

# Linacotide treatment reduces endometriosis-associated vaginal hyperalgesia and mechanical allodynia through viscerovisceral cross-talk

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## Abstract

Endometriosis, an estrogen-dependent chronic inflammatory disease, is the most common cause of chronic pelvic pain. Here, we investigated the effects of linacotide, a Food and Drug Administration–approved treatment for IBS-C, in a rat model of endometriosis. Eight weeks after endometrium transplantation into the intestinal mesentery, rats developed endometrial lesions as well as vaginal hyperalgesia to distension and decreased mechanical hind paw withdrawal thresholds. Daily oral administration of linacotide, a peripherally restricted guanylate cyclase-C (GC-C) agonist peptide acting locally within the gastrointestinal tract, increased pain thresholds to vaginal distension and mechanical hind paw withdrawal thresholds relative to vehicle treatment. Furthermore, using a cross-over design, administering linacotide to rats previously administered vehicle resulted in increased hind paw withdrawal thresholds, whereas replacing linacotide with vehicle treatment decreased hind paw withdrawal thresholds. Retrograde tracing of sensory afferent nerves from the ileum, colon, and vagina revealed that central terminals of these afferents lie in close apposition to one another within the dorsal horn of the spinal cord. We also identified dichotomizing dual-labelled ileal/colon innervating afferents as well as colon/vaginal dual-labelled neurons and a rare population of triple traced ileal/colon/vaginal neurons within thoracolumbar DRG. These observations provide potential sources of cross-organ interaction at the level of the DRG and spinal cord. GC-C expression is absent in the vagina and endometrial cysts suggesting that the actions of linacotide are shared through nerve pathways between these organs. In summary, linacotide may offer a novel therapeutic option not only for treatment of chronic endometriosis-associated pain, but also for concurrent treatment of comorbid chronic pelvic pain syndromes.

**Keywords:** Central sensitization, Chronic pelvic pain syndrome, Endometriosis, Guanylate cyclase-C, Peripheral sensitization, Visceral hypersensitivity

## 1. Introduction

Chronic pelvic pain (CPP) is a complex, multifaceted condition that can originate from different organs/tissues located inside the

pelvic cavity. Among CPP syndromes, endometriosis, an estrogen-dependent chronic inflammatory disease defined by the presence of endometrial glands and stroma outside the uterus, is the most common cause.<sup>27,34,53</sup> Dysmenorrhea, dyspareunia, nonmenstrual pelvic pain, and infertility are the most severe symptoms of endometriosis.<sup>27</sup> Further aggravating and complicating this disease are the high prevalence of comorbid pain syndromes including irritable bowel syndrome (IBS), vulvodynia, and somatic pain, and increased incidences of depression and anxiety.<sup>27,33,47,49</sup>

Pharmacologic therapy for endometriosis is centered on ameliorating the severity and frequency of pain symptoms.<sup>2,14,34</sup> Nonsteroidal anti-inflammatory drugs are commonly used despite a lack of evidence in clinical studies that they effectively manage endometriosis-associated pain.<sup>9</sup> Hormonal suppression to induce a hypoestrogenic state and subsequent amenorrhea include combined oral contraceptives, progestin monotherapy, and gonadotropin-releasing hormone agonists.<sup>10,50</sup> Furthermore, aromatase inhibitors and gonadotropin-releasing hormone antagonists are in late-stage clinical development.<sup>23</sup> Although hormonal therapies provide temporary relief from endometriosis-associated pain, side effects due to systemically low estrogen levels are common. A recent survey of opioid prescribing practices among US obstetrician-gynecologists revealed that 24% typically prescribe opioids for chronic endometriosis-associated pelvic pain despite their serious side effects,

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reiterating the need for novel, safe, and efficacious therapies for treatment of chronic endometriosis-associated pain.<sup>38</sup>

Linaclotide, a minimally absorbed synthetic 14-amino acid agonist peptide of guanylate cyclase-C (GC-C) expressed predominantly on the intestinal epithelium, is Food and Drug Administration-approved for treatment of adult patients with constipation-predominant IBS (IBS-C).<sup>18,45</sup> GC-C activation by linaclotide stimulates production and secretion of intracellular cyclic guanosine-3',5' monophosphate (cGMP) into the lumen and submucosa. Activation of the GC-C/cGMP pathway is linked to increased secretion and accelerated transit.<sup>11–13,51</sup> Analgesic effects of linaclotide in rodent models of inflammation and stress-induced colonic hypersensitivity are mediated through a pathway that functions independently from improvements in bowel function.<sup>16,22,36</sup> This pathway directly links cGMP secreted into the submucosa to inhibition of colonic nociceptors, resulting in peripheral analgesia.<sup>16,31</sup> Moreover, antinociceptive effects of chronic linaclotide treatment have recently been demonstrated in models of colon-bladder cross-sensitization, in which chemically induced inflammation either in the colon or bladder induced hypersensitivity and organ dysfunction not only in the insulted organ, but also in the otherwise uninflamed organ.<sup>31,41</sup> These data support the concept that cross-organ sensitization underlies the development and maintenance of comorbid pelvic pain syndromes.<sup>30,31,41</sup> Correspondingly, pharmaceutical treatments that inhibit afferents innervating the gut may also improve other comorbid symptoms through common sensory pathways.<sup>31</sup>

Here, we investigated whether linaclotide, acting locally on GC-C within the gastrointestinal tract, reduces endometriosis-associated vaginal hyperalgesia and mechanical hind paw allodynia in a rat model. Peripheral and central cross-organ sensitization pathways were explored to develop a mechanistic understanding of linaclotide-mediated analgesia. Because both endometrial cysts driving increased pain perception and target organs/tissues of secondary hyperalgesia and allodynia are located outside the gastrointestinal tract, our findings suggest broad utility of linaclotide treatment for endometriosis-associated pain and concurrent comorbid CPP syndromes.

## 2. Methods

### 2.1. Animals

Six-week-old female Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 125 to 130 g at the start of the study were housed individually in a controlled environment, with access to food and water ad libitum. All animals were maintained in a temperature-controlled room (21 ± 4°C) with a 12:12-hour light/dark cycle (6:30 AM–6:30 PM). Rats were acclimated to their environment for a minimum of 5 days before initiation of a study. The estrous cycle was monitored daily by cytological examination of vaginal lavage following traditional nomenclature (2 hours after lights on).<sup>3</sup> All studies were approved by the Ironwood Institutional Animal Care and Use Committee (O15-008) and are consistent with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals."

For retrograde tracing experiments, female rats aged 6 to 8 weeks were used. Rats were acquired from an in-house breeding (Sprague-Dawley; originally purchased from Charles River Laboratories, United Kingdom) within the South Australian Health and Medical Research Institute's (SAHMRI's) specific and opportunistic pathogen-free animal care facility. Rats were group housed before retrograde tracing (3 rats per cage) and individually housed after tracing, within individual ventilated GM900 cages, filled with chip coarse dust-free aspen bedding. Cages were stored on IVC racks within a temperature-controlled environment

of 22°C and a 12-hour light/12-hour dark cycle. Rats had free access to Lab Diet JL Rat and Mouse chow and autoclaved reverse osmosis water. Experiments were approved by the SAHMRI animal ethics committee, which conformed to regulatory standards and the ARRIVE guidelines.

### 2.2. Endometriosis (ENDO) and SHAM surgeries

ENDO and SHAM surgeries were performed as previously described.<sup>6,42</sup> Briefly, rats were anesthetized with an injection of ketamine hydrochloride (73 mg/kg, intraperitoneal [i.p.]) and xylazine (8.8 mg/kg, i.p.). Following aseptic conditions, a small midline abdominal incision was made to expose the uterus, and a small segment (approximately 1 cm) of the center part of left uterine horn was clamped between 2 hemostats and excised. Four 2 × 2-mm pieces of uterine horn tissue (similarly sized pieces of abdominal fat during SHAM surgeries) were sutured on alternate mesenteric cascade arteries that supply the small intestine. Muscle and skin were sutured separately. Throughout the surgery and during the recovery period, animals were kept on a heating pad to maintain body temperature. Rats received Buprenex (0.03 mg/kg, i.p.) before surgery to alleviate postoperative pain. After recovery from surgery, animals were monitored daily for postsurgical complications. No signs of distress or unusual pain behaviors were observed. The regular estrous cycle resumed within approximately 7 days of surgery. Photographs of endometrial cysts growing ectopically on mesenteric arteries taken 8 weeks after ENDO surgery, and the mesentery of an age-matched naive female rat, are shown in Supplementary Fig. 1 (available at <http://links.lww.com/PAIN/A847>).

### 2.3. Behavioral training

Five weeks after ENDO surgery or SHAM surgery, rats were subjected to 2 to 3 training sessions (1–2 days in between sessions) in a restraining chamber (a transparent plexiglass chamber: 65 mm wide × 75 mm high × 220 mm long that gently, but not aversively, restrained the rats) to acclimate the animals to this device (stress reduction) used in the behavioral testing scheme to measure referred vaginal hyperalgesia. Training sessions lasted for 10 minutes each day and were initially conducted without a distensible latex balloon (8-mm long by 1.5-mm wide, uninflated) inserted into the vaginal canal. For the final behavioral training session, the latex balloon was inserted into the vaginal canal, but was not inflated.

### 2.4. Telemetric probe implantation

Seven weeks after ENDO surgery or SHAM surgery, under aseptic conditions and ketamine hydrochloride (73 mg/kg, i.p.) and xylazine (8.8 mg/kg, i.p.) anesthesia, a wireless telemetric probe (Stellar Implantable Transmitter, Type BTA-XS; TSE Systems, Inc, Chesterfield, MO) was implanted under the skin on the back, slightly posterior to the scapulae and secured with 4-0 silk suture using the loops on both sides of the probe to attach to the hypodermis. Electrodes were separately tunneled under the skin using a 14-gauge trocar and implanted in the left external oblique muscle. The skin was then sutured. Postsurgical recovery and monitoring of animals proceeded as described above.

### 2.5. Visceromotor response

The experimental design is shown in schematic form in **Figure 1A**. Visceromotor response (VMR) measurements in naive rats (age-matched), and in SHAM surgery and ENDO

surgery rats (ENDO rats) were initiated 8 weeks after surgery (in ENDO rats, sensory and sympathetic innervation of cysts and vaginal hyperalgesia are fully developed 6–8 weeks after ENDO surgery).<sup>40</sup> Stable VMR baselines and VMR thresholds were recorded in proestrus, when pain threshold levels are lowest across the 4-day estrous cycle.<sup>15,21,42</sup> Rats with stable baseline VMR were assigned to the following study groups: group 1: naive (N = 4); group 2: SHAM surgery (N = 8); group 3: ENDO surgery, vehicle (sterile water, p.o.) (N = 9); and group 4: ENDO surgery, linaclotide (3  $\mu$ g/kg/day, p.o.) (N = 14). In previous studies, this dose of linaclotide was shown to elicit potent antinociceptive effects in several rodent models of inflammation and stress-induced visceral pain.<sup>16,22,31,36,41</sup> Visceromotor response measurements (2–3/animal) were performed 2 hours after treatment on day 1 and 5 (in proestrus), and on day 9 (in proestrus) 4 days after the last dose either of vehicle or linaclotide. For VMR measurements, conscious rats were placed in the restraining chamber located close to the receiver, and a small balloon (~10-mm diameter when fully inflated) connected to a pressure transducer was inserted into the middle of the vaginal canal. Following establishment of a stable baseline, the balloon was inflated (water) using an infusion pump (0.3 mL/minute, 1 mL maximum), and changes in electromyographic (EMG) activity of the external oblique musculature in response to vaginal distension (surrogate endpoint for vaginal pain) were relayed to the Stellar Telemetry System, consisting of the receiver and antenna (TSE Systems Inc, Chesterfield, MO) and synchronized with the amplified and digitalized signal from the pressure transducer. The integral of the rectified EMG signal was calculated in 100-ms intervals using the analysis module of the AcqKnowledge ACK100-STL Biopac Software (TSE Systems, Inc). The threshold for discriminating activity from baseline EMG activity was preset at 200% of maximum amplitude of the EMG baseline activity and calculated as reported previously.<sup>42</sup> All measurements were performed by the same experimenter who was blinded to the condition and treatment status of the animals during all measurements.

## 2.6. Mechanical hind paw allodynia using electronic von Frey

The experimental design is shown in schematic form in **Figure 1B**. Mechanical hind paw allodynia was measured using an electronic von Frey anesthesiometer with a rigid tip (up to 800 g) (IITC Life Science, Woodland Hills, CA). Unrestrained rats were placed in a Perspex clear chamber on a wire mesh grid (IITC Life Science) and allowed to acclimate for at least 15 minutes before testing. For measurement of hind paw withdrawal thresholds, the electronic von Frey rigid tip was applied to the center of the plantar skin of the hind paw, and hind paw withdrawal thresholds were measured and automatically recorded during 3 measurements on each hind paw. The force at which a withdrawal response was observed was taken as the nociceptive threshold. Mean baseline values were established for each group, based on average values corresponding to 3 measurements for each animal, starting 8 weeks after surgery. Study groups were naive (N = 17), SHAM surgery (N = 10), and ENDO surgery (N = 18). To determine the effects either of vehicle (sterile water, p.o.) or linaclotide treatment (3  $\mu$ g/kg/day, p.o.) in SHAM surgery and ENDO rats, animals were then divided into vehicle (SHAM surgery: N = 5; ENDO surgery: N = 10) or linaclotide treatment groups (SHAM surgery: N = 5; ENDO surgery: N = 8). Mechanical hind paw allodynia was determined in proestrus on days 1, 5, 13, and 17. The day 13 timepoint was added to determine whether the effects of chronic linaclotide administration could be sustained over a prolonged treatment period

spanning 8 additional days (2 estrous cycles) beyond day 5. On days 1 and 5, measurements were performed 1 hour and 2 hours after dosing either of vehicle or linaclotide, and after 1, 2, and 4 hours on day 13. SHAM surgery and ENDO rats received no treatment between days 13 and 17 (treatment withdrawal period), and final mechanical hind paw withdrawal measurements were performed on day 17. All measurements were performed by the same experimenter who was blinded to the condition and treatment status of the rats during all measurements.

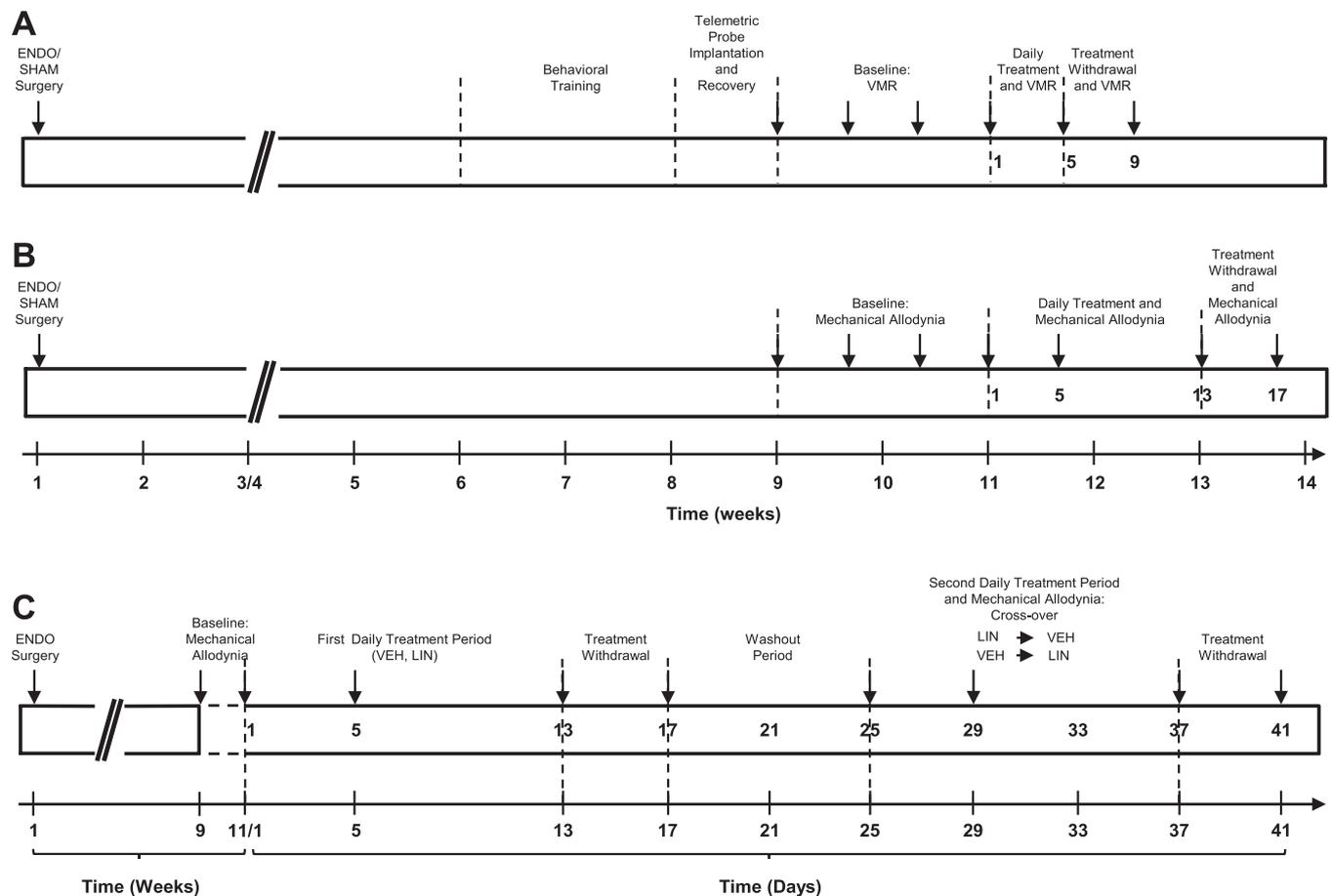
The experimental design for the cross-over study is shown in schematic form in **Figure 1C**. A separate cohort of female Sprague-Dawley rats underwent ENDO surgery and baseline values for hind paw withdrawal thresholds were established 8 weeks after surgery as described above. Rats were then assigned to 2 treatment groups: linaclotide (3  $\mu$ g/kg/day, p.o.) (N = 10) or vehicle (sterile water, p.o.) (N = 9). During the first treatment period (day 1–13), mechanical hind paw allodynia was determined on day 1, 5, and 13, and on day 25, 29, and 37 during the second treatment period (day 25–37). Timepoints for measurement of hind paw withdrawal thresholds were informed by data obtained from the study described in **Figure 1B**. All measurements were performed 1 and 2 hours after dosing either of vehicle or linaclotide. Both the first and second treatment periods were followed by a 4-day withdrawal period from treatment (day 13–17 and day 37–41, respectively), and hind paw withdrawal thresholds were measured at the end of each withdrawal period from treatment (day 17 and 41). To further exclude the possibility of any residual linaclotide activity remaining after the 4-day withdrawal period after the first treatment period, an 8-day washout period spanning 2 estrous cycles was added (day 17–25) before initiation of the second treatment period. All measurements were performed by the same experimenter who was blinded to the condition and treatment status of the rats during all measurements.

## 2.7. Quantitative RT-PCR for detection of GC-C mRNA expression

Rat tissue total RNA from ileum, endometrial cysts, colon, vagina, and uterus (N = 5–8), taken 2 hours after the last dose either of vehicle (sterile water, p.o.) or linaclotide (3  $\mu$ g/kg/day, p.o.) on day 5, was extracted using RNeasy Plus Mini Kit (Cat # 74134; Qiagen, Valencia, CA). cDNAs were synthesized using a high-capacity cDNA reverse-transcription kit (Cat # 4368814; ThermoFisher Scientific, Waltham, MA). Twenty nanogram of cDNA was used for TaqMan quantitative real-time polymerase chain reaction (qRT-PCR) gene expression analysis. Rat TaqMan cDNA probes specific for *Gucy2c* (Cat # Rn00565895\_m1; ThermoFisher Scientific) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cat # 4352338E; ThermoFisher Scientific) (housekeeping gene, control) were obtained from ThermoFisher Scientific. The  $2^{-\Delta\Delta Ct}$  method was used to calculate relative mRNA expression.<sup>37</sup>

## 2.8. Detection of *Gucy2c* expression by in situ hybridization

In situ detection of rat guanylate cyclase-C (*Gucy2c*) transcripts in formalin-fixed paraffin-embedded ileum, endometrial cysts, colon, vagina, and uterus was performed using the RNAscope 2.5 LS assay (Advanced Cell Diagnostics, RNAscope 2.5 LS Reagent Kit—BROWN, Cat No. 436598) according to the manufacturer's instructions (RNAscope LS 2.5 Brown User Manual). Briefly, 5- $\mu$ m formalin-fixed paraffin-embedded ileum, endometrial cysts, colon, vagina, and uterus sections, taken 2 hours after the last



**Figure 1.** Experimental design to measure vaginal hyperalgesia (A) and mechanical hind paw allodynia (B and C). (A) Five weeks after ENDO surgery (during this time, endometrial cysts develop pathological features that closely resemble those seen in human endometriosis), SHAM surgery, or naive rats were subjected to behavioral training sessions to habituate the animals to the chamber used to measure vaginal hyperalgesia. Two weeks later, rats were surgically implanted a wireless telemetry probe. Behavioral tests to record baselines of visceromotor responses (VMR) to vaginal balloon distension started 7 days after implantation and lasted over a period of 2 weeks. Rats with stable baselines were then assigned to vehicle or linaclotide treatment groups. Visceromotor responses were recorded on days 1 and 5 after treatment, and after a 4-day withdrawal period from treatment on day 9. (B) Eight weeks after SHAM or ENDO surgery, and in naive rats, stable hind paw withdrawal baselines in response to pressure using electronic von Frey tips were established over a 2-week period, and rats with stable baselines were then assigned to either the vehicle or linaclotide treatment group. Hind paw withdrawal thresholds were measured on days 1, 5 and 13 after treatment, and after a 4-day withdrawal period from treatment on day 17. (C) In the cross-over design, ENDO rats were assigned to vehicle or linaclotide treatment groups for the first treatment period (day 1-13) followed by a 4-day withdrawal period from treatment (day 13-17). The 4-day withdrawal period from treatment was followed by an 8-day washout period spanning 2 estrous cycles (days 17-25) before initiation of the second treatment period to further exclude the possibility of any remaining residual linaclotide activity. Animals that had received vehicle during the first treatment period were then switched to linaclotide during the second treatment period (day 25-37) and conversely, animals that had received linaclotide during the first treatment period were switched to vehicle. The second treatment was followed by another 4-day withdrawal period from treatment (day 37-41). Hind paw withdrawal thresholds were measured on day 1, 5, and 9 during the first treatment period, and day 25, 29, and 37 during the second treatment period, and on the last day of the withdrawal period after each treatment period. Arrows indicate days of VMR (A) or hind paw withdrawal threshold (B and C) measurements. LIN, linaclotide; VEH, vehicle.

dose either of vehicle (sterile water, p.o.) or linaclotide (3  $\mu\text{g}/\text{kg}/\text{day}$ , p.o.) on day 5, were deparaffinized, and pretreated with protease digestion followed by hybridization for 2 hours with the target probe for *Gucy2c* (Cat No. 313528 RNAscope LS 2.5 Probe-Rn-*Gucy2c*, Advanced Cell Diagnostics). Thereafter, an HRP-based signal amplification system was hybridized to the target probe before color development with 3,3'-diaminobenzidine tetrahydrochloride. Positive staining for *Gucy2c* was defined by the presence of brown dots. The dihydrodipicolinate reductase (*DapB*) gene (Cat No. 312038, RNAscope 2.5 LS Negative Control Probe\_*dapB*, Advanced Cell Diagnostics) was used as a negative control. Photographs of representative sections from the ileum, colon, cyst, vagina, and uterus were taken using a Nikon Eclipse E400 microscope and NIS Elements Version 4.40 Imaging Software (Micro Video Instruments (MVI), Avon, MA).

### 2.9. Retrograde tracing to label afferent neurons and central terminals of ileal, colonic, and vaginal afferents

An aseptic abdominal incision was made in anaesthetized (isoflurane 2%-4% in oxygen) rats, and the caecum was located and exteriorized along with proximal 1 cm of the small intestine (distal ileum) into which 10  $\mu\text{L}$  of 0.5% Cholera Toxin subunit B (CTB) conjugated to AlexaFluor555 (AF555) (CTB-555; #C22843, ThermoFisher Scientific) was injected into the wall over 4 lateral injection sites.<sup>17</sup> This was followed by injections of CTB conjugated to AlexaFluor488 (AF488) (CTB-488; #C22843, ThermoFisher Scientific) into the wall of the distal colon injected (10  $\mu\text{L}$  in total) at 4 to 5 lateral and midline sites covering a 1-cm region proximal to the pelvic bone.<sup>5,31,46</sup> After this, the uterine horns and uterus were located and slightly pulled proximally, at the base of the uterus (cervix), the syringe was inserted 0.5 cm distally into the wall of the proximal vagina, into which CTB

conjugated to AlexaFluor647 (AF647) (CTB-647; #C34778, ThermoFisher Scientific) was injected (5  $\mu$ L over 2 injections). All injections were made using separate and AlexaFluor-dedicated Hamilton syringes (5  $\mu$ L, 65RN-7633-01; Hamilton, NV) attached to either a 25- or 30-gauge needle (RN custom point 4, Hamilton). The abdominal incision was sutured closed, and rats were allowed to recover and monitored for 5 days for dorsal root ganglia (DRG) labelling (N = 4) or 10 days for spinal cord labelling (N = 4) before undergoing transcardial perfuse fixation.

### 2.10. Transcardial perfuse fixation and processing of dorsal root ganglia and spinal cord

Rats were given an overdose of Lethobarb (250 mg/mL, 200  $\mu$ L i.p.; Virbac Pty Limited, Milperra, NSW, Australia) followed by opening of the chest cavity to exposure of the heart. Heparin saline was injected into the left ventricle, into which the perfusion needle was inserted and then the right atrium was cut. Warm 0.1M phosphate buffer was then infused until the fluid exiting the right atrium was clear, at which perfusate was switched to 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in 0.1M phosphate buffer. Using ribs as anatomical markers, individual DRG (thoracic T7 to sacral S1) or spinal cord (segments thoracic T7-T9, thoracic T10-T12, thoracolumbar T12-L1, or lumbosacral [L2 and below]) were removed and postfixed for 18 hours in 4% paraformaldehyde/0.1M phosphate buffer at 4°C. Tissue was then cryoprotected for 24 hours at 4°C in 30% sucrose/0.1M phosphate buffer. After this, DRG were snap frozen in Optimal Cutting Compound (OCT; C361603E, VWR Biostrategy, Victoria, Australia), whereas the spinal cord went a further 72 hours of cryoprotection in a mixture of 30% sucrose/OCT after which they too were snap frozen in OCT. Tissue was then cryosectioned in entirety at 15- $\mu$ m thick for DRG and 50- $\mu$ m thick for spinal cord and placed on gelatin-coated slides. Sections were then air dried before washing with 0.2% Triton X100/phosphate buffered saline and mounted with Prolong Gold antifade (P36935; ThermoFisher Scientific) and allowed to dry before visualization.

### 2.11. Microscopy

Dorsal root ganglia and spinal cord sections were visualized using standard fluorescence microscopy (Olympus BX51) and confocal microscopy (Leica TCS SP8X, Wetzlar, Germany). For confocal microscopy, sequential scanning with a white light laser (70% power) tuned to excite AF488 (495 nm laser 47%), AF555 (532 nm 29% intensity), and AF647 (633 nm 53%) was used. Detectors were tuned to emission ranges of 504 to 542 nm for AF488, 550 to 597 nm for AF555, and 649 to 750 nm for AF647. Images (1024  $\times$  1024 pixels) were taken with an oil immersion X20 or X40 objective with a frame average of 6. Spinal cord slices for CTB-labelled projections were optically sectioned (2- $\mu$ m thick sections) and projected images were reconstructed (50  $\mu$ m). Images were processed and analyzed using LAS Lite (Leica Microsystems, Wetzlar, Germany), Image J software (NIH), and CorelDraw 2017 (CorelDraw Graphic Suite 2017, Corel) software. Other than making moderate adjustments for contrast and brightness, the images were not manipulated in any way.

### 2.12. Quantification of labelled dorsal root ganglia neurons

Neuronal counts were analyzed from previously saved digital photomicrographs, with only neurons with intact nuclei counted. The number of neurons traced was counted in 5 to 10 sections per DRG per animal, with N = the number of animals and n = the

total number of neurons counted. The mean ( $\pm$ SEM) number of neurons labelled from ileal, colon, and vaginal tracing and those colabelled with ileal/colon, colon/vaginal, or ileal/vaginal per DRG section at each DRG level from T7 to S1 was then obtained. This was used to determine the mean ( $\pm$ SEM) percentages of the total traced neurons labelled from the 3 regions and those colabelled across N = 4 rats with the percentage data presented in the following DRG groups: T7-T9, T10-T12, T13-L1, and L6-S1.

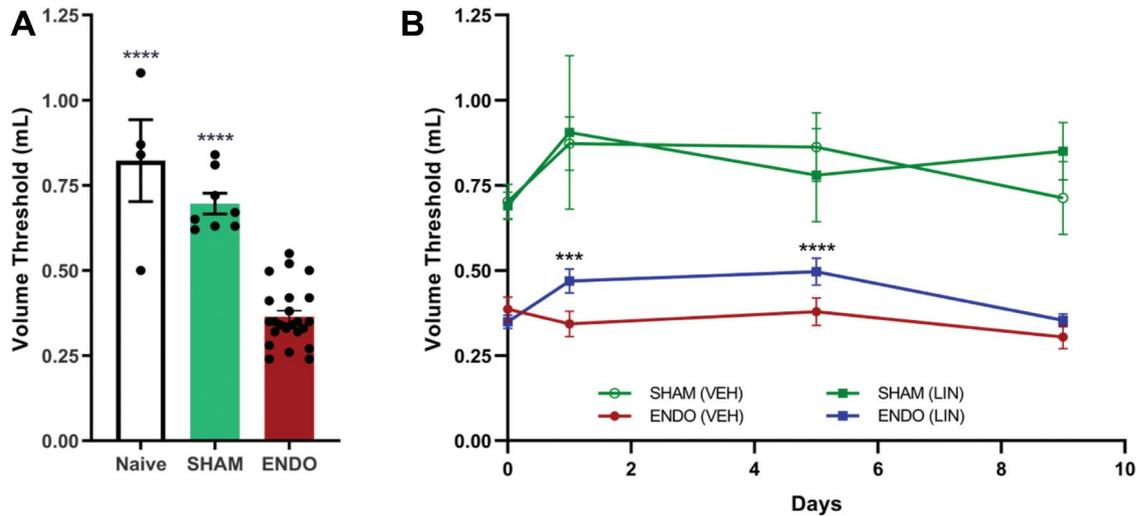
### 2.13. Statistical analysis

Two-way repeated-measures analysis of variance followed by Tukey multiple comparison test was used in longitudinal studies. One-way analysis of variance followed by Tukey multiple comparison test was used for comparison across treatment groups. The use of each statistical test is denoted in the individual figure legends. Differences were considered statistically significant at \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, and \*\*\*\* $P$  < 0.0001. Averaged data values are expressed as mean  $\pm$  SEM. Figures were prepared in GraphPad 7.02. Software (San Diego, CA). N equals number of animals per group, whereas n equals the number of neurons or independent observations.

## 3. Results

### 3.1. Daily oral linaclotide treatment reduces endometriosis-associated vaginal hyperalgesia

Previous studies have shown that oral linaclotide reversed visceral hypersensitivity in rodent models of colon-bladder cross-sensitization.<sup>31,41</sup> These findings prompted us to investigate whether oral linaclotide reduces referred vaginal hyperalgesia in an established rat model of endometriosis in which vaginal hyperalgesia is driven by ectopically growing endometrial cysts through mechanisms involving peripheral and central sensitization.<sup>6–8,15</sup> The effects either of vehicle or linaclotide were measured through changes in evoked VMR responses to vaginal distension on day 1 (acute) and day 5 (chronic) 2 hours after the last dose either of vehicle or linaclotide and compared for each group at all timepoints to their baselines to follow treatment effects over time. Furthermore, the effects either of vehicle or linaclotide on day 1 (acute) and day 5 (chronic) 2 hours after the last dose either of vehicle or linaclotide for each individual animal, compared at all timepoints to its own baseline, are shown in Supplementary Fig. 2 (available at <http://links.lww.com/PAIN/A847>). At baseline, vaginal volume thresholds in ENDO rats were significantly lower compared to those of SHAM surgery rats and naive rats ( $P$  < 0.0001), consistent with previous reports (Fig. 2A).<sup>20,21,42</sup> By contrast, volume thresholds between SHAM surgery rats and naive rats were not significantly different, confirming that SHAM surgery is not associated with vaginal hyperalgesia (Fig. 2A).<sup>21</sup> Administration of linaclotide on day 1 significantly increased the volume threshold to vaginal distension in ENDO rats, compared with baseline ( $P$  < 0.001) (Fig. 2B). Increased volume thresholds in ENDO rats were sustained after chronic linaclotide treatment (5-day treatment), suggesting that both acute and chronic linaclotide treatments elicit antinociceptive effects in response to vaginal distension (chronic:  $P$  < 0.0001, Fig. 2B). This increase in vaginal volume thresholds expressed as percent change from baseline was significant both after acute linaclotide treatment on day 1 (35%,  $P$  < 0.001) and chronic linaclotide treatment on day 5 (42%,  $P$  < 0.0001). After a 4-day withdrawal period after the last dose of linaclotide on day 5, the volume threshold returned to baseline on day 9, whereas no changes in volume thresholds were evident in vehicle-treated



**Figure 2.** Daily oral linaclotide treatment reduces endometriosis-associated vaginal hyperalgesia. (A) Vaginal volume thresholds in naive, SHAM surgery, and ENDO rats. (B) The effects of linaclotide (3  $\mu$ g/kg/day, p.o.) (N = 14, day 1 and 5; N = 12, day 9) or vehicle (sterile water, p.o.) treatment (N = 9) on vaginal volume thresholds in ENDO rats and SHAM surgery rats (linaclotide [3  $\mu$ g/kg/day, p.o., N = 4]; vehicle [sterile water, p.o., N = 4]) were measured as EMG recordings of evoked visceromotor responses (VMR) to vaginal distension 2 hours after the last dose either of linaclotide or vehicle on day 1 and day 5, and on day 9 after a 4-day withdrawal period from treatment after the last dose either of vehicle or linaclotide on day 5. All measurements were performed in proestrus. Data are expressed as the mean  $\pm$  SEM. (A) \*\*\*\* $P$  < 0.0001: naive and SHAM surgery rats vs ENDO rats; (B) \*\*\* $P$  < 0.001, \*\*\*\* $P$  < 0.0001 ENDO rats (LIN) vs baseline. Two-way repeated-measures ANOVA followed by Tukey multiple comparison test (ENDO rats and SHAM surgery rats): linaclotide and vehicle treatment (day 1, 5) vs baseline, and day 9 after a 4-day withdrawal period from treatment vs baseline; one-way ANOVA followed by Tukey multiple comparison test: naive rats and SHAM surgery rats vs ENDO rats; one-way ANOVA followed by Tukey multiple comparison test: naive rats vs SHAM surgery rats. ANOVA, analysis of variance; LIN, linaclotide; VEH, vehicle.

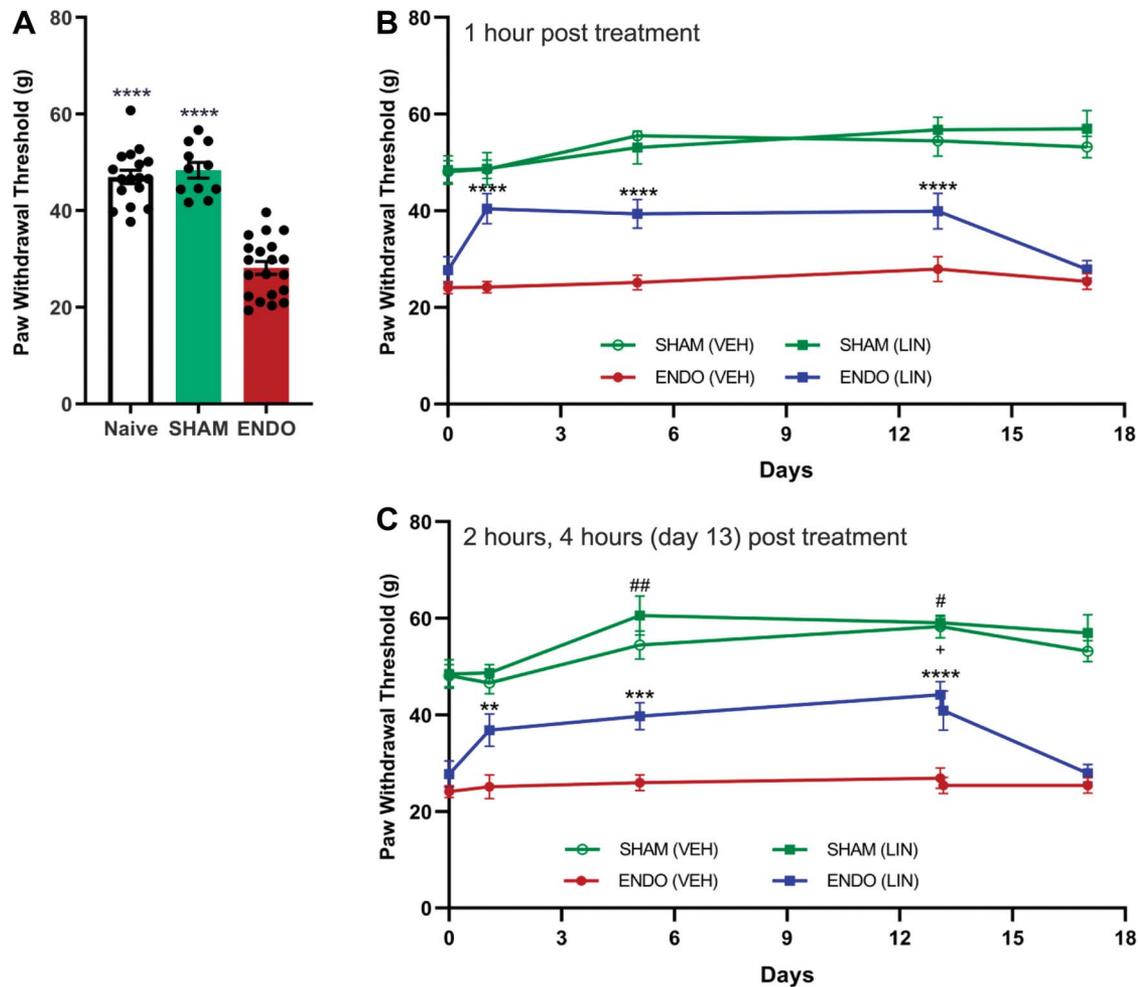
animals after a 4-day withdrawal period after the last dose of vehicle on day 9, compared with baseline (**Fig. 2B**). By contrast, neither vehicle nor linaclotide treatment significantly altered volume thresholds in SHAM surgery animals at all timepoints, compared with baseline (**Fig. 2B**). Volume thresholds of linaclotide-treated SHAM surgery rats were significantly higher than those of linaclotide-treated ENDO rats on day 1 ( $P$  < 0.01), but not on day 5 (Supplementary Fig. 3, available at <http://links.lww.com/PAIN/A847>). Representative VMR recordings from SHAM surgery rats, ENDO rats (baseline), or ENDO rats on day 5, 2 hours after the last dose either of vehicle or linaclotide, are shown in Supplementary Fig. 4 (available at <http://links.lww.com/PAIN/A847>).

### 3.2. Daily oral linaclotide treatment reduces endometriosis-associated mechanical hind paw allodynia

In endometriosis patients, symptoms are often aggravated by the presence of comorbid pain syndromes, including somatic pain.<sup>49</sup> Here, we assessed the effects of either vehicle or linaclotide treatment on referred somatic pain by measuring mechanical hind paw withdrawal thresholds during probing with electronic von Frey filaments and compared the effects for each group at all timepoints to their own baselines to follow treatment effects over time. Similarly, the effects either of vehicle or linaclotide treatment for each individual animal were compared at all timepoints to its own baseline (Supplementary Fig. 5, available at <http://links.lww.com/PAIN/A847>). At baseline, hind paw withdrawal thresholds in ENDO rats were significantly lower compared with those of SHAM surgery rats and naive rats ( $P$  < 0.0001) (**Fig. 3A**). By contrast, hind paw withdrawal thresholds between SHAM surgery and naive rats were not significantly different (**Fig. 3A**). A single dose of linaclotide, at 1 or 2 hours after dosing, significantly increased hind paw withdrawal thresholds after 1 hour ( $P$  < 0.0001) (**Fig. 3B**) and 2 hours

( $P$  < 0.01 in ENDO rats (**Fig. 3C**), compared with baseline, but had no effect on hind paw withdrawal thresholds in SHAM surgery rats, compared with baseline (**Figs. 3B and C**). These findings suggest that acute linaclotide administration can transiently reduce endometriosis-associated mechanical hind paw allodynia.

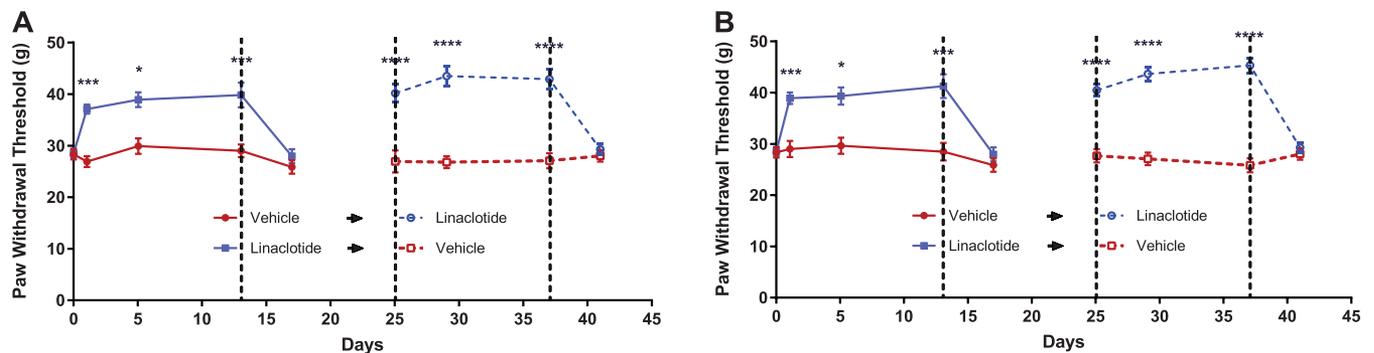
We then determined the effects of chronic linaclotide dosing on hind paw withdrawal thresholds in ENDO rats and SHAM surgery rats on day 5 and day 13, 1 hour (**Fig. 3B**) and 2 hours (**Fig. 3C**) after the last dose, and 4 hours after the last dose on day 13 (ENDO rats) (**Fig. 3C**). Here, we observed that chronic linaclotide treatment significantly increased withdrawal thresholds in ENDO rats at all timepoints (day 5:  $P$  < 0.0001, 1 hour;  $P$  < 0.001, 2 hours; and day 13:  $P$  < 0.0001, 1 hour;  $P$  < 0.0001, 2 hours;  $P$  < 0.0001, 4 hours) (**Figs. 3B and C**), compared with baseline. By contrast, no significant changes in hind paw withdrawal thresholds were observed in vehicle-treated ENDO rats at all timepoints, compared with baseline (**Figs. 3B and C**). Few changes in hind paw withdrawal thresholds were observed in SHAM surgery rats after treatment either with vehicle or linaclotide, except on day 5 (2 hours,  $P$  < 0.001, LIN vs baseline) and day 13 (2 hours,  $P$  < 0.05, LIN vs baseline; VEH vs baseline) (**Fig. 3C**). After a 4-day withdrawal period after the last dose of either vehicle or linaclotide on day 13, hind paw withdrawal thresholds in linaclotide-treated ENDO rats returned to baseline on day 17, whereas those of vehicle-treated ENDO rats on day 17 were not different from baseline (**Fig. 3B**). Hind paw withdrawal thresholds of vehicle- and linaclotide-treated SHAM surgery rats on day 17 remained unchanged, compared with baseline (**Fig. 3C**). Hind paw withdrawal thresholds of linaclotide-treated SHAM surgery rats were significantly higher than those of linaclotide-treated ENDO rats (day 1, 2 hours:  $P$  < 0.05; day 5, 1 hour:  $P$  < 0.05, 2 hours:  $P$  < 0.0001; day 13, 1 hour:  $P$  < 0.05, 2 hours:  $P$  < 0.05), except on day 1, 1 hour after linaclotide treatment (Supplementary Fig. 6, available at <http://links.lww.com/PAIN/A847>).



**Figure 3.** Daily oral linaclotide treatment reduces endometriosis-associated mechanical hind paw allodynia. (A) Hind paw withdrawal thresholds in naive, SHAM surgery, and ENDO rats. The effects of linaclotide (3  $\mu\text{g}/\text{kg}/\text{day}$ , p.o.) or vehicle (sterile water, p.o.) treatment in ENDO rats and SHAM surgery rats were measured as hind paw withdrawal thresholds in response to applied force (g) using electronic von Frey. Measurements were performed (B) 1 hour and (C) 2 hours after a single dose either of linaclotide or vehicle on day 1, 5, and 13, and on day 13, 4 hours after the last dose either of linaclotide or vehicle (ENDO rats). The effects of a 4-day withdrawal period from treatment after the last dose of either linaclotide or vehicle on day 13 were measured on day 17. Naive: N = 17, vehicle-treated SHAM surgery rats: N = 5, linaclotide-treated SHAM surgery rats: N = 5, vehicle-treated ENDO rats: N = 10, linaclotide-treated ENDO rats: N = 8. Data are expressed as the mean  $\pm$  SEM. (A) \*\*\*\* $P$  < 0.0001: naive and SHAM surgery rats vs ENDO rats; (B) \*\*\*\* $P$  < 0.0001: ENDO rats (LIN) vs baseline; (C) \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, \*\*\*\* $P$  < 0.0001: ENDO rats (LIN) vs baseline; # $P$  < 0.05, ## $P$  < 0.01: SHAM surgery rats (LIN) vs baseline; + $P$  < 0.05: SHAM surgery rats (VEH) vs baseline. Two-way repeated-measures ANOVA followed by Tukey multiple comparison test (ENDO rats and SHAM surgery rats): linaclotide and vehicle treatment (day 1, 5, 13) vs baseline, and day 17 after a 4-day withdrawal period from treatment vs baseline; one-way ANOVA followed by Tukey multiple comparison test: naive rats and SHAM surgery rats vs ENDO rats (baseline); one-way ANOVA followed by Tukey multiple comparison test: linaclotide-treated ENDO rats vs linaclotide-treated SHAM surgery rats; ANOVA, analysis of variance; LIN, linaclotide; VEH, vehicle.

We next used a cross-over, longitudinal design in which ENDO rats crossed over from either vehicle or linaclotide treatment to the other during the course of the study to further confirm that the previously observed beneficial effects on mechanical allodynia (1) are linaclotide dependent and sustainable when initiated at different periods of the study and (2) are sustained when measured either 1 hour (Fig. 4A) or 2 hours (Fig. 4B) after treatment with linaclotide. To exclude the possibility of any remaining residual linaclotide activity after the 4-day withdrawal period from treatment (day 13–17), an extra 8-day washout period spanning 2 estrous cycles was added (days 17–25) before initiation of the second treatment period. When animals that had received vehicle during the first treatment period (day 1–13) were switched to linaclotide during the second treatment period (day 25–37), sustained increases in hind paw withdrawal thresholds were observed with levels of improvement similar to those

exhibited by linaclotide-treated animals during the first treatment period (Figs. 4A and B). During the 4-day withdrawal period (day 37–41), hind paw withdrawal thresholds gradually decreased again to levels shown by vehicle-treated animals during the first treatment period. Conversely, when animals that had received linaclotide during the first treatment period were switched to vehicle during the second treatment period sustained decreases in hind paw withdrawal thresholds were observed similar to levels exhibited by the vehicle group during the first treatment period (Figs. 4A and B). The differences in hind paw withdrawal thresholds between linaclotide- and vehicle-treated animals during the second treatment period were significant at all timepoints and comparable when measured either 1 hour (Fig. 4A) or 2 hours after treatment (Fig. 4B) ( $P$  < 0.0001: day 25, 29, and 37). These data confirm that initiation of chronic linaclotide treatment at different timepoints during the study induced rapid



**Figure 4.** Cross-over study: daily oral linaclotide treatment reduces endometriosis-associated mechanical hind paw allodynia during each treatment period. Hind paw withdrawal thresholds in ENDO rats were measured 1 hour (A) and 2 hours (B) after administration either of linaclotide (3  $\mu$ g/kg/day, p.o.) or vehicle (sterile water, p.o.) on days 1, 5, and 13 during the first treatment period (day 1-13) and on days 25, 29, and 37 during the second treatment period (day 25-37). Hind paw withdrawal thresholds were also measured after 4-day withdrawal periods from treatment at the end of each treatment period (day 17, A, and day 41, B). Vehicle treatment: N = 9, linaclotide treatment: N = 10. All measurements were performed in proestrus. Data are expressed as the mean  $\pm$  SEM. \* $P$  < 0.05, \*\* $P$  < 0.001, \*\*\*\* $P$  < 0.0001. One-way ANOVA Tukey multiple comparison test: vehicle-treated vs linaclotide-treated ENDO rats. ANOVA, analysis of variance.

improvements in hind paw withdrawal thresholds and sustained beneficial treatment effects during the course of treatment.

### 3.3. Triple tracing from the ileum, colon, and vagina identifies shared sensory pathways

Our previous studies investigating colon-bladder cross-organ sensitization show that innervation of these organs by common sensory pathways is a key component to their concurrent sensitization and the reversal of this sensitization by linaclotide treatment.<sup>31</sup> Here, we investigated whether the ileum, colon, and vagina also share common sensory innervations and where these interactions take place.

Previous studies in the rat show that spinal afferent neurons projecting into the ileum are localized within DRG at spinal levels T8-L1, with a peak occurring at T10-T13.<sup>17</sup> In comparison, colonic afferent neurons are most abundant in DRG T13-L1 and L6-S1,<sup>19</sup> whereas vaginal afferent neurons have been located in DRG T12-L3 and L6-S2, peaking in T13-L1 and S1 ganglia.<sup>32</sup> Given this distribution of the spinal afferent neurons supplying the ileum, colon, and vagina, we wanted to determine the degree of overlap between the afferent input, if any, at different spinal levels and the existence of dichotomizing afferent neurons that may act as neuronal substrate for organ cross-sensitization. To do this, we used simultaneous retrograde tracing from the ileum, colon, and vagina and localized afferent neurons in DRG and projections within the spinal cord dorsal horn.

At the DRG level of T7-T9 (**Fig. 5Ai**), the majority (84  $\pm$  4.8%) of traced neurons were from the ileum, with a small number of traced neurons from the colon (15.9  $\pm$  4.8%). No dual ileal/colonic traced neurons were observed at any DRG level in T7 to T9. Moreover, no neurons traced from the vagina were observed in T7 to T9 DRG (**Figs. 5Aii and Aiii**). Correspondingly, in the T7 to T9 spinal cord, ileal traced projections were found within lamina I of the dorsal horn (**Fig. 5Aiv**).

In DRG levels T10 to T12 (**Fig. 5B**), the majority of neurons traced were from the colon (60.7  $\pm$  5%, **Figs. 5Bi and Bii**). We observed a small population of dual colonic/ileal traced neurons (5.5  $\pm$  0.9%) indicative of dichotomizing ileal/colon innervating afferents (**Figs. 5Bi-Biii**). By contrast, we did not observe any vaginal traced neurons in these levels. In the T10-T12 spinal cord, colonic traced projections were also found within lamina I of the dorsal horn (**Fig. 5Biv**).

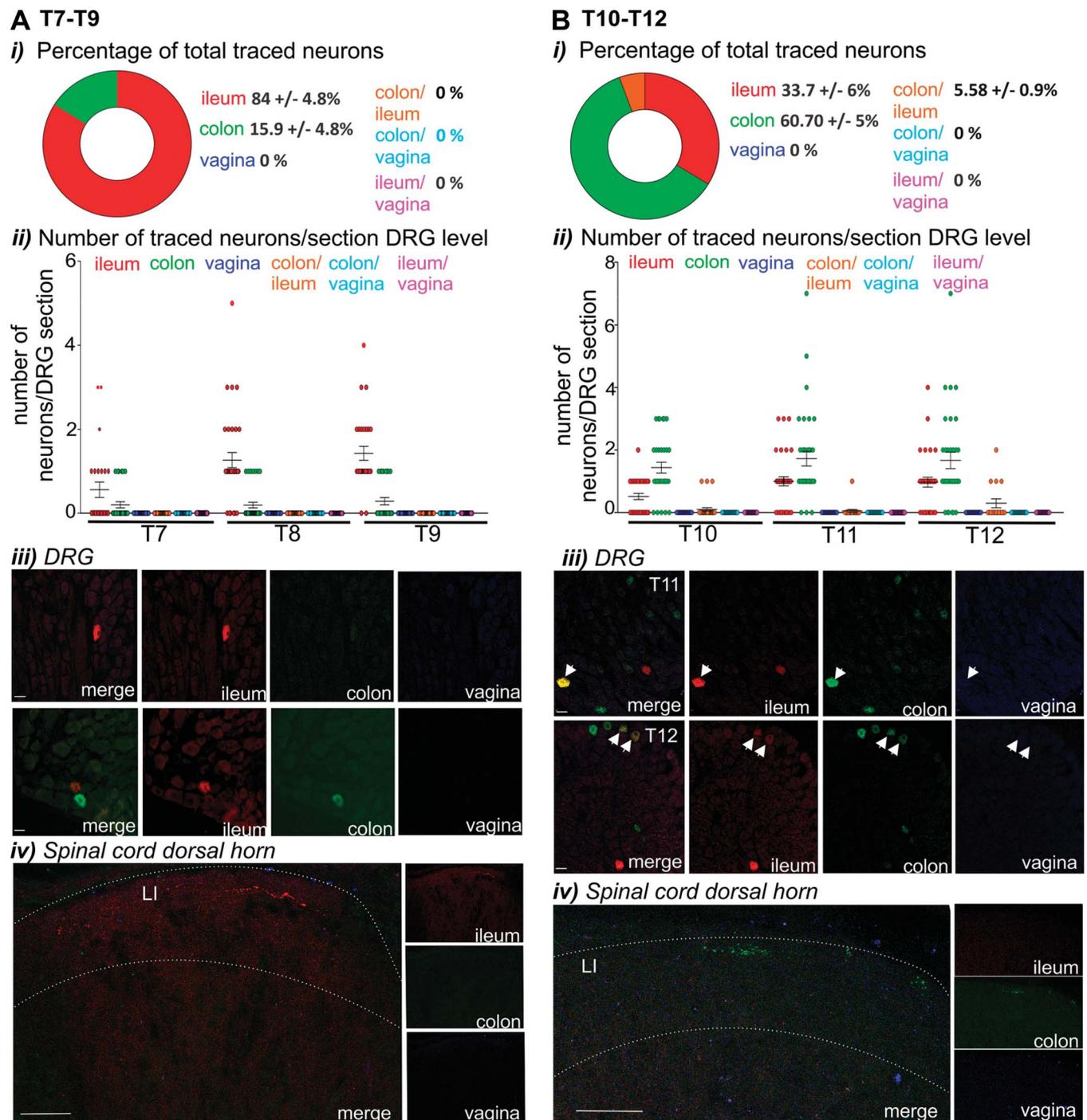
In DRG levels T13-L1 (**Fig. 6A**), DRG neurons traced from the ileum, colon, and vagina were found (**Figs. 6Ai and Aii**). The

majority of the traced neurons were colon-only (78.4  $\pm$  2%) labelled (**Fig. 6Ai**) within minor populations of ileal-only (8.9  $\pm$  2.7%), vaginal-only (3.5  $\pm$  2.1%), and colonic/ileal (6.4  $\pm$  1.2%) colabelled neurons. A small number of colon/vaginal dual-labelled neurons (2.2  $\pm$  0.6%) were observed in L1 (**Figs. 6Aii and Aiii**). We did also observe very rare dual ileal/vaginal labelled neurons, which were also often colonic traced (**Figs. 6Aii and Aiii**). In the spinal cord (**Fig. 6Aiv**), we observed projections from the ileum and colon in close apposition within the dorsal horn lamina I.

In DRG levels L6-S1 (**Fig. 6B**), similar proportions of colon- (46.8  $\pm$  3.3%) and vaginal-only (44.6  $\pm$  4.5%) neurons were observed (**Fig. 6Bi**), with no ileal traced neurons observed (**Figs. 6Bi and Bii**). A small population of colon/vaginal dual traced neurons was observed in both L6 and S1 DRG (8.6  $\pm$  1.4%) (**Figs. 6Bii and Biii**). In the spinal cord (**Fig. 6Biv**), colonic labelled projections were present in lamina I and within lateral collateral tracts projecting into regions of the lateral spinal nuclei and sacral parasympathetic nuclei. Vaginal traced projections were observed in lamina I and in midline and lateral tracts.

### 3.4. Linaclotide effects are not mediated through direct effects on the vagina or endometrial cysts

Although orally administered linaclotide has very low bioavailability and GC-C is expressed predominantly on the apical surface of intestinal epithelial cells, we wanted to confirm that the effects of linaclotide observed in this study were not mediated through direct effects on the vagina or endometrial cysts. Using qRT-PCR, abundant GC-C mRNA expression was detected in the ileum and colon (**Fig. 7A**), consistent with our previous findings.<sup>16,48</sup> Expression levels of GC-C mRNA in the ileum and colon were abundant and did not significantly differ between control rats (naive, SHAM surgery) and ENDO rats treated either with vehicle or linaclotide for 5 days (**Fig. 7A**). Furthermore, GC-C mRNA expression levels in the ileum and colon were not significantly different between vehicle- and linaclotide-treated ENDO rats (**Fig. 7A**). By contrast, GC-C mRNA expression was absent in both the vagina and in endometrial cysts irrespective of treatment condition (**Fig. 7A**). Similarly, GC-C expression was absent in the uterus under all treatment conditions, demonstrating that GC-C mRNA expression is not differentially regulated in normal uterine tissue after transplantation onto mesenteric arteries and grown into endometrial cysts (Supplementary Fig. 7A, available at <http://links.lww.com/PAIN/A847>). In situ hybridization studies



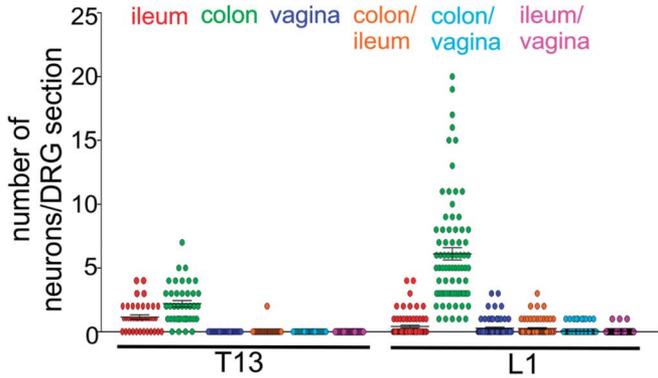
**Figure 5.** Retrograde tracing from the ileum, colon, and vagina show little shared sensory innervation in thoracic spinal levels. Ileum-, colon-, vaginal-innervating dorsal root ganglion (DRG) neurons and central projections within the spinal cord. Retrograde tracing from the ileum (CTB-555; red), colon (CTB-488; green), and vagina (CTB-647; blue) and assessment of labelling in (A) T7-T9 and (B) T10-T12 spinal cord levels of DRG afferent neurons (i, ii, and iii) and central projections in the spinal cord dorsal horn (iv). (A) Thoracic 7-thoracic 9 (T7-T9) spinal levels. (i) Quantitative data of the percentage of ileum-only, colon-only, vaginal-only, or dual-traced neurons as a proportion of total labelled neurons within T7-T9 DRG collectively (N = 4 rats, n = 115 neurons). (ii) Quantitative data of the number of ileum-only, colon-only, vaginal-only, or dual-traced neurons per DRG section within individual T7, T8, and T9 DRG (N = 4 rats, n = 25-36 DRG sections/DRG). Data indicate that few neurons were labelled in T7-T9 DRG, with the most abundant being from the ileum and no colabelled neurons. (iii) Representative image of an ileal traced (red) neuron in DRG T8. (iv) Projections of ileal spinal afferents were observed in the spinal cord dorsal horn lamina I (LI). Scale bars: Aiii = 20  $\mu$ m, Aiv = 50  $\mu$ m. (B) Thoracic 10-thoracic 12 (T10-T12) spinal levels. (i) Quantitative data of the percentage of ileum-only, colon-only, vaginal-only, or dual-traced neurons as a proportion of total labelled neurons within T10-T12 DRG collectively (N = 4 rats, n = 280 neurons). (ii) Quantitative data of the number of ileum-only, colon-only, vaginal-only, or dual-traced neurons per DRG section within individual T10, T11, and T12 DRG (N = 4 rats, 27-36 DRG sections/DRG). Data indicate prevalence of colonic-traced neurons and that a small number of neurons were colonic/ileal colabelled. (iii) Representative images of ileal- (red), colon- (green), and dual- (yellow; arrows) labelled neurons in T11 and T12 DRG. (iv) Projections of colonic spinal afferents were observed in the spinal cord dorsal horn lamina I (LI). Scale bars: Scale bars: Biii = 20  $\mu$ m, Biv = 50  $\mu$ m.

**A T13-L1**

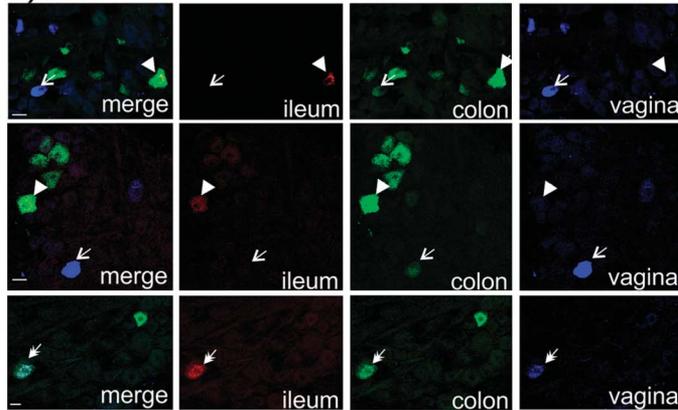
**i) Percentage of total traced neurons**



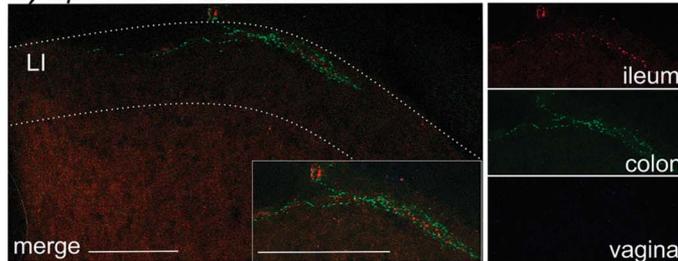
**ii) Number of traced neurons/section DRG level**



**iii) DRG**

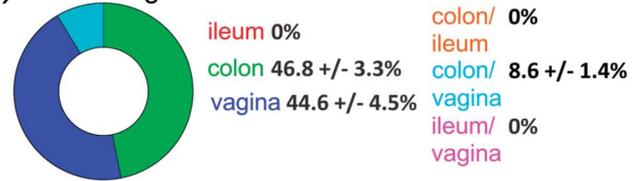


**iv) Spinal cord dorsal horn**

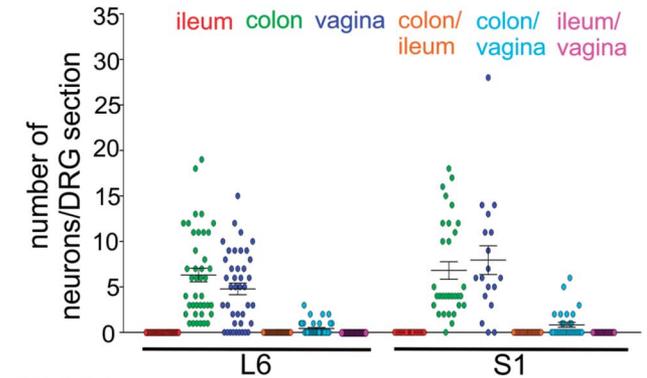


**B L6-S1**

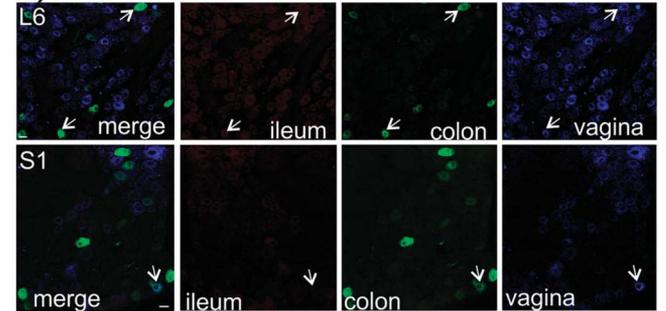
**i) Percentage of total traced neurons**



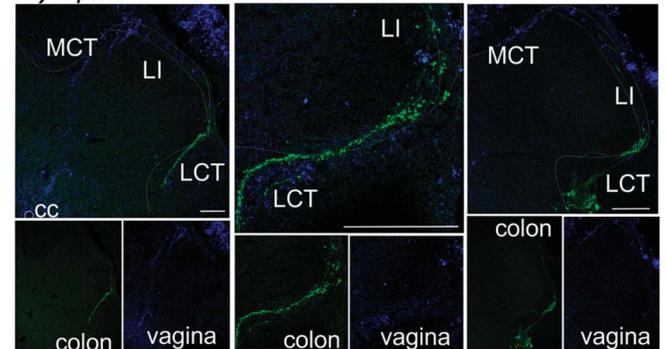
**ii) Number of traced neurons/section DRG level**



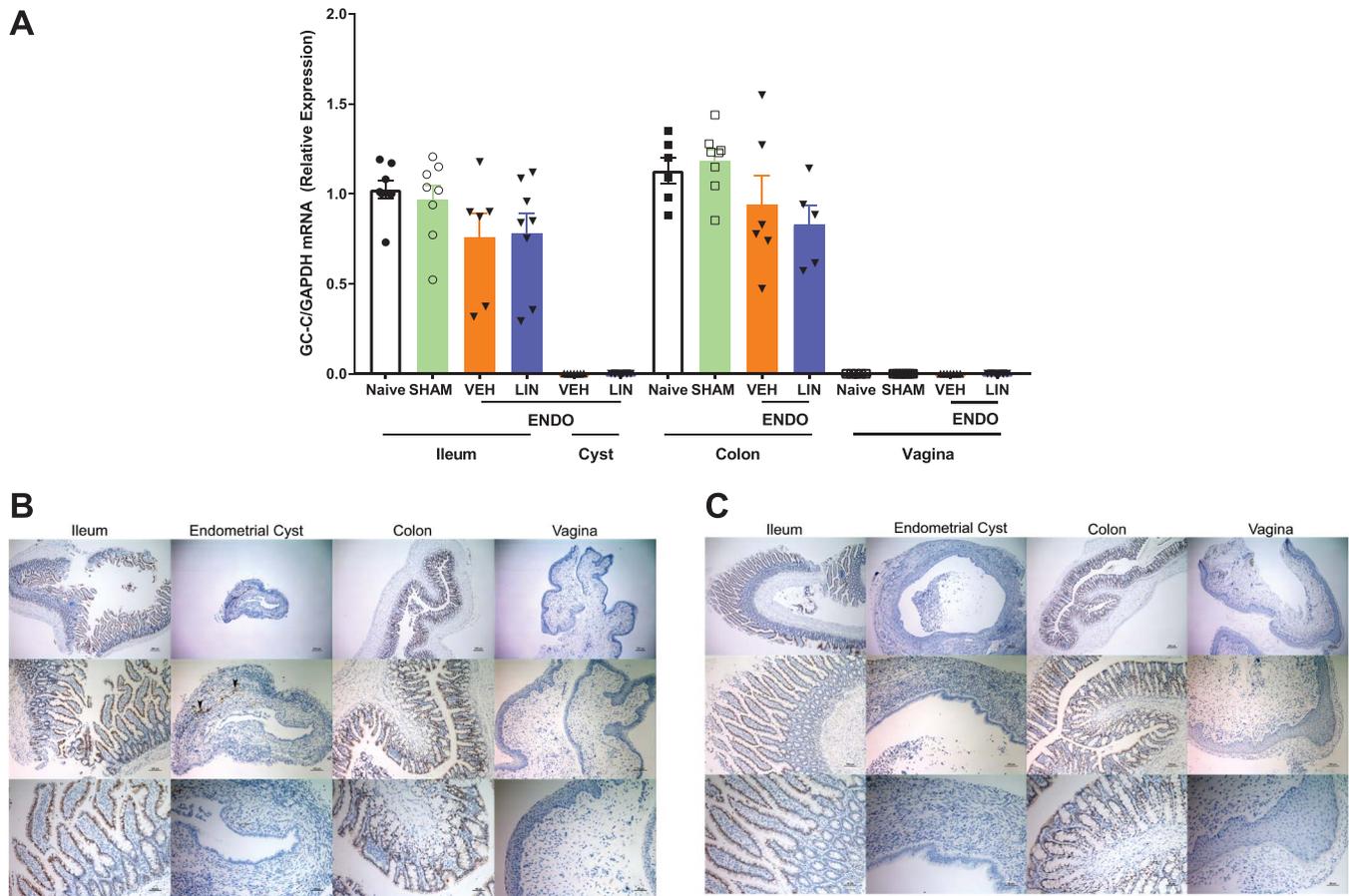
**iii) DRG**



**iv) Spinal cord dorsal horn**



**Figure 6.** Retrograde tracing from the ileum, colon, and vagina show shared sensory innervation in thoracolumbar and lumbosacral spinal levels. Ileum-, colon-, vaginal-innervating dorsal root ganglion (DRG) neurons and central projections within the spinal cord. Retrograde tracing from the ileum (CTB-555; red), colon (CTB-488; green), and vagina (CTB-647; blue) and assessment of labelling in (A) thoracolumbar (T13-L1) and (B) lumbosacral (L6-S1) spinal cord levels of DRG afferent neurons (i, ii, and iii) and central projections in the spinal cord dorsal horn (iv). (A) Thoracic 13-lumbar 1 (T13-L1) spinal levels. (i) Quantitative data of the percentage of ileum-only, colon-only, vaginal-only, or dual-traced neurons as a proportion of total labelled neurons within T13-L1 DRG collectively (N = 4 rats, n = 432 neurons). (ii) Quantitative data of the number of ileum-only, colon-only, vaginal-only, or dual-traced neurons per DRG section within individual T13 and L1 DRG (N = 4 rats, 40-78 DRG sections). Data indicate that the majority of traced neurons were colonic in origin, but ileal-only traced and vaginal-only traced neurons are also observed in T13-L1, with a small number of colonic/vaginal and ileal/vaginal colabelled neurons occurring within L1. (iii) Representative images of ileal- (red), colon- (green), and vaginal- (blue) labelled neurons in L1 DRG showing colon/ileal (white arrow heads), colonic/vaginal (white arrows), and colonic/vaginal/ileal (double arrowhead) colabelled neurons. (iv) Projections of colonic and ileal spinal afferents in close apposition were observed in the spinal cord dorsal horn lamina I (LI). Scale bars: Aiii = 20  $\mu$ m, Aiv = 50  $\mu$ m. (B) Lumbar 6-sacral 1 (L6-S1) spinal levels. (i) Quantitative data of the percentage of ileum-only, colon-only, vaginal-only, or dual-traced neurons as a proportion of total labelled neurons within L6-S1 DRG collectively (N = 3 rats, n = 684 neurons). (ii) Quantitative data of the number of ileum-only, colon-only, vaginal-only, or dual-traced neurons per DRG section (N = 4 rats, 31-41 DRG sections). Data indicate the equal prevalence of colonic-only and vaginal-only labelled neurons and a small number of colabelled neurons. No ileal traced neurons were observed. (iii) Representative images of colon- (green) and vaginal- (blue) labelled neurons in L6 and S1 DRG, with a small number of colonic/vaginal (white arrows) colabelled neurons in both DRG. (iv) Projections of colonic (green) and vaginal (blue) spinal afferents in the spinal cord dorsal horn. Colonic projections are present in lamina I (LI), in lateral collateral tracts (LCT) projecting into regions of lateral spinal nuclei (LSN) and sacral parasympathetic nuclei. Vaginal projections appeared in lamina I and within midline (MCT) and lateral tracts. Scale bars: Biii = 20  $\mu$ m, Biv = 50  $\mu$ m.



**Figure 7.** Linacotide effects are not mediated through direct effects on the vagina or endometrial cysts. (A) Using qRT-PCR, GC-C mRNA expression was measured in the ileum, endometrial cysts, colon, and vagina of ENDO rats after daily oral treatment either with vehicle (sterile water) or linacotide (3  $\mu$ g/kg/day) for 5 days and compared to GC-C mRNA expression in naive and SHAM surgery rats (N = 5–8). Abundant GC-C mRNA expression was detected in the ileum and colon of naive rats, consistent with our previous data.<sup>48</sup> No significant changes in GC-C mRNA expression levels were observed independent of treatment condition in the ileum and colon. By contrast, GC-C mRNA expression was absent in the vagina and endometrial cysts under all treatment conditions. Data are expressed relative to expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) In situ hybridization showing representative sections of the ileum, endometrial cysts, colon, and vagina at 40 $\times$  (top), 100 $\times$  (middle), and 200 $\times$  (bottom) magnification from vehicle-treated (left panel) and (C) linacotide-treated ENDO rats (right panel). These data further confirm abundant GC-C mRNA expression in the epithelial layer of the ileum and colon, whereas GC-C mRNA expression is absent in the vagina and endometrial cysts. The staining in the endometrial cyst, indicated by arrowheads, is caused by nonspecific staining of hemosiderin-laden macrophages, a common histologic finding in endometrial lesions in rodent endometriosis models and in endometriosis patients.<sup>4,28,43</sup> Scale bars: 200  $\mu$ m (40 $\times$ ), 100  $\mu$ m (100 $\times$ ), and 50  $\mu$ m (200 $\times$ ) magnification.

further confirmed these findings, displaying abundant GC-C mRNA expression in the epithelium of the ileum and colon (Fig. 7B). By contrast, no GC-C mRNA expression was evident in the vagina, endometrial cysts, and uterus under all treatment conditions (Fig. 7B; Supplementary Fig. 7B, available at <http://links.lww.com/PAIN/A847>).

#### 4. Discussion

Endometriosis is the most common cause of CPP, and greater than 50% of women diagnosed with endometriosis suffer from at least one concurrent comorbid pain syndrome.<sup>26,49</sup> Here, we provide evidence that linacotide, a peripherally restricted, minimally absorbed peptide acting locally within the gastrointestinal tract on GC-C, reduces symptoms of CPP in a rat model of endometriosis with underlying pathology that closely resembles human disease. The analgesic effects of linacotide on referred vaginal hyperalgesia and comorbid somatic pain seem mediated through engagement of neuronal cross-talk mechanisms. Support for this concept derives from the fact that both endometrial cysts driving increased pain perception and target organs/tissues

of secondary hyperalgesia and allodynia are anatomically located outside the gastrointestinal tract, distant from the site of action of linacotide. The effects of linacotide on symptoms of endometriosis-associated pain and comorbid somatic pain may provide a gateway for broader clinical utility of linacotide in the treatment of CPP syndromes.

Development of new therapies to manage pelvic pain conditions in the absence of side effects remains challenging. Therefore, having a therapeutic that is localized to and acts within the gastrointestinal tract to inhibit specific subtypes of nociceptors may be useful as an adjunct to treat other comorbidities. Linacotide has previously been shown to elicit analgesic effects in several rodent models of inflammation and stress-induced colonic pain.<sup>16,22,31,36,41</sup> Recent studies have further explored the mechanism by which linacotide alleviates pain using a mouse model of chronic visceral hypersensitivity in which hypersensitivity of colonic afferent pathways, spinal cord neuroplasticity, and colonic pain persist in the postinflammatory state. In these studies, chronic linacotide treatment reversed colonic afferent hypersensitivity and altered spinal cord connectivity, associated with alleviation of colonic pain, analogous to data from phase III

IBS-C trials with linaclotide.<sup>18,31,45</sup> Moreover, recent findings in rodent models of colon-bladder cross-organ sensitization support an even broader therapeutic potential of linaclotide in the treatment of CPP.<sup>31,41</sup> Chronic visceral hypersensitivity mice with colitis-induced changes of colonic afferent pathways develop concurrent bladder dysfunction and afferent hypersensitivity to distension, closely resembling symptoms in patients with interstitial cystitis/bladder pain syndrome.<sup>31</sup> Chronic linaclotide treatment restored normal bladder voiding patterns and reversed bladder afferent hypersensitivity via reduced nociceptive signaling from the colon mediated through common sensory pathways. These findings provided evidence supporting the concept that linaclotide acts on neuronal mechanisms involved in cross-organ sensitization.<sup>31</sup>

In the current study, using a rat endometriosis model in which the association between endometriosis and increased pelvic nociception is firmly established, we show that acute, but particularly chronic oral linaclotide treatment significantly decreased referred vaginal hyperalgesia. These findings further expand our understanding of linaclotide-mediated pain inhibition in models of CPP in which the underlying pathology involves mechanisms of peripheral and central sensitization. Central sensitization in this model has been argued because sensitized afferent fibers from endometrial cysts enter the spinal cord through the splanchnic nerve in thoracic segments T8-T12, anatomically distant from spinal segments receiving input from the vaginal canal that enter the spinal cord primarily through the pelvic nerve in segments L6-S1.<sup>7,8,15</sup> Thus, intersegmental neuronal processing is believed to involve modification of nociceptive information received from L6-S1 lumbosacral segments in thoracic segments T8-T12 through descending intraspinal and supraspinal interactions.<sup>6-8,42</sup>

To further identify shared sensory pathways that could account for the significantly increased vaginal pain thresholds elicited by linaclotide treatment in this model, we performed triple retrograde tracing studies concurrently from the ileum, colon, and vagina. First, although the majority of T10 to T12 and T13-L2 innervating DRG neurons contain only tracer injected into the colon, or to a lesser degree the ileum, a small subpopulation of dichotomizing neurons with axons projecting to the colon and ileum was detected. Correspondingly, we postulate the presence of a subpopulation of dichotomizing DRG neurons in endometriosis rats that branch to supply the colon and endometrial cysts. This concept would offer a clear mechanism for the analgesic effects of linaclotide by decreasing the hyperexcitability of DRG neurons and is consistent with findings reported previously in the chronic visceral hypersensitivity model of colon-bladder cross-sensitization.<sup>31</sup> A similar mechanism could also be postulated for dichotomizing neurons within L6-S1, which have axons projecting to both the colon and vagina. However, due to their respective small numbers, it is unlikely this effect of linaclotide on dichotomizing neurons alone fully explains its analgesic effects in this model. In addition to dichotomizing neurons, we also observe that the central terminals of ileal and colon innervating afferents reside within close apposition within the T13-L1 spinal cord, whereas colon and vaginal afferent central terminals also reside in close apposition within the L6-S1 spinal cord. These observations thereby provide a potential source of cross-organ interaction at the level of the spinal cord. Second, we show that DRG neurons innervating the ileum project to thoracic spinal cord segments (T7-T12). Direct sensory innervation of endometrial cysts occurs through newly sprouted nerve fibers derived from preexisting nerve fibers that innervate adjacent regions, connecting the cysts to midthoracic spinal segments (T8-T12) through the splanchnic nerve.<sup>6-8</sup> Thus, it is conceivable that linaclotide, by acting on nociceptors within the small intestinal wall,

further contributes to the desensitization of convergent spinal afferents from the cyst. Indirect evidence supporting this mechanism derives from both nonclinical and clinical studies in which orally administered linaclotide was shown to reduce visceral hypersensitivity and to improve bowel symptoms.<sup>11,12,16,18,22,31,35,36,41,45</sup> However, a direct association between GC-C activation in the small intestine and the reduction of visceral hypersensitivity has not yet been shown. Finally, no GC-C expression was detected in the vagina and endometrial cysts, further confirming that linaclotide's ability to reverse endometriosis-associated hyperalgesia and allodynia is not mediated through direct actions on these organs/tissues. Thus, we hypothesize that linaclotide-induced analgesia occurs as a consequence of its actions on colonic nociceptors and the subsequent desensitization of cross-organ neuronal pathways.<sup>16,31</sup> Moreover, our data further suggest that the beneficial effects linaclotide in this model may also involve direct actions on small intestinal nociceptors.<sup>48</sup>

Clinical studies in patients diagnosed with endometriosis have revealed a high prevalence of comorbid pain syndromes, such as IBS, vulvodynia, and somatic pain.<sup>26,47,49</sup> The beneficial effects of linaclotide treatment in the comorbid endometriosis-induced mechanical hind paw allodynia may therefore have important clinical implications. Somatic afferents innervating the rat hind paw travel through the sciatic nerve pathway, entering at spinal cord segments L4 to L6. Importantly, spinal cord segment L6 is also entered by colon-innervating and vagina-innervating sensory afferent DRG neurons. Therefore, colonic, vaginal, and somatic innervating afferents reside within a shared nerve pathway. Because mechanisms of central cross-organ sensitization underlie endometriosis-induced vaginal hyperalgesia and somatic mechanical allodynia through "engaging" viscerosomatic convergence, it is tempting to speculate that linaclotide operates to "disengage" these pathways. Notably, studies in other models of pelvic pain, including mouse models of neonatal vaginal irritation and endometriosis, have shown that pharmacological intervention with agents targeting well-characterized pathways involved in pain perception, such as the HPA axis and the prostaglandin E<sub>2</sub> signaling pathway, respectively, elicited beneficial treatment effects on primary and referred hyperalgesia but importantly, also reduced referred mechanical hind paw allodynia in these models.<sup>29,44</sup> Thus, these findings, in addition to linaclotide being localized to and acting on GC-C expressed within the gastrointestinal tract seem to further illustrate a differentiated mechanism of action of linaclotide for treatment in CPP.

Moreover, clinical studies have shown that endometriosis patients with comorbid urinary calculosis also suffer from more painful menstrual cycles and urinary colics as well as referred lumbar and abdominal muscle hyperalgesia.<sup>24,26</sup> These symptoms have been closely reproduced in ENDO rats with experimental ureteral calculosis and hypothesized to involve sensitization of viscerovisceral and viscerosomatic convergent sensory neurons.<sup>25</sup> Under those conditions, it is conceivable that linaclotide treatment could prove beneficial for endometriosis patients suffering from comorbid urinary calculosis because spinal sensory afferents projecting into the ureter (mostly T11-L2) and colon (mostly T13-L1 and L6-S1) broadly overlap, enabling linaclotide-mediated desensitization of cross-organ neuronal pathways.

Together, our findings provide evidence that suggests linaclotide-mediated inhibition of viscerovisceral and viscerosomatic pain, which presents a major step forward in our understanding of alleviating pain in CPP states. Moreover, because the severity of endometriosis-induced vaginal hyperalgesia in cycling rats is known to parallel circulating levels of estradiol, which are highest in proestrus, our data further confirm

that the analgesic effects of linaclotide occur independently of peripheral and central effects of estradiol that promote pain in this model. Thus, the improved understanding of cross-talk mechanisms involved in endometriosis-associated pain should help to further shift our therapeutic focus from the endometrial lesion towards pain, the cardinal symptom of endometriosis.<sup>1,39,42,52</sup>

In summary, our findings that linaclotide reduces chronic endometriosis-associated pain and comorbid somatic pain provide new insights into mechanisms of cross-organ sensitization in CPP. The concept of therapeutic targeting the GC-C/cGMP pathway has already translated in the clinic, with linaclotide Food and Drug Administration–approved for treatment of IBS-C. Consequently, combined with the favorable safety profile of this drug, linaclotide may offer a novel, nonopioid based alternative not only for treatment of chronic endometriosis–associated pain, but also for concurrent treatment of multiple comorbid CPP syndromes.

### Conflict of interest statement

S.M. Brierley received grant support from Ironwood Pharmaceuticals and Allergan. P. Ge, J. Ren, and G. Hannig are employees of Ironwood Pharmaceuticals and own stock/stock options of Ironwood Pharmaceuticals. The remaining authors have no conflicts of interest to declare.

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Author contributions: J. Ren and P. Ge performed ENDO and SHAM surgeries. P. Ge, J. Ren, and G. Hannig designed, performed, and analyzed VMR to vaginal distension studies and mechanical hind paw withdrawal threshold data in response to electronic von Frey filaments. P. Ge designed, performed, and analyzed qRT-PCR and in situ hybridization studies.

A.M. Harrington, S.M. Brierley, L. Grundy, and J. Castro designed, performed, and analyzed retrograde tracing studies. All authors contributed to the discussion. G. Hannig, P. Ge, S.M. Brierley, and A.M. Harrington wrote the manuscript with contributions from all authors. S.M. Brierley is a National Health and Medical Research Council of Australia (NHMRC) R.D. Wright Biomedical Research Fellow (APP1126378).

### Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at <http://links.lww.com/PAIN/A847>.

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