats underwent to the wrap partial restraint stress. *Neurogastroenterol Motil* **28**:1172–1185.
Tran L, Chaloner A, Sawalha AH, and Greenwood Van-Meerveld B (2013) Importance of epigenetic mechanisms in visceral pain induced by chronic water avoidance stress. *Psychoneuroendocrinology* **38**:898–906.
Tran L, Wiskur B, and Greenwood-Van Meerveld B (2012) The role of the anteriolateral bed nucleus of the stria terminalis in stress-induced nociception. *Am J Physiol Gastrointest Liver Physiol* **302**:G1301–G1309.
Tyler K, Moriceau S, Sullivan RM, and Greenwood-van Meerveld B (2007) Long-term colonic hypersensitivity in adult rats induced by neonatal unpredictable vs predictable shock. *Neurogastroenterol Motil* **19**:761–768.
Utsumi D, Matsumoto K, Tsukahara T, Amagase K, Tominaga M, and Kato S (2018) Transient receptor potential vanilloid 1 and transient receptor potential ankyrin 1 contribute to the progression of colonic inflammation in dextran sulfate sodium-induced colitis in mice: links to calcitonin gene-related peptide and substance P. *J Pharmacol Sci* **136**:121–132.
van Tilburg M (2011) Childhood abuse is not only a case of bruises and broken bones: role of abuse in unexplained GI symptoms in children. *J Pediatr Gastroenterol Nutr* **53** (Suppl 2):S40–S41.
Walsh DA, Mapp PI, and Kelly S (2015) Calcitonin gene-related peptide in the joint: contributions to pain and inflammation. *Br J Clin Pharmacol* **80**:965–978.
Wang Z, Chang HH, Gao Y, Zhang R, Guo Y, Holschneider DP, and Rodriguez LV (2017) Effects of water avoidance stress on peripheral and central responses during bladder filling in the rat: a multidisciplinary approach to the study of urologic chronic pelvic pain syndrome (MAPP) research network study. *PLoS One* **12**:e0182976.
Whitehead WE, Holtkotter B, Enck P, Hoelzl R, Holmes KD, Anthony J, Shabsin HS, and Schuster MM (1990) Tolerance for rectosigmoid distention in irritable bowel syndrome. *Gastroenterology* **98**:1187–1192.
Wolf M, Schrödl F, Neuhuber W, and Brehmer A (2007) Calcitonin gene-related peptide: a marker for putative primary afferent neurons in the pig small intestinal myenteric plexus? *Anat Rec (Hoboken)* **290**:1273–1279.

Address correspondence to: Ehsan Noor-Mohammadi, O'Donoghue Research Bldg. Rm. 332, 1122 NE 13th St., Oklahoma City, OK 73117. E-mail: Ehsan-mohammadi@ouhsc.edu