Linaclotide Inhibits Colonic Nociceptors and Relieves Abdominal Pain via Guanylate Cyclase-C and Extracellular Cyclic Guanosine 3′,5′-Monophosphate

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BACKGROUND & AIMS: Linaclotide is a minimally absorbed agonist of guanylate cyclase-C (GUCY2C or GC-C) that reduces symptoms associated with irritable bowel syndrome with constipation (IBS-C). Little is known about the mechanism by which linaclotide reduces abdominal pain in patients with IBS-C. **METHODS:** We determined the effects of linaclotide on colonic sensory afferents in healthy mice and those with chronic visceral hypersensitivity. We assessed pain transmission by measuring activation of dorsal horn neurons in the spinal cord in response to noxious colorectal distention. Levels of *Gucy2c* messenger RNA were measured in tissues from mice using quantitative reverse transcription polymerase chain reaction and in situ hybridization. We used human intestinal cell lines to measure release of cyclic guanosine-3′,5′-monophosphate (cGMP) by linaclotide. We performed a post-hoc analysis of data from a phase III, double-blind, parallel-group study in which 805 patients with IBS-C were randomly assigned to groups given an oral placebo or 290 μg linaclotide once daily for 26 weeks. We quantified changes in IBS-C symptoms, including abdominal pain. **RESULTS:** In mice, linaclotide inhibited colonic nociceptors with greater efficacy during chronic visceral hypersensitivity. Intra-colonic administration of linaclotide reduced signaling of noxious colorectal distention to the spinal cord. The colonic mucosa, but not neurons, was found to express linaclotide's target, GC-C. The downstream effector of GC-C, cGMP, was released after administration of linaclotide and also inhibited nociceptors. The effects of linaclotide were lost in *Gucy2c*$^{-/-}$ mice and prevented by inhibiting cGMP transporters or removing the mucosa. During 26 weeks of linaclotide administration, a significantly greater percentage of patients (70%) had at least a 30% reduction in abdominal pain compared with patients given placebo (50%). **CONCLUSIONS: We have identified an analgesic mechanism of linaclotide: it activates GC-C expressed on mucosal epithelial cells, resulting in the production and release of cGMP. This extracellular cGMP acts on and inhibits nociceptors, thereby reducing nociception. We also found that linaclotide reduces chronic abdominal pain in patients with IBS-C.**

Keywords: CVH; CRD; Signaling Transduction; Analgesia.

Irritable bowel syndrome (IBS) is a prevalent chronic functional gastrointestinal disorder affecting 7%–14% of the North American population.[1] IBS is characterized by abdominal pain or discomfort associated with altered bowel habits and is subclassified as IBS with constipation (IBS-C), IBS with diarrhea, and alternating/mixed IBS.[2] Up to 33% of IBS patients have IBS-C, which places a considerable financial burden on society[3] and negatively impacts the quality of life of those affected.[4] Abdominal pain is the key clinical feature and the most difficult symptom to treat in patients with IBS.[5] Given the limited treatments currently available for patients with IBS-C, additional therapeutic options for abdominal pain relief are urgently needed.

Linaclotide, a synthetic, minimally absorbed, 14-amino acid peptide, is a guanylate cyclase-C (GC-C) agonist related to guanylin and uroguanylin, members of a family of naturally occurring peptide hormones (Supplementary Figure 1).[6] These hormones regulate intestinal fluid and electrolyte homeostasis and, thereby, bowel function through GC-C–mediated production of cyclic-guanosine-3′,5′-monophosphate (cGMP).[7] Linaclotide acts via the same mechanism as the endogenous hormones, through binding and activating GC-C located on the luminal surface of intestinal epithelial cells. This interaction elevates intracellular and extracellular levels of cGMP, inducing fluid secretion and accelerating intestinal transit in animal models.[8–10] In addition, linaclotide has been shown to elicit anti-hyperalgesic effects in several animal models of visceral pain.[11] These pharmacological effects of linaclotide have

Abbreviations used in this paper: cGMP, cyclic guanosine-3′,5′-monophosphate; CRD, colorectal distention; CVH, chronic visceral hypersensitivity; DH, dorsal horn; GC-C, guanylate cyclase-C; IBS, irritable bowel syndrome; IBS-C, irritable bowel syndrome with constipation; IR, immunoreactivity; pERK, phosphorylated MAP kinase ERK 1/2; TNBS, trinitrobenzene sulfonic acid.

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translated into the clinic. In phase II and phase III studies, linaclotide accelerated colonic transit and improved abdominal pain and constipation associated with IBS-C[12–14] and chronic idiopathic constipation.[15–17]

However, the exact mechanism by which linaclotide reduces abdominal pain remains unclear. In preclinical studies, anti-nociceptive actions have not been previously described for either guanylin or uroguanylin, and the anti-hyperalgesic effects of linaclotide exhibited in several distinct models of visceral pain are not attributable to alterations in colonic compliance.[11] Although the pathophysiology of IBS is not completely understood, hallmarks of IBS include allodynia and hyperalgesia to mechanical events within the intestine.[18–20] As mechanical hypersensitivity of colonic afferents is implicated in the development and maintenance of visceral pain in IBS,[19–21] we hypothesized that linaclotide and its downstream effector, intestinal epithelial cell–derived cGMP, might be responsible for the inhibition of colonic nociceptors.

We specifically targeted high-threshold nociceptive afferents in the splanchnic pathway, as we have shown they normally respond to noxious levels of colonic distention/contraction.[22,23] They also become hypersensitive[23] and hyperexcitable[24,25] in models of chronic visceral pain, which translates to increased signaling of noxious colorectal distention (CRD) within the thoracolumbar spinal cord.[26] We have shown that specific functional deficits in these afferents translate to reduced sensory responses to noxious CRD in whole-animal studies.[22,27] Most recently, we have shown that alterations in peripheral blood mononuclear cell supernatants from IBS patients correlate with abdominal pain intensity and frequency, and evoke mechanical hypersensitivity of colonic nociceptors.[21]

Here, our data show that linaclotide inhibits colonic nociceptors in vitro and in vivo, and that the efficacy of this inhibitory effect is greatest during chronic visceral hypersensitivity (CVH). Correspondingly, in a new post-hoc analysis of data from a 26-week phase III clinical trial, we show that oral administration of linaclotide significantly increases the percentage of patients with clinically meaningful improvement in abdominal pain, as specified in the recent US Food and Drug Administration guidance for IBS clinical trials[28] compared with placebo. Overall, our data reveal a unique analgesic mechanism of action that suggests linaclotide is able to exert beneficial effects on abdominal sensory symptoms, independent of improvements in bowel frequency.

Methods

For detailed descriptions of the methodology used, please see the Supplementary Material.

Model of CVH

Intra-colonic trinitrobenzene-sulfonic acid (TNBS; 130 μL/mL in 30% ethanol, 0.1-mL bolus) was administered as described previously.[23] TNBS-treated mice were allowed to recover for 28 days, at which stage inflammation had resolved and chronic colonic afferent mechanical hypersensitivity was evident.[23] These mice are termed *CVH mice*.

In Vitro Electrophysiology and Pharmacology

Splanchnic colonic afferents recordings were made from C57BL/6 healthy, CVH mice or GC-C null (*Gucy2c*$^{-/-}$) mice[29] using standard protocols.[22,23,30] High-threshold splanchnic nociceptors were investigated in intact colonic preparations and in those where the mucosa had been removed.

CRD and Phosphorylated MAP Kinase ERK 1/2 Immunohistochemistry

Mice received an enema of either saline or linaclotide (1000 nM). Five minutes later, under anesthesia, a 4-cm CRD balloon catheter was inserted transanally into healthy or CVH mice.[26] After regaining consciousness, CRD was performed (80 mmHg for 10 seconds, then deflated for 5 seconds and repeated 5 times). After sacrifice via anesthetic overdose, mice underwent fixation by transcardial perfusion and the thoracolumbar (T10–L1) spinal cord was removed and cryoprotected. Frozen sections were cut and incubated with monoclonal rabbit anti–phosphorylated MAP kinase ERK 1/2 (pERK) with AlexaFluorR488 used for visualization.

Determination of GC-C Expression With Quantitative Reverse Transcription Polymerase Chain Reaction and In Situ Hybridization

Quantitative polymerase chain reaction was performed using mouse-specific *Gucy2c* and glyceraldehyde-3-phosphate dehydrogenase Taqman probes on complementary DNAs synthesized from total RNAs extracted from a panel of mouse tissues. For in situ hybridization, sections were hybridized overnight at 55°C with either ^{35}S-labeled complementary RNA anti-sense or sense probes to *Gucy2c*.

cGMP Efflux Studies in Human Intestinal Caco-2 Cells

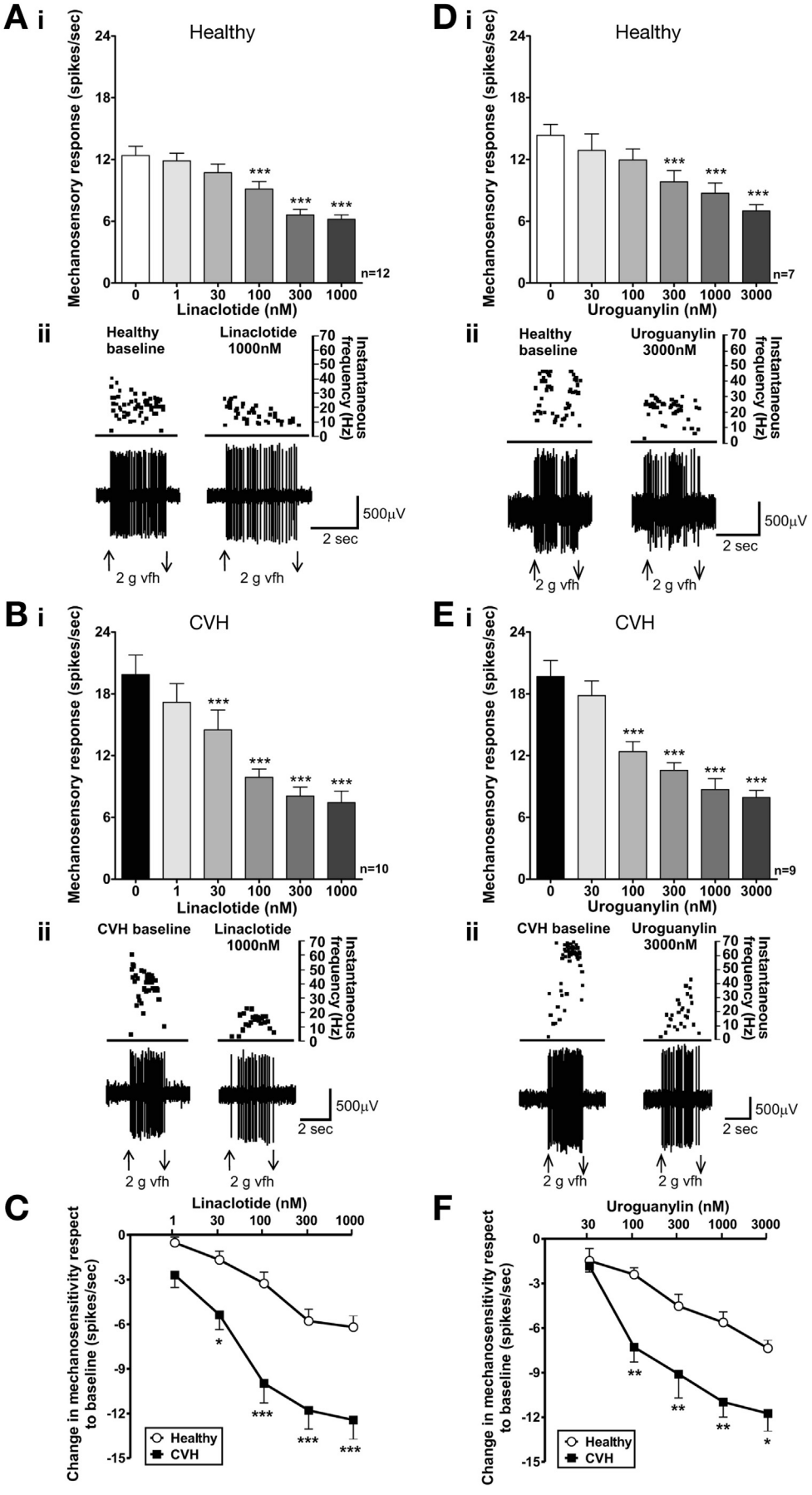
Cells were grown in monolayers and stimulated for 1 hour with linaclotide (1000 nM) in the presence or absence of the cGMP transporter inhibitor probenecid (0.5 mM or 2 mM). Samples from the basolateral chambers were collected and cGMP concentrations determined by liquid chromatography mass spectrometry.

Contractility Studies

Electrical field stimulation was applied to colonic tissues in the presence and absence of linaclotide or membrane permeable 8-bromo-cGMP. Contraction amplitude was compared between each condition.

Phase III Clinical Trial

The current results are from a post-hoc efficacy analysis of a phase III, double-blind, parallel-group, placebo-controlled trial that randomized 805 IBS-C patients to placebo or 290 μg oral linaclotide once daily for a 26-week treatment period. The current efficacy analysis are based on a responder end point for abdominal pain, specified as part of a co-primary end point recommended in the May 2012 US Food and Drug Administration final guidance for industry on the clinical evaluation of products for IBS,[28] defined as a ≥30% improvement from


A i
Healthy
Mechanosensory response (spikes/sec)
Linaclotide (nM)
n=12
ii
Healthy baseline
Linaclotide 1000nM
Instantaneous frequency (Hz)
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2 sec
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D i
Healthy
Uroguanylin (nM)
n=7
ii
Healthy baseline
Uroguanylin 3000nM
B i
CVH
n=10
ii
CVH baseline
Linaclotide 1000nM
E i
CVH
n=9
ii
CVH baseline
Uroguanylin 3000nM
C
Linaclotide (nM)
Change in mechanosensitivity respect to baseline (spikes/sec)
Healthy
CVH
F
Uroguanylin (nM)

baseline in average daily worst abdominal pain score.[28] We present, for the first time, an evaluation of this abdominal pain responder end point for each week of the 26-week treatment period, comparing treatment and control groups.

Results

Linaclotide Inhibits Colonic Nociceptors With Greater Efficacy in CVH

We hypothesized that linaclotide reduces abdominal pain in IBS-C patients[17] via an inhibitory action on colonic nociceptors. In order to test this hypothesis in mice, we performed in vitro single-unit afferent recordings. First, we investigated if linaclotide affected the mechanosensitivity of colonic nociceptors from healthy mice. We found that 100−1000 nM linaclotide applied to the surface of the mucosal epithelium significantly and dose-dependently decreased healthy colonic nociceptor activity, reducing responses to mechanical stimulation by 49% at the highest concentration tested (Figure 1*Ai, Aii*). Nociceptor mechanosensitivity was similarly reduced by linaclotide in response to noxious circular stretch (Supplementary Figure 2*A, B,* and *C*).

We then asked if these linaclotide-induced anti-nociceptive effects were maintained, or indeed augmented in chronic visceral pain, such as that suffered by IBS patients.[19] This question was assessed in an animal model of chronic visceral pain, where colonic nociceptor mechanical hypersensitivity[23] and colonic mechanical hyperalgesia and allodynia are evident long after resolution of TNBS-induced colitis.[31,32] We found that colonic nociceptors in the CVH model displayed pronounced mechanical hypersensitivity and that linaclotide significantly reduced their mechanosensitivity (Figure 1*Bi* and *Bii*), showing significant reductions at 30 nM and reversing the chronic visceral mechanical hypersensitivity, with a maximal reduction of 63% at 1000 nM (Figure 1*Bi* and *Bii*). Linaclotide's inhibitory effect was greatly enhanced in CVH compared with healthy nociceptors (Figure 1*C*).

Endogenous GC-C Agonist Uroguanylin Also Inhibits Colonic Nociceptors

In order to determine whether these anti-nociceptive effects were specific to linaclotide or could be induced by other GC-C agonists, we also studied the endogenous hormone uroguanylin. Application of uroguanylin to the colonic mucosal surface caused significant, dose-dependent inhibition of healthy colonic nociceptors (Figure 1*Di* and *Dii*). This effect was greatly enhanced in CVH (Figure 1*Ei, Eii,* and *F*). Overall, these findings indicate the GC-C agonists linaclotide and uroguanylin are able to inhibit colonic nociceptor function and reverse CVH.

Linaclotide Reduces Processing of Noxious CRD in the Thoracolumbar Spinal Cord In Vivo

Because linaclotide inhibits colonic nociceptors, as shown here, and inhibits pain responses in vivo,[11] we hypothesized this inhibition should correspondingly reduce signaling of noxious CRD within the spinal cord in vivo. We identified activated neurons in the dorsal horn (DH) of the thoracolumbar spinal cord in response to noxious CRD by pERK immunoreactivity (IR).[26] In healthy mice, intra-colonic administration of 1000 nM linaclotide resulted in significantly fewer pERK-IR DH neurons in the thoracolumbar spinal cord after noxious CRD compared with saline administration (Figure 2*A*, *D*, and *E*).

In response to noxious CRD, CVH mice displayed greater numbers of pERK-IR DH neurons than healthy mice, which corresponds with the extent of colonic nociceptor mechanical hypersensitivity observed in vitro. In CVH mice, linaclotide pretreatment resulted in a dramatic reduction in the number of pERK-IR DH neurons in the thoracolumbar spinal cord after noxious CRD (Figure 2*B*, *D*, and *F*). Overall, these results suggest that linaclotide reduces nociceptive signaling and reverses chronic visceral mechanical hypersensitivity in vivo. This finding correlates with our in vitro nociceptor findings and potentially

Figure 1. Linaclotide, a GC-C agonist, inhibits colonic nociceptors with greater efficacy in CVH. (*A*) (*i*) Healthy colonic nociceptor mechanosensitivity is significantly reduced after increasing concentrations of linaclotide applied to the mucosal epithelium for 5 minutes at each concentration. Linaclotide at 100 nM, 300 nM, and 1000 nM significantly reduced colonic nociceptor mechanosensitivity compared with baseline (***$P < .001$; n = 12 afferents, one-way analysis of variance [ANOVA], Bonferroni-post hoc). (*ii*) Single-unit recording showing action potential discharge of a healthy mouse colonic nociceptor in response to mechanical application of a 2-g von Frey hair filament (vfh) at baseline. Afferent responsiveness is reduced after 5-minute application of linaclotide (1000 nM). *Top panel* shows the instantaneous frequency of action potential firing. *Arrows* indicate application and removal of the vfh. (*B*) (*i*) In a model of CVH, nociceptors are potently and concentration-dependently inhibited by linaclotide. Concentrations of linaclotide at 30 nM, 100 nM, 300 nM, and 1000 nM significantly reduced the mechanical response of colonic nociceptors compared with baseline responses (***$P < .001$, n = 10 afferents, one-way ANOVA, Bonferroni post-hoc tests). (*ii*) Single-unit recordings showing potent inhibition of a CVH nociceptor after application of linaclotide (1000 nM). (*C*) Change in mechanosensitivity induced by linaclotide in healthy and CVH nociceptors compared with their respective baseline responses. Linaclotide caused significantly more inhibition at 30 nM (*$P < .05$), 100 nM, 300 nM, and 1000 nM (***$P < .001$) in CVH nociceptors than healthy nociceptors (n = 12 healthy, n = 10 CVH, 2-way ANOVA, Bonferroni post-hoc tests). (*D*) Uroguanylin inhibits colonic nociceptors with greater efficacy in CVH. (*i*) The endogenous GC-C agonist uroguanylin applied to the mucosal epithelium for 5 minutes at each concentration significantly reduces healthy colonic nociceptor mechanosensitivity at 300 nM,1000 nM, and 3000 nM compared with baseline (***$P < .001$, n = 7 afferents). (*ii*) Inhibition of healthy murine colonic nociceptor responsiveness after uroguanylin (3000 nM) administration. (*E*) CVH nociceptors are concentration-dependently inhibited by uroguanylin at 100 nM, 300 nM, 1000 nM, and 3000 nM (***$P < .001$, n = 9 afferents). (*ii*) Inhibition of CVH murine colonic nociceptor responsiveness after uroguanylin (3000 nM). (*F*) Uroguanylin causes significantly more inhibition at 100nM (**$P < .01$), 300 nM (**$P < .01$), 1000 nM (**$P < .01$), and 3000 nM (*$P < .05$) in CVH nociceptors relative to healthy nociceptors compared with their respective baseline responses (n = 7 healthy, n = 9 CVH).

explains improvements in abdominal pain in our IBS-C clinical trial analysis.

Expression of GC-C in Mouse Tissue

To further elucidate the site and mechanism of action of linaclotide, we used quantitative reverse transcription polymerase chain reaction and found that GC-C is predominantly expressed within the gastrointestinal tract, with low or undetectable expression in other tissues (Figure 3*A*). We confirmed these findings with in situ hybridization (Figure 3*B*) and found abundant GC-C expression in the colonic mucosa (Figure 3*C*). Although previous studies have also shown GC-C expression localized to specific midbrain neurons,[33] we found that GC-C expression was not detectable in key sensory structures, such as dorsal root ganglia and spinal cord neurons (Figure 3*D*). In order to confirm that inhibition of colonic nociceptors by linaclotide was GC-C dependent, we performed mechanosensitivity studies using GC-C$^{-/-}$ mice. Baseline colonic nociceptor responses were similar to those observed in normal healthy mice; however, the linaclotide-induced inhibition was completely lost (Figure 4*A*). Taken together, these data suggest that the anti-nociceptive effect of linaclotide is dependent on local activation of GC-C on intestinal epithelial cells. We also show that linaclotide does not alter colonic muscle contractility, and the membrane permeably 8-bromo-cGMP does reduce contractility (Figure 4*B*).

Linaclotide Stimulates cGMP Release, Which Inhibits Colonic Nociceptors

Linaclotide, like other GC-C agonists, elevates intracellular cGMP, which acts as a second messenger in the downstream mediation of intestinal fluid secretion.[6,34,35] Linaclotide acts locally with very low systemic bioavailability,[34] so is unlikely to activate intestinal nociceptors directly. However, cGMP is released from intestinal epithelial cells upon GC-C activation,[9,10] and could serve as a downstream mediator for linaclotide-induced effects on colonic nociceptors. In order to further investigate this role of extracellular cGMP, we used a human intestinal Caco-2 cell line, which is known to express GC-C, and stimulated the cells with linaclotide. This stimulation resulted in a significant transporter-dependent basolateral release of cGMP out of the cells, which was concentration-dependently decreased by the cGMP transporter inhibitor, probenecid (Figure 4*C*). Correspondingly, in colonic nociceptor recordings, linaclotide-induced inhibition of mechanosensitivity (Figure 4*Di*) was prevented by probenecid pretreatment (Figure 4*Dii*). This finding suggests extracellular cGMP derived from intestinal epithelial cells mediates linaclotide-induced inhibition of colonic nociceptors. To confirm this hypothesis, colonic nociceptor recordings were performed in preparations where the mucosal epithelium had been removed, to abolish the source of GC-C. In these studies, baseline nociceptor mechanosensitivity was normal, however, linaclotide-induced inhibition was significantly diminished in preparations from both healthy (Figure 4*Ei*) and CVH mice (Figure 4*Eii*).

In healthy colonic preparations, application of exogenous cGMP to the colonic mucosa replicated the inhibitory effects (Figure 5*Ai* and *Aii*) evoked by both linaclotide and uroguanylin. In addition, exogenous cGMP caused greater inhibition of CVH nociceptors (Figure 5*Bi* and *Bii*). In preparations where the colonic mucosa had been removed, the inhibitory effect of exogenous non−cell permeant cGMP was more potent, dose-dependent, and occurred at lower concentrations of cGMP (Figure 6*A*, *B*, and *C*).

Oral Linaclotide Reduces Abdominal Pain in Patients With IBS-C in a Phase III Clinical Trial

We include a new post-hoc longitudinal responder analysis, using the US Food and Drug Administration's recommended abdominal responder criterion,[28] from a 26-week phase III trial of oral, once-daily administration of linaclotide vs placebo in 805 IBS-C patients. The percentage of patients achieving at least a 30% reduction in abdominal pain compared with baseline was statistically significant and clinically meaningful for each of the 26 weeks of treatment with linaclotide compared with the placebo. A ≥30% reduction in abdominal pain compared with baseline was reported by >50% of linaclotide-treated patients by week 3, increased to >60% of linaclotide-treated patients by week 7, and was sustained at approximately 70% of linaclotide-treated patients for the remainder of the 26 weeks of treatment (Figure 7*A*).

Discussion

This study provides strong evidence for a direct analgesic mechanism of action, whereby linaclotide inhibits colonic nociceptors via a GC-C/extracellular cGMP pathway, to reduce colonic nociception and abdominal pain. This novel, previously unreported, pathway suggests linaclotide is able to exert its beneficial effects directly on abdominal sensory symptoms, independent of improvements in bowel movement frequency and function.

We have demonstrated that linaclotide inhibits the mechanical responsiveness of splanchnic colonic nociceptors, which have high-activation thresholds to mechanical stimuli. This finding is important, as these afferents have endings distributed throughout the length of the colon,[30] express large quantities of algesic channels and receptors,[21,22,27,36,37] and become mechanically hypersensitive[23] and hyperexcitable[24,25] in various preclinical models of chronic visceral pain. These in vitro findings translate in vivo as mice administered linaclotide have a reduced capacity to detect noxious CRD, as indicated by the reduction in activated DH neurons within the thoracolumbar spinal cord. In particular, we observed fewer activated neurons in the

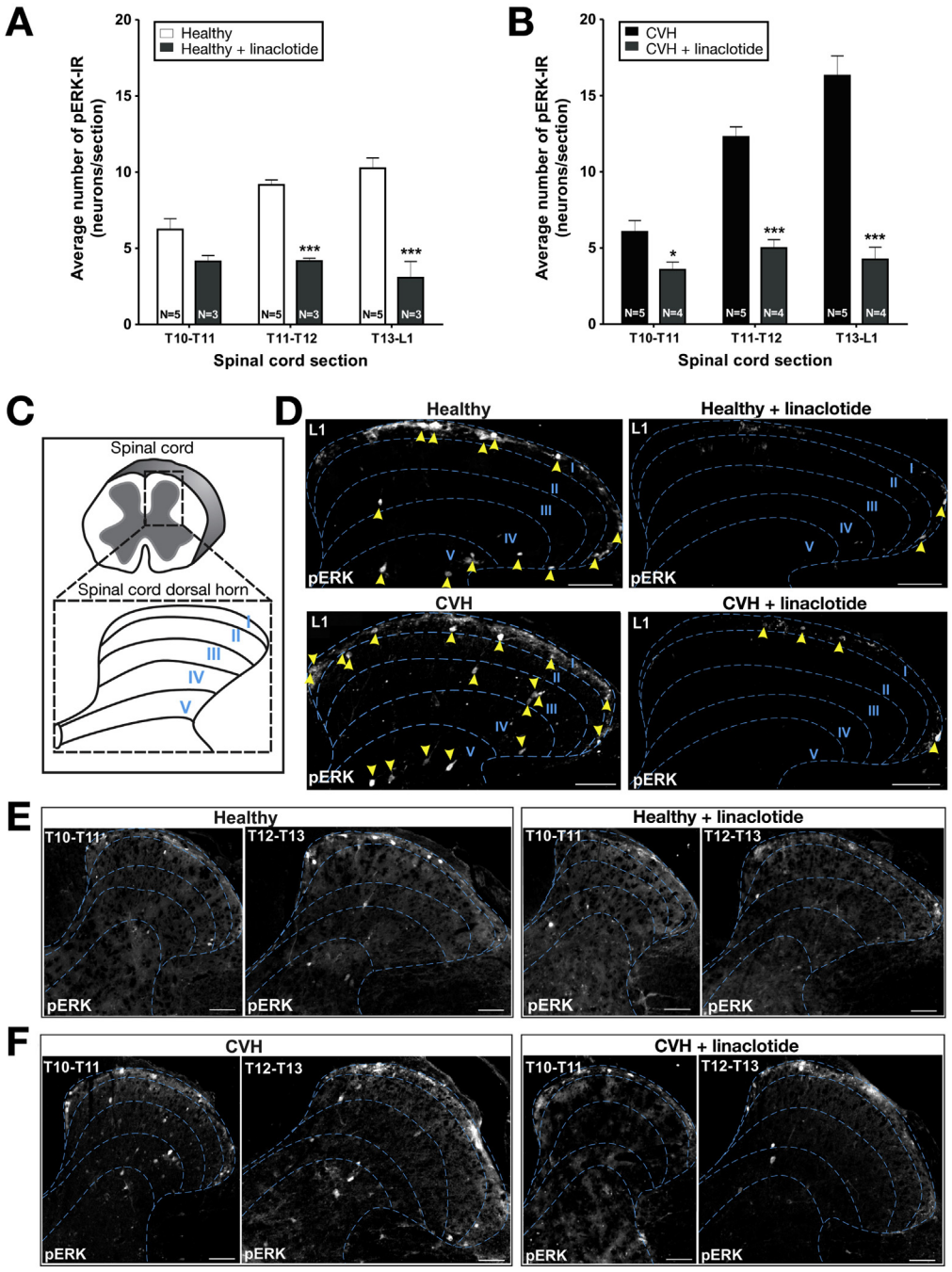

Figure 2. Intra-colonic administration of linaclotide reduces nociceptive signaling in the dorsal horn of the spinal cord in response to noxious CRD. (*A*) Noxious CRD (80 mmHg) in healthy mice results in activation of DH neurons in the thoracolumbar spinal cord, as indicated by pERK-IR (*open columns*). In mice pretreated with intra-colonic linaclotide (1000 nM; *shaded columns*), significantly fewer DH neurons are activated in the T11–T12 (***$P < .001$) and T13–L1 (***$P < .001$) spinal cord. One-way analysis of variance (ANOVA), healthy plus saline (N = 5), healthy plus 1000 nM linaclotide (N = 3). (*B*) In CVH mice more DH neurons are activated by noxious CRD at baseline (*closed columns*). However, pretreatment with intra-colonic linaclotide (1000 nM) significantly reduces the number of pERK-IR DH neurons in T10–T11 (*$P < .05$), T11–T12 (***$P < .001$), and T13–L1 (***$P < .001$) spinal cord (*shaded columns*). CVH plus saline (N = 5), CVH plus 1000 nM linaclotide (N = 4). (*C*) Schematic representation of laminae I–V in the dorsal horn of the thoracolumbar spinal cord. (*D*) *Upper panels*: In healthy mice, after noxious CRD, pERK-IR (*yellow arrows*) neurons were predominantly located in lamina I and lamina V. In healthy mice pretreated with linaclotide (1000 nM) significantly fewer pERK-IR neurons are evident after noxious CRD (see [*E*] for more examples). *Lower panels*: In CVH mice, after noxious CRD, pERK-IR neurons were predominantly located in the superficial DH laminae I–II and throughout laminae III–V. In CVH mice pretreated with linaclotide (1000 nM) significantly fewer pERK-IR neurons are evident after noxious CRD (see [*F*] for more examples).

superficial lamina of the DH, which is the major termination zone for nociceptive afferents and consists of nociception-specific neurons responding to noxious inputs from afferent fibers. Notably, the potency of these in vitro and in vivo inhibitory effects are greatest in a model of CVH, where linaclotide fully reversed the chronic mechanical hypersensitivity in vitro, and linaclotide pretreatment in vivo reduced signaling of

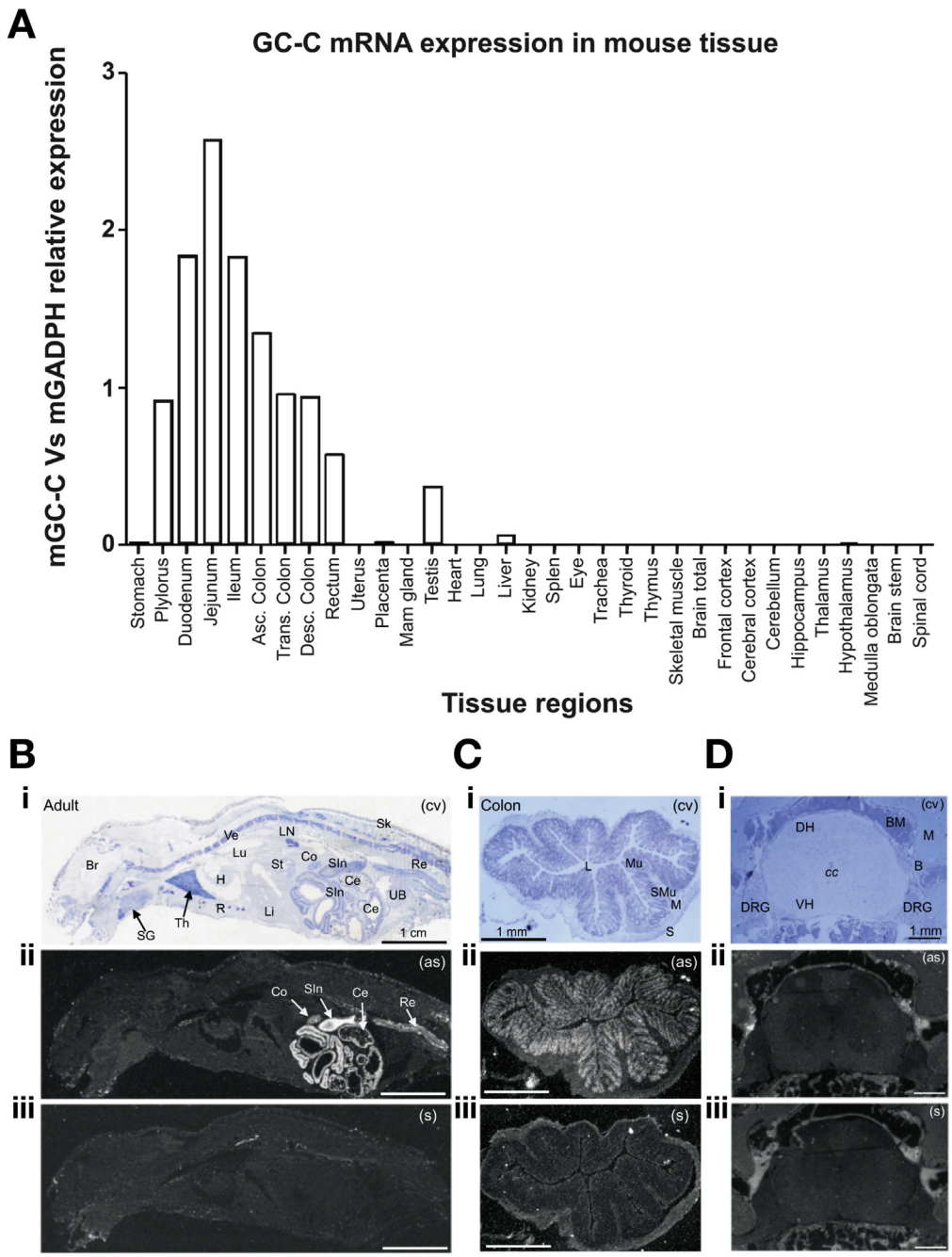

Figure 3. (*A*) GC-C messenger RNA (mRNA) expression in mouse tissues. Determined by quantitative reverse transcription polymerase chain reaction normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The vast majority of GC-C expression is localized in the gastrointestinal tract. (*B*) GC-C in situ hybridization in adult mouse tissues. (*i*) Cresyl violet (cv) staining in sections of adult mouse. (*ii*) In situ hybridization x-ray film autoradiography with GC-C receptor anti-sense (as) probes, showing a gut-specific GC-C mRNA distribution pattern in the adult mouse. GC-C staining is abundant in the small intestine (SIn), cecum (Ce), colon (Co), and rectum (Re). (*iii*) Use of complementary sense (s) probes as negative controls, revealing no labeling above background. Br, brain; H, heart; K, kidney; L, lumen; Li, liver; LN, lymph node; Lu, lung; Mu, mucosa; R, ribs; Re, rectum; SG, salivary gland; Sk, skin; Smu, submucosa; St, stomach; Th, thymus; UB, urinary bladder; Ve, vertebrae. (*C*) GC-C in situ hybridization in adult mouse colon. (*i*) Cresyl violet staining in a section of colon. (*ii*) In situ hybridization with GC-C anti-sense probes, showing GC-C mRNA expression in the colonic mucosa. (*iii*) Negative control using complementary sense probes. L, lumen, Mu, mucosa, Smu, submucosa; M, muscle; S, serosa. (*D*) Lack of GC-C staining in adult spinal cord and dorsal root ganglia (DRG). (*i*) Cresyl violet staining. (*ii*) In situ hybridization with GC-C anti-sense probes, showing a lack of GC-C mRNA in the spinal cord and DRG. (*iii*) Negative control complementary sense probes. B, bone; BM, bone marrow; cc, central canal; DH, dorsal horn; M, muscle; VH, ventral horn.

noxious CRD within the thoracolumbar spinal cord to normal, healthy levels.

These anti-nociceptive actions of linaclotide correlate well with one previous preclinical study, which showed linaclotide reduces colonic hypersensitivity in different models of noninflammatory and inflammatory visceral pain.[11] The remarkable aspect of these findings was the ability of linaclotide to reverse the visceral hypersensitivity

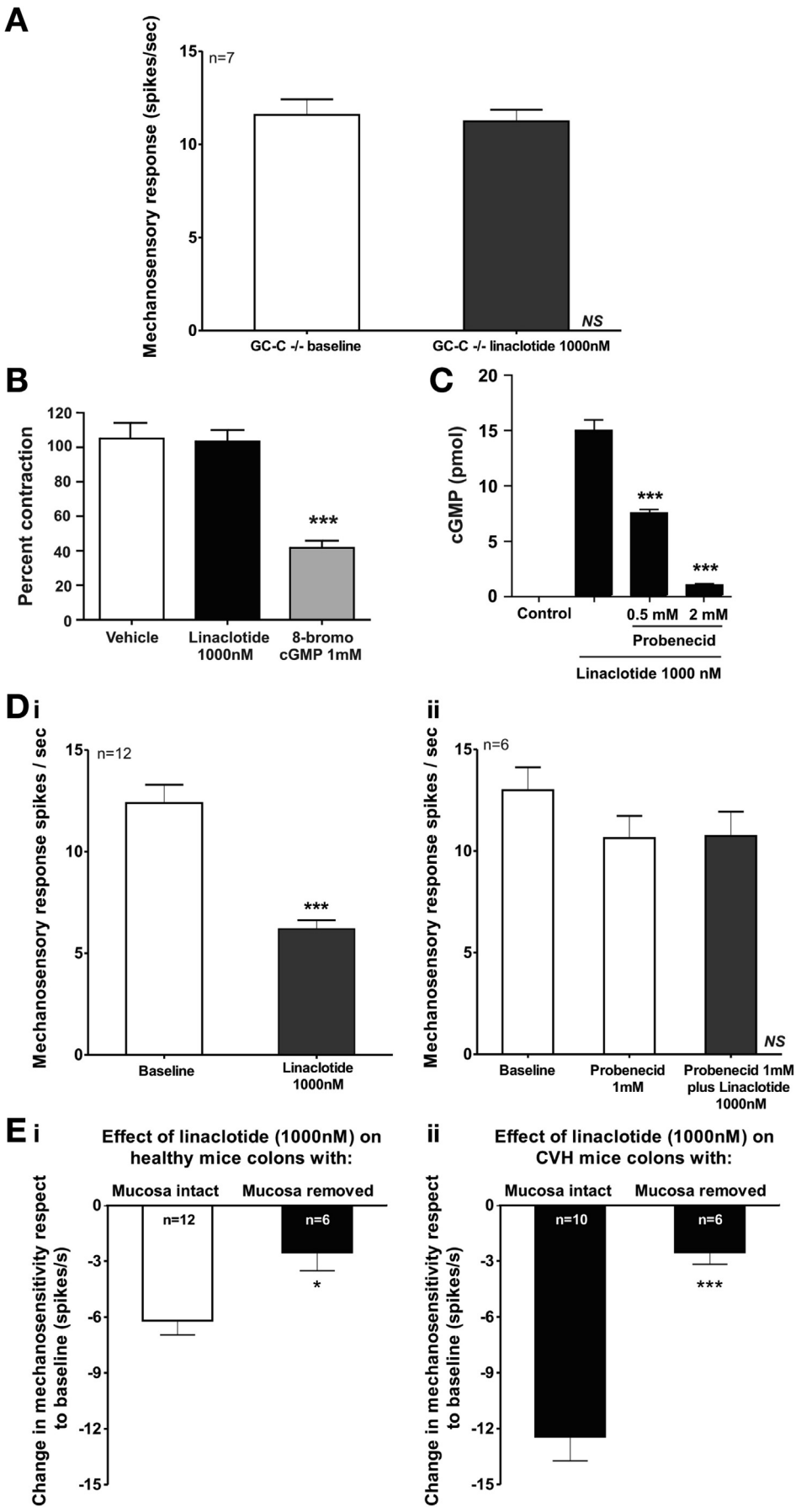

Figure 4. (*A*) Linaclotide-induced inhibition of colonic nociceptors is lost in GC-C$^{-/-}$ mice. Colonic nociceptors from GC-C$^{-/-}$ mice display normal mechanosensory responses; however, linaclotide-induced inhibition is completely lost (NS; $P > .05$, n = 7, paired *t* test). (*B*) Linaclotide does not affect electrical field stimulation (EFS) –induced contractions of rat colonic tissues. EFS (8 Hz, 0.5 milliseconds, 10 V, 3 trains of 10-second width, 2-minute delay between trains) induced colonic contractions were not significantly altered by prior incubation of vehicle (n = 6) or linaclotide (1000 nM; n = 6) for 5 minutes. In contrast, membrane permeable 8-bromo-cGMP significantly reduced contractility (***$P < .001$). (*C*) Linaclotide stimulates release of cGMP from human intestinal Caco-2 cells. Cells grown in monolayers were stimulated for 1 hour with linaclotide (1000 nM) in the presence or absence of the cGMP transporter inhibitor probenecid (0.5 mM or 2 mM; triplicate for each experiment). Linaclotide stimulated cGMP production and cGMP transport from the basolateral membrane, an effect that was concentration-dependently blocked by probenecid treatment (***$P < .001$, 1-way analysis of variance [ANOVA]). (*D*) Inhibiting the cGMP transporter prevents linaclotide-induced inhibition of colonic nociceptors. (*i*) Nociceptors are potently inhibited by a single dose of 1000 nM linaclotide (***$P < .001$, n = 12 afferents, paired *t* test). (*ii*) Prior addition of the cGMP transporter inhibitor, probenecid (1 mM), prevented linaclotide-induced inhibition of colonic nociceptors (NS, $P > .05$, n = 6, 1-way ANOVA). (*E*) Linaclotide-induced inhibition of colonic nociceptors requires an intact mucosal epithelium. (*i*) A single dose of linaclotide (1000 nM) causes pronounced nociceptor inhibition in intact preparations from healthy mice. This inhibitory effect is significantly diminished in preparations where the mucosal epithelium has been removed (*$P < .05$, n = 12 mucosa intact, n = 6 mucosa removed, unpaired *t* test). (*ii*) Similarly, in CVH mice, nociceptor inhibition by linaclotide is significantly reduced in preparations where the mucosa has been removed (***$P < .001$, n = 10 mucosa intact, n = 6 mucosa removed, unpaired *t* test).

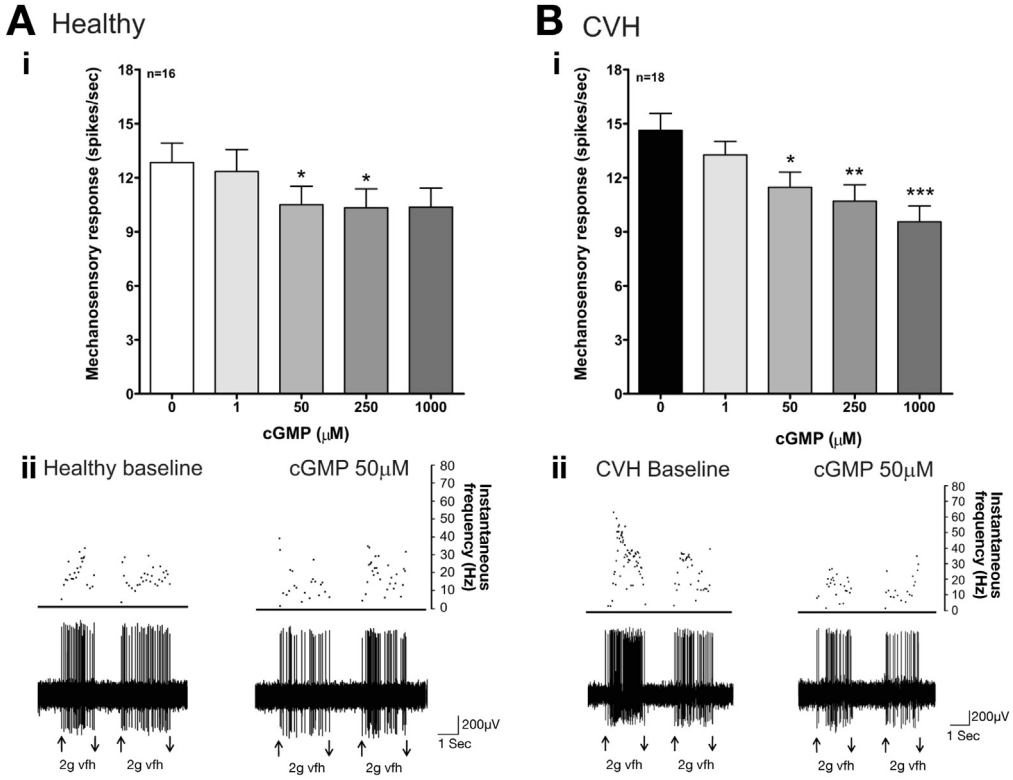

Figure 5. (*A*) (*i*) cGMP inhibits healthy colonic nociceptors. Exogenous application of non–cell permeant cGMP to the mucosal epithelium inhibits healthy colonic nociceptors (*$P < .05$ at 50 μM and 250 μM, n = 16 afferents, 1-way analysis of variance [ANOVA], Bonferroni post-hoc tests). (*ii*) Healthy murine colonic nociceptor mechanical response to a von Frey hair filament (vfh) is reduced after a 5-minute application of cGMP (50 μM). (*B*) (*i*) cGMP causes greater inhibition of CVH colonic nociceptors. CVH nociceptors are potently inhibited by exogenous application of cGMP to the mucosal epithelium at 50 μM (*$P < .05$), 250 μM (**$P < .01$), and 1000 μM (***$P < .001$, n = 18 afferents, 1-way ANOVA, Bonferroni post-hoc tests). (*ii*) Reduced CVH murine colonic nociceptor mechanical response to a vfh after cGMP (50 μM).

evoked in models of water-avoidance stress, acute-restraint stress, and TNBS-induced colitis.[11] Our results also confirm that linaclotide does not act to alter colonic contractile activity in response to electrical field simulation. Overall, our newly identified mechanism of action of linaclotide provides a rationale for linaclotide's antihyperalgesic effects in mechanistically distinct models of visceral pain via linaclotide-induced inhibition of colonic nociceptor peripheral endings.

These preclinical findings have translated into the clinic; in a new analysis of a 26-week phase III clinical trial using the recently published US Food and Drug Administration abdominal pain responder criterion,[28] >50% of linaclotide-treated IBS-C patients at week 3 reported a ≥30% reduction in abdominal pain compared with baseline. This level of analgesic effect increased to >60% of linaclotide-treated patients at week 7 and was sustained at approximately 70% of linaclotide-treated patients for the remainder of the 26 weeks of treatment. Previous analysis of these data showed the mean absolute and percent changes in abdominal pain for the linaclotide and placebo groups over time.[12] In the present study, we have evaluated the percent of patients with at least 30% improvement in abdominal pain on a weekly basis, data that have not been shown previously. These findings are important, as abdominal pain strongly correlates with IBS severity and is one of the most difficult symptoms to treat.[38] Our current findings of reduced colonic nociceptor mechanosensitivity in response to linaclotide provide a potential mechanism of action underlying the improvement of abdominal pain in patients after linaclotide treatment.

Our mechanistic studies have confirmed previous studies showing that GC-C expression is found predominantly on the gastrointestinal mucosa. In addition, we have found there is little or no GC-C expression in sensory dorsal root ganglia neurons, and the inhibitory effect of linaclotide on colonic nociceptors was lost in GC-C$^{-/-}$ mice. These results confirm that linaclotide inhibits colonic nociceptors by acting on intestinal epithelial cells via a GC-C–dependent mechanism. This linaclotide-induced nociceptor inhibition was significantly attenuated by prior removal of the colonic mucosa in both healthy and CVH states and by the cGMP transporter inhibitor probenecid, which blocked linaclotide-stimulated cGMP release from human intestinal Caco-2 cells. We also found that exogenously applied cGMP causes nociceptor inhibition with greatest efficacy in CVH, although at higher concentrations than linaclotide. However, by improving accessibility to nociceptor endings, exogenous cGMP has increased efficacy, exerting its antinociceptive effects at lower concentrations, where dose-dependence is also most evident. Taken together, these findings suggest that linaclotide, rather than acting directly on colonic nociceptors, binds and activates GC-C on the luminal surface of intestinal epithelial cells, resulting in increased intracellular cGMP production. cGMP is then actively transported across the basolateral epithelial cell membrane into the submucosal space, where it exerts its action on nociceptors located on blood vessels[30,39] to inhibit their function (Figure 7*B*). Although active mechanisms for transport of cGMP out of cells have been described, cGMP is poorly diffused across cell

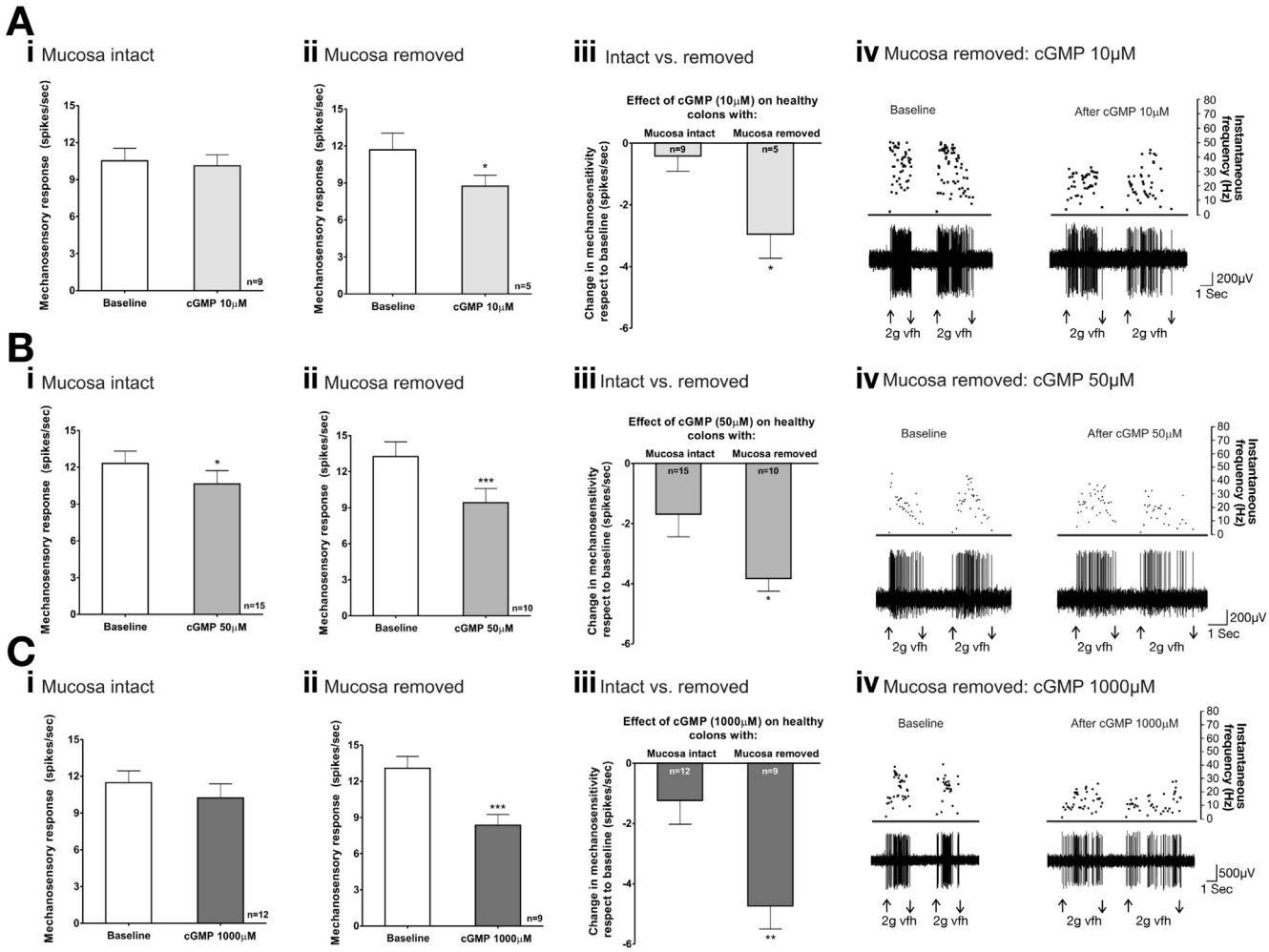

Figure 6. Exogenous non–cell permeant cGMP causes greater inhibition at lower doses in healthy preparations where the mucosal epithelium has been removed. (*i*) Exogenous application of cGMP at (*Ai*) 10 μM (NS, n = 9, paired *t* test), (*Bi*) 50 μM (*$P < .05$, n = 15), and (*Ci*) 1000 μM (NS, n = 14, paired *t* test) to the mucosal epithelium has a minimal effect on healthy nociceptors in intact preparations. (*ii*) In preparations where the mucosal epithelium had been removed, where accessibility of cGMP to nociceptors is improved, cGMP caused significant inhibition at (*Aii*) 10 μM (* $P < .05$, n = 5, paired *t* test), (*Bii*) 50 μM (***$P < .01$, n = 10, paired *t* test), and (*Cii*) 1000 μM (***$P < .01$, n = 9, paired *t*-test). (*iii*) cGMP-induced inhibition is significantly increased in mucosa removed preparations compared with intact preparations at (*Aiii*) 10 μM (*$P < .05$), (*Biii*) 50 μM (*$P < .05$), and (*Ciii*) 1000 μM (**$P < .01$). (*iv*) Healthy colonic nociceptor recordings in preparations where the colonic mucosa had been removed. Trace shows the mechanical responsiveness at baseline and after 5-minute application of cGMP (*Aiv*: 10 μM, *Biv*: 50 μM, *Civ*: 1000 μM).

membranes passively and is not actively transported back into cells.[40] Therefore, we believe the effects of cGMP on colonic nociceptors are acting through an extracellular or membrane target. We believe this report is the first to show that extracellular cGMP alters intestinal nociceptor function and mediates peripheral analgesia. This pathway is independent of the NO/soluble guanylate cyclase mechanism and the resulting effects of increasing neuronal intracellular cGMP that have been reported previously using different pharmacological agents,[41,42] including membrane permeable cGMP (8-bromo-cGMP or CPT-cGMP).[43] Additional studies to elucidate the molecular target for extracellular cGMP are ongoing.

In addition to linaclotide, the endogenous GC-C agonist uroguanylin also inhibited colonic nociceptors. These findings are not only consistent with those of linaclotide, but uncover a previously unidentified antinociceptive effect of uroguanylin, suggesting sensory signaling from the colon can be modulated endogenously via GC-C activation. A principal task of the digestive system is to solubilize nutrients for absorption, and also regulate fluid secretion. The guanylate cyclase system is conserved across vertebrate, nonvertebrate, and more distant phylogenetic species.[44] As uroguanylin and guanylin are released after a meal, we suggest this system might have evolved to facilitate digestion by assuring a fluid environment, while suppressing pain evoked by food-induced distention and naturally occurring high-amplitude intestinal contractions. We speculate that patients with IBS-C might have alterations in the GC-C signaling pathway, which is currently under investigation.

In conclusion, our findings demonstrate linaclotide inhibits colonic nociceptors via a novel GC-C/extracellular cGMP pathway to reduce nociception and abdominal pain. These results also advance our understanding of how the release of mediators, like cGMP, from the

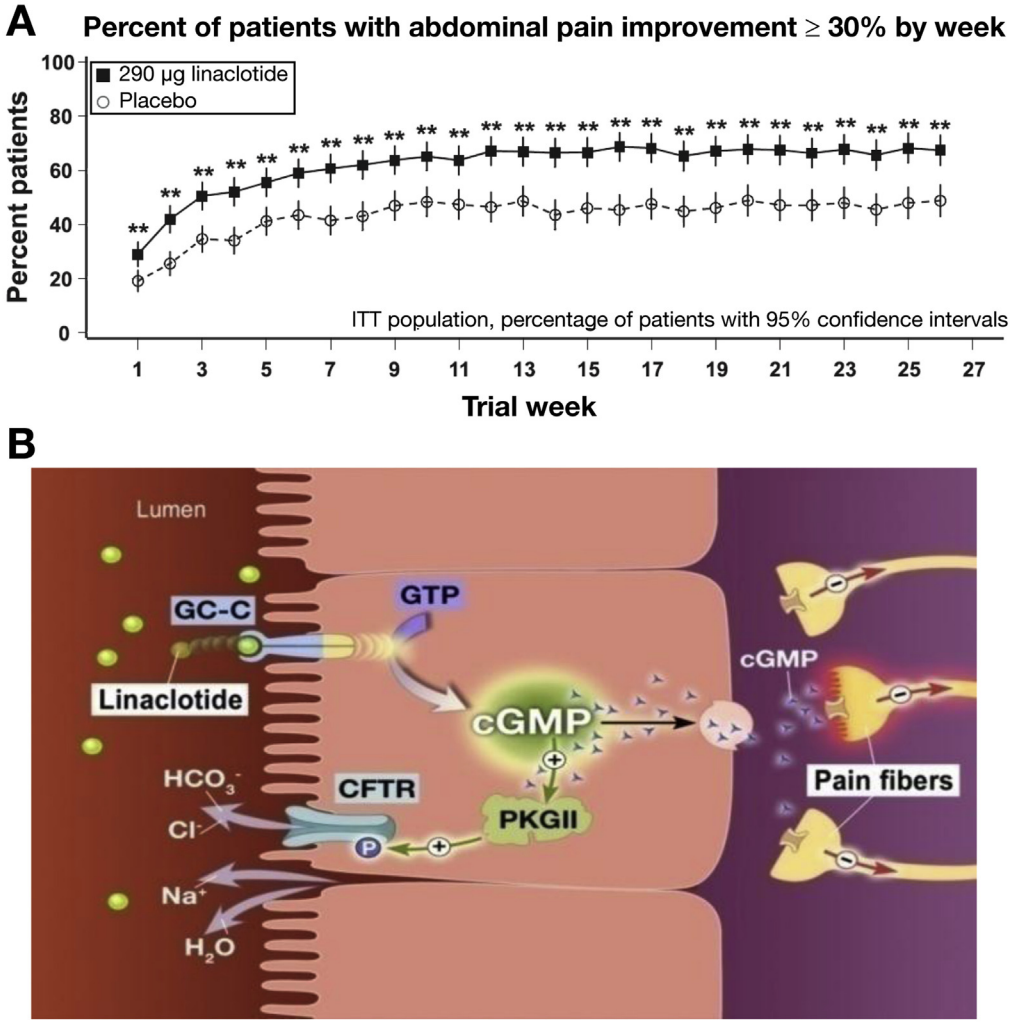

Figure 7. (*A*) Linaclotide significantly improves abdominal pain in patients with IBS-C. In a 26-week phase III clinical trial involving 805 IBS-C patients, oral administration of linaclotide (290 μg) resulted in a significant increase in the percentage of patients with a ≥30% improvement in abdominal pain over baseline (US Food and Drug Administration abdominal pain responder criterion) compared with placebo each week for 26 weeks of treatment. The percentage of patients with this level of improvement in abdominal pain was evident and separated from placebo during the first week of treatment, continued to increase over the ensuing 10–12 weeks and was maintained throughout the remainder of the 26-week treatment period, with a maximal sustained response in approximately 70% of linaclotide-treated patients versus approximately 50% of placebo-treated patients (**$P <$ .005 for each of the 26 weeks). One-way analysis of covariance at each week. (*B*) Proposed anti-nociceptive mechanism of linaclotide. Linaclotide, a minimally absorbed peptide GC-C agonist, binds to GC-C expressed on the apical surface of the mucosal epithelium, leading to cGMP production. The increase in cGMP within intestinal epithelial cells triggers a signal transduction cascade activating the cystic fibrosis transmembrane conductance regulator (CFTR) and inhibiting isoform 3 of the sodium/hydrogen exchanger. These processes lead to secretion of chloride and bicarbonate into, and retention of sodium within the intestinal lumen, resulting in increased luminal fluid secretion and an acceleration of intestinal transit. cGMP is also released, via a transporter dependent mechanism, through the basolateral membrane of intestinal epithelial cells. This extracellular, non–cell permeant cGMP can then act on high-threshold colonic nociceptors, which are located on blood vessels, to inhibit their mechanosensitivity and lead to reduced nociception and reduced abdominal pain. GTP, guanosine triphosphate; PKGII, Protein kinase II.

mucosal epithelium in the gastrointestinal tract influences visceral perception. This analgesic mechanism of action of linaclotide suggests that improvements in abdominal pain can occur independently of improvements in bowel function. These findings further support the therapeutic use of linaclotide as a new option for chronic abdominal pain in patients with IBS-C.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2013.08.017.

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Acknowledgments

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Conflicts of interest

These authors disclose the following: Mark G. Currie, Pei Ge, Gerhard Hannig, Hong Jin, Sarah Jacobson, Jeffrey M. Johnston, Caroline B. Kurtz, Bernard J. Lavins, James E. MacDougall, Courtney Shea, and Inmaculada Silos-Santiago are employees of Ironwood Pharmaceuticals and own stock/stock options in Ironwood Pharmaceuticals. Stuart M. Brierley and L. Ashley Blackshaw both received grant funding from Ironwood Pharmaceuticals, which funded this study in part. The remaining authors disclose no conflict.

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Supplementary Methods

Preclinical Studies

Experiments involving animals were approved by the Animal Ethics Committees of the Institute of Veterinary and Medical Science and University of Adelaide.

CVH Model

Male C57 BL/6 mice were used in all experiments. Colitis was induced by administration of TNBS, as described previously.[1] Briefly, 13-week-old mice were administered an intracolonic enema of 0.1 mL TNBS (130 μg/mL in 30% EtOH) via a polyethylene catheter inserted 3 cm from the anus of isoflurane-anesthetized mice. Mice were then individually housed and observed daily for changes in body weight, physical appearance, and behavior. Histological examination of mucosal architecture, cellular infiltrate, crypt abscesses, and goblet cell depletion confirmed TNBS-induced significant damage by day 3 post treatment, largely recovered by day 7, and fully recovered at the day 28 time point. High-threshold nociceptors from mice at the 28 day time point display significant mechanical hypersensitivity, lower mechanical activation thresholds,[1] and display hyperalgesia and allodynia.[2] As such, they are therefore termed *chronic visceral hypersensitivity* (CVH) mice.

In Vitro Mouse Colonic Primary Afferent Preparation, Recording, and Drug Addition

Age-matched mice were euthanized by CO_2 inhalation at days 0 (healthy) or 28 days post TNBS administration (CVH). In both cases, the colon, rectum, and attached mesentery were removed and afferent recordings from the splanchnic nerves performed as described previously.[1,3–5] Briefly, tissue was removed and pinned flat, mucosal side up, in a specialized organ bath. The colonic compartment was superfused with a modified Krebs solution (in mM: 117.9 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.3 NaH_2PO_4, 1.2 $MgSO_4$ $[H_2O]_7$, 2.5 $CaCl_2$, and 11.1 *D*-glucose) and bubbled with carbogen (95% O_2, 5% CO_2) at a temperature of 34°C. In vitro single-unit extracellular recordings were performed of action potentials generated by mechanical stimuli applied to afferent fiber receptive fields.

Mechanoreceptor Classification

Receptive fields were identified by systematically stroking the mucosal surface with a still brush to activate all subtypes of mechanoreceptors. Categorization of afferent properties was in accordance with our previously published classification system.[1,3–6] In short, splanchnic serosal afferents, which are also termed *vascular afferents*, have high-mechanical activation thresholds responding to noxious distension (40 mmHg), stretch ($\geq$7 g), or von Frey hair filaments (2 g), but do not respond to fine mucosal stroking (10 mg von Frey hair).[1,4,5] They express large quantities of algesic channels and receptors (TRPV1,[7] TRPA1,[3,8] TRPV4,[5] $P2X_3$,[7] B1,[6] $Na_V1.8$,[9] TNFR1,[10]), become mechanically hypersensitive in models of chronic visceral pain,[1] have nociceptor phenotypes, and are therefore referred to as "nociceptors" in this study.[4,11] Baseline mechanosensitivity was determined in response to a 2-g von Frey hair probe stimulation of the receptive field for 3 seconds, which was then removed. This process was repeated 3–4 times, separated each time by 10 seconds. Mechanosensitivity was then retested after application of linaclotide (1, 30, 100, 300, 1000 nM), uroguanylin (30, 100, 300, 1000, 3000 nM), and cGMP (1, 50, 250, 1000 μM). All doses of linaclotide, uroguanylin, and cGMP were applied for 5 minutes each. We have calculated that oral treatment of linaclotide (290 μg) in humans equates approximately to the highest dose of linaclotide we used in the preclinical studies (Supplementary Figure 1). Probenecid was incubated for 10 minutes. Linaclotide, uroguanylin, and cGMP were provided by Ironwood Pharmaceuticals Inc. Probenecid was purchased from Sigma-Aldrich Inc. (Sydney, Australia).

In all cases, test compounds were applied to the mucosal surface of the colon for a period of 5 minutes at each concentration via a small metal ring placed over the receptive field of interest. In some experiments, nociceptor mechanosensitivity was determined in response to 11-g circular stretch[1,5] applied across the receptive field in the absence and presence of linaclotide (1000 nM) (Supplementary Figure 2). In some experiments, before recordings commenced, the mucosal epithelium was microdissected away from the underlying muscle layers to determine the contribution of mucosal epithelium GC-C expression on nociceptor mechanosensitivity in the presence and absence of GC-C agonists and cGMP. Some recordings were also performed in GC-C null mutant (−/−) mice.[12] In some experiments, the cGMP efflux blocker, probenecid (1 mM), was incubated for 10 minutes before linaclotide incubation. Action potentials were analyzed off-line using the Spike 2 wavemark function and discriminated as single units on the basis of distinguishable waveform, amplitude, and duration. Data are presented as spikes/second or total number of action potentials per response. Data are expressed as mean ± SEM. In some cases, data are presented as the change from baseline. This is calculated by determining the change in mechanosensitivity of individual afferents between their normal baseline response in healthy or CVH conditions compared with their respective mechanical response after drug addition. This difference is then averaged across all afferents tested in the respective study set, for a final mean ± SEM change in response from baseline (n indicates the number of individual afferents). Data were analyzed using Prism 5 software (GraphPad Software, San Diego, CA), where appropriate, using either paired or unpaired *t* tests and 1-way or 2-way analysis of variance, followed by Bonferroni post-hoc tests.

Differences between specific drug concentrations and baseline responses were considered significant at a level of $*P < .05$; $**P < .01$; and $***P < .001$.

CRD

Healthy or CVH mice were fasted overnight with free access to water and a 5% glucose solution, ensuring fecal pellets were absent from the colorectum for CRD. Twenty-four hours later, mice were briefly anesthetized with isoflurane anesthetic. Mice received either a single 0.2-mL intracolonic enema of saline or linaclotide (1000 nM in 0.1M phosphate buffered saline [PBS]) via a polyethylene catheter inserted 3 cm from the anus. Five minutes after saline or linaclotide were administered, a 4-cm balloon catheter was inserted into the perianal canal and secured to the tail so that the start of the balloon sat 0.5 cm from the anal opening. The tube was secured to the tail. The balloon catheter was attached to a sphygmomanometer pressure gauge and a 20-mL syringe via a 3-way stopcock. Mice were removed from the isofluorane chamber and, on regaining consciousness, the balloon was distended for 10 seconds to a pressure of 80 mmHg applied via the syringe. This pressure was released and the balloon deflated (0 mmHg) for 5 seconds. This process was repeated 5 times, as per our previous studies.[13] After the fifth distension, mice were injected with an anesthetic overdose (0.2 mL/250 g sodium pentobarbitone) and within 4 minutes underwent transcardial perfuse fixation with ice-cold 4% paraformaldehyde fixative. Control mice received intracolonic administration of 0.1M PBS before the CRD protocol (n indicates the number of animals used per study). Our previous histological studies demonstrate that this protocol does not overtly damage the colonic epithelium.

Tissue Processing for Immunohistochemistry

Within 2 minutes after the final deflation, mice were administered an anesthetic overdose (0.125 mL/250 g sodium pentobarbitone) and underwent transcardial perfuse fixation. Mice were transcardial perfused with warm saline (0.85% physiological sterile saline) followed by ice-cold 4% paraformaldehyde in 0.1M phosphate buffer (Sigma-Aldrich, St Louis, MO). After transcardial perfusion, thoracolumbar (T10–L1) spinal cord was removed, using the lowest rib as an anatomical marker of T13, and post-fixed for 16 hours at 4°C in 4% paraformaldehyde in 0.1M phosphate buffer. After fixation, dorsal root ganglion and spinal cord were cryoprotected in 30% sucrose/phosphate buffer (Sigma-Aldrich) overnight at 4°C and then placed in 50% Optimal Cutting Temperature compound (Tissue-Tek, Sakura Finetek, Torrance, CA) in 30% sucrose/phosphate buffer solution for 7 hours, before block freezing in 100% Optimal Cutting Temperature compound. Frozen sections (15 μm) were cut using a cryostat and placed onto saline-coated slides for fluorescent immunohistochemistry.

pERK Immunohistochemistry

Immunohistochemistry for pERK was performed in a paired fashion, with tissue from healthy and CVH mice exposed to linaclotide or saline run simultaneously. After 20 minutes of air drying, sections were flushed 3 times with PBS and incubated with 5% normal chicken serum/0.2% Triton-X 200 (Sigma-Aldrich) in PBS (0.2% TX-PBS) for 30 minutes at room temperature to block nonspecific binding of secondary antibodies. Sections were then incubated for 18 hours at room temperature with monoclonal anti-sera rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (pERK; 1:200; #4370; Cell Signaling Technology, Danvers, MA) diluted in 0.2% TX-PBS. Sections were then washed 3 times with 0.2% TX-PBS before being incubated for 1 hour at room temperature with secondary antibody chicken anti-rabbit AlexaFluorR488 (AF-488). Negative controls were prepared as mentioned here with the primary antibody omitted.[13]

Expression of GC-C in Mouse Tissue

Quantitative reverse transcription polymerase chain reaction. Total RNAs from mouse peripheral tissues were purchased from Clontech Laboratories. Brain regions, dorsal root ganglion, esophagus, stomach, small and large intestinal segments, bladder, and vaginal tissue were collected from C57Bl6 mouse. Total RNA from these tissues was extracted using Trizol (cat. no. 15596018; Life Technologies, Carlsbad, CA) and further purified with the RNeasy Mini Kit (cat. no. 74106; Qiagen, Valencia, CA). Complementary DNAs were synthesized using a high-capacity cDNA reverse transcription kit (cat. no. 4368813; Life Technologies) according to the manufacturer's protocol (cms_04557; Life Technologies). Five hundred nanograms of complementary DNA was used for Taqman quantitative reverse transcription polymerase chain reaction gene expression analysis. Mouse-specific GC-C (Gucy2c) (cat. no. Mm01267705_m1; Life Technologies) and housekeeping glyceraldehyde-3-phosphate dehydrogenase Taqman probes (cat. no. 4352339E; Life Technologies) were obtained from Life Technologies. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative messenger RNA expression.[14]

GC-C Receptor In Situ Hybridization in Adult Mouse Tissue

Tissue preparation and pretreatment. Tissues were frozen in isopentane cooled to −35°C. Cryostat sections, 6–10 μm, were mounted on gelatin-coated slides or Fisherbrand Superfrost Plus slides (cat. no. 12-550-15; Fisher Scientific, Waltham, MA) and stored at −80°C. Before in situ hybridization, sections were fixed in 4% formaldehyde (freshly made from paraformaldehyde; P6148; Sigma Aldrich) in PBS, treated with triethanolamine/acetic anhydride, washed, and dehydrated in a series of ethanol washes. For distal gut tissues, the rectum

(tissue up to 1 cm up from the anus) was dissected free from the colon. The colon (from the cecum to the rectum) was divided into 3 equally sized pieces and termed the *ascending*, *transverse*, and *descending* colon, respectively.

Complementary RNA probe preparation. The sequences of GC-C (*Gucy2c* Mus Musculus) primers were obtained with PrimerQuest and positioned within exons 7 and 11 of the transcripts NM_001127318.1 and NM_145067.3 (see Appendix) coding a fragment of 622 bp.

Forward primer: TTTGAGGAGAACACCACAGCTCCT

The T7 RNA polymerase recognition sequence (T7 = 5′-GCGCTATAATACGACTCACTATAGGGAGA-3′) was included adjacent to the forward primer.

Reverse primer: TGATGTGGTTGGTCTCGTTGGTCT

The SP6 RNA polymerase recognition sequence (SP6 = 5′-GCATTAATTTAGGTGACACTATAGAAGCG-3′) was included adjacent to the reverse primer. The complementary RNA probe template was amplified by polymerase chain reaction. Before in situ hybridization, cold complementary RNA probes were produced in vitro using SP6 and T7 RNA polymerases to verify if the templates would yield probes of the appropriate length. After successful validation, antisense and sense radiolabeled probes were synthesized in vitro according to manufacturer's specifications (Ambion, Carlsbad, CA) and labeled with ^{35}S-UTP (>1000 Ci/mmol; cat. no. NEG039H; PerkinElmer LAS Canada, Inc., Woodbridge, Ontario, Canada).

Hybridization and washing procedures. Sections were hybridized overnight at 55°C in 50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM NaH_2PO_4, 10% dextran sulfate, 1× Denhardt's, 50 μg/mL total yeast RNA, and 50−80,000 cpm/μL ^{35}S-labeled complementary RNA probe. The tissues were subjected to stringent washing at 65°C in 50% formamide, 2× standard saline citrate, 10 mM dithiothreitol, and washed in PBS before treatment with 20 μg/mL RNAse A at 37°C for 30 minutes. After washes in 2× standard saline citrate and 0.1× standard saline citrate for 10 minutes at 37°C, the slides were dehydrated and exposed to Kodak BioMaxMR x-ray film for 5 days then dipped in Kodak NTB nuclear track emulsion and exposed in light-tight boxes with desiccant at 4°C for 14 days.

Imaging

Photographic development was carried out in Kodak D-19 and fixed in 35% sodium thiosulfate solution. Sections were counterstained lightly with cresyl violet and analyzed using brightfield and darkfield microscopy. Sense (control) riboprobes established the level of background signal.

Contractility Studies

Colonic tissue from male Sprague-Dawley rats was divided into 2-cm segments and transferred to an Schuler Organ Bath. Colon segments were suspended in tissue bath vessels containing Krebs solution (117.9 mmol/L NaCl, 4.7 mmol/L KCl, 25 mmol/L $NaHCO_3$, 1.3 mmol/L NaH_2PO_4, 1.2 mmol/L $MgSO_4[H_2O]$, 2.5 mmol/L $CaCl_2$, and 11.1 mmol/L D-glucose) continuously aerated with 95% O_2, 5% CO_2 and maintained at a temperature of 37°C. Tissue resting tension was 0.5 g. Colonic tissues were stimulated at a fixed frequency (8 Hz, 0.5-millisecond duration, 10 V, 3 trains of 10-second width, with a 2-minute delay between trains). Two control stimulations were performed and tissues were allowed to recover for 10 minutes. After the recovery period, tissues were treated either with vehicle (n = 6), linaclotide (1000 nM; n = 6), or membrane permeable 8-bromo-cGMP (100 μM; n = 6) at a volume of 100 μL and electrical field stimulation at the fixed frequency was repeated. Contraction amplitude is expressed as percent baseline amplitude of the second set of 8-Hz control stimulations. The amplitude of stimulations before treatment is considered 100% amplitude. Treatment with test article was initiated 5 minutes before electrical field simulation or pharmacological stimulation. Data are expressed as the mean ± SEM.

Basolateral cGMP Accumulation After Linaclotide Activation of GC-C in Caco-2 Cells

Human cell line and culture reagents. The human intestinal cell line, Caco-2, was acquired from American Type Culture Collection (ATCC, Manassas, VA, catalog # HTB-37 L/N 57850025). Dulbecco's modified Eagle's medium, L-glutamine, and 0.25% trypsine-EDTA were purchased from VWR (Bridgeport, NJ). Heat-inactivated fetal bovine serum, nonessential amino acids solution, Hank's balanced salt solution, and hydroxyethylpiperazine-N′-2-ethanesulfonic acid were obtained from Gibco (Grand Island, NY). Probenecid, cGMP, dimethyl sulfoxide, and collagen solution were purchased from Sigma-Aldrich Inc.

Preparation of Caco-2 cell monolayers. Caco-2 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% glutamine, and 1% nonessential amino acids solution, in a humidified atmosphere (37°C ± 2°C, 5% ± 1% CO_2). The culture medium was changed 3 times weekly, and the cell growth was observed by light microscopy. When the cells reached confluency, they were harvested by trypsinization. The collected cells were then seeded to collagen-coated polyethylene terephthalate membrane inserts in 24-well plates (0.33 cm^2 insert area, 0.4-μm pore size; BD Biosciences, San Jose, CA) to grow cell monolayers. The seeding density was ~2×10^4 cells per well. Plates were placed in a humidified incubator (37°C ± 2°C, 5% ± 1% CO_2), and the culture medium was changed for each well every other day until use. Cells cultured in the plates for 21−28 days were used. The transepithelial electric resistance was measured to ensure monolayer integrity using an epithelial tissue volt ohmmeter (World Precision Instruments Inc., Sarasota, FL). Cell monolayers with resistance values of >250 Ωcm^2 were used for experiments.

cGMP efflux inhibition study. Caco-2 cell monolayers were first washed twice with prewarmed transport assay buffer at pH 7.0 for apical side and 7.4 for basolateral side. The transport assay buffer was Hank's balanced salt solution containing 10 mM hydroxyethylpiperazine-N′-2-ethanesulfonic acid and 15 mM glucose. Transport buffer containing 1% dimethyl sulfoxide or transporter inhibitor probenecid in dimethyl sulfoxide (1% final concentration) was added to both apical and basolateral chambers and incubated at 37°C. After 15-minute incubation, cells (in triplicate) received treatments (Table 1) and were incubated at 37°C for 60 minutes.

After the treatments, 0.2-mL samples from basolateral chambers were collected and cyclic GMP (cGMP) concentrations were determined by liquid chromatography mass spectrometry. To determine the levels of intracellular cGMP in the Caco-2 cells after treatments, cells were washed once with transporter assay buffer and then lysed by treating each well with 0.1 mL 0.1 M HCl on ice for 15 minutes, followed by centrifugation to pellet cell debris. The resulting supernatants were transferred in 90-μL aliquots to 96-well plates and neutralized with 90 μL of 1M ammonium acetate. The centrifuged and neutralized (pH 7) Caco-2 cell lysates and aliquot from the basolateral compartments were analyzed using liquid chromatography mass spectrometry. The method outlined in Table 2 was used to quantify the concentration of cGMP in each sample. A standard curve of cGMP was prepared in transporter buffer at pH 7.0 for apical samples, at pH 7.4 for basolateral samples, and 0.1 M HCl for cell lysates, followed by neutralization with an equal volume of ammonium acetate, resulting in standards ranging from 1 nM to 1024 nM.

Human Phase III Clinical Trials. This phase III, double-blind, parallel-group, placebo-controlled trial randomized 805 IBS-C patients to placebo or 290 μg of oral linaclotide once daily for a 26-week treatment period. Trial design and details have been published previously[15] (Supplementary Figure 3) and conform to Consolidated Standards of Reporting Trials guidelines (ClinicalTrials.gov number: NCT00938717).

The current results are from a post-hoc analysis of this trial and are an efficacy analysis based on a responder end point for abdominal pain, specified as part of a co-primary end point recommended in the May 2012 US Food and Drug Administration final guidance for industry on the clinical evaluation of products for IBS,[16] defined as a $\geq$30% improvement from baseline in average daily worst abdominal pain score.[16] We present, for the first time, an evaluation of this abdominal pain responder end point for each week of the 26-week treatment period, comparing treatment and control groups. All authors had access to the study data and had reviewed and approved the final manuscript.

Trial Design

This 26-week, multicenter, randomized, double-blind, placebo-controlled, parallel-group trial was conducted at 102 clinical centers in the United States between July 2009 (first patient enrolled) and September 2010 (last patient completed). The trial was designed, conducted, and reported in compliance with the principles of Good Clinical Practice guidelines. At each center, an informed consent form approved by an Institutional Review Board was reviewed and signed by all patients before their participation in the trial.

During an initial screening period of up to 21 days, patients provided blood and urine for routine testing and were instructed to discontinue any prohibited medications (eg, anticholinergics, narcotics) for at least 14 days (24 hours for nonsteroidal anti-inflammatory drugs, if taken for abdominal pain, and for laxatives) before the start of baseline assessments. Patients meeting the inclusion and exclusion criteria then entered the 14-day baseline period, during which they used an interactive voice response system to provide daily and weekly symptom assessments. Patients eligible for the 26-week treatment period were randomized (1:1) to receive 290 μg linaclotide or placebo, administered as an oral capsule once daily at least 30 minutes before breakfast. This dose-strength designation reflects specific linaclotide content rather than the total peptide content expressed in previously reported studies, as linaclotide content is a more accurate and precise indication of dose strength. The actual amount of linaclotide received by patients did not change throughout the trial.

In addition to a screening visit, study site visits occurred at the start of the baseline period (day 14) and throughout the treatment period (days 1, 15, 29, 57, 85, 113, 141, and 183). All personnel involved in the design and implementation of the trial remained blinded until the database was locked.

Trial Patients

Patients were men and women aged 18 years or older who met modified Rome II criteria for IBS-C.[15] In the 12 months before the screening visit, eligible patients reported for at least 12 weeks (which need not be consecutive) abdominal pain or abdominal discomfort that had $\geq$2 of the following features: relief with defecation; onset associated with a change in frequency of stool; and/or onset associated with a change in form (appearance) of stool, before starting chronic treatment with tegaserod or lubiprostone (if patients had taken these medications) and <3 spontaneous bowel movements (SBMs, defined as bowel movements [BMs] occurring in the absence of laxative, enema, or suppository use during the 24 hours before the BM) per week and $\geq$1 of the following symptoms: straining during >25% of BMs, lumpy or hard stools during >25% of BMs, and a sensation of incomplete evacuation during >25% of BMs, before starting chronic treatment with tegaserod, lubiprostone, polyethylene glycol 3350, or any laxative (if patients had taken these medications). To be eligible for randomization, patients had to report

during the baseline period an average weekly score of ≥3 for daily abdominal pain at its worst (worst abdominal pain) on an 11-point numeric rating scale (0 = no abdominal pain, 10 = severe abdominal pain), and an average of <3 complete SBMs (defined as SBMs accompanied by patient self-reporting of a feeling of complete evacuation) per week and ≤5 SBMs per week.

Exclusion criteria included the following: loose (mushy) or watery stools reported in the absence of laxatives for >25% of BMs during the 12 weeks preceding the trial; mushy stool (Bristol Stool Form Scale[14] score of 6) for >1 SBM, or a watery, liquid stool (Bristol Stool Form Scale score of 7) for any SBM during the baseline period; a history of surgery to remove a segment of the gastrointestinal tract or bariatric surgery for obesity at any time; appendectomy/cholecystectomy within 2 months or other abdominal operations within 6 months before entry into the trial; history of diverticulitis or any chronic condition that could be associated with abdominal pain or discomfort and could confound the assessments in this trial; or a history of laxative abuse. In general, patients were excluded if they were taking drugs that could cause constipation (eg, narcotics), however, patients taking certain drugs for IBS that might be constipating (eg, tricyclic antidepressants) were eligible, provided that they were on a stable dose for at least 30 days before the screening visit and there was no plan to change the dose after the screening visit. Colonoscopy requirements were based on the American Gastroenterological Association guidelines.[17]

A total of 805 patients were randomized and received at least 1 dose of trial medication. A total of 599 patients completed the entire 26-week treatment period. The treatment groups were well balanced with respect to demographics and baseline bowel and abdominal symptoms, with the exception that the placebo group had a greater percentage of men than did the linaclotide group (12.7% vs 8.2%). During the baseline period, 87% of patients experienced abdominal pain every day and 76% of patients had no complete SBMs. Mean compliance with study-drug dosing (assessed by counting pills returned at study visits) up to study discontinuation/completion during the 26-week treatment period was 97.2% and 96.8% for the placebo and linaclotide groups, respectively.

Efficacy Assessments

Daily symptoms recorded using interactive voice response system included worst abdominal pain (measured using an 11-point numerical rating scale), the number of BMs, and whether rescue medication (ie, per protocol use of bisacodyl 5-mg tablets or suppositories) was used. Each BM was assessed for sensation of complete bowel emptying (yes/no).

The efficacy assessments evaluated during the 26 weeks of treatment were the following: improvement of ≥30% from baseline in average daily worst abdominal pain score and increase of ≥1 complete SBM from baseline.

Statistical Methods and Data Analysis

Patients were randomized by a computer-generated schedule to 1 of the 2 treatment groups (1:1) and were balanced within each site using a block size of 4. The sponsor staff, patients, and trial center personnel were blinded to trial treatment allocation.

The overall family-wise type I error rate for testing the primary efficacy end points was controlled at the .05 significance level using a 5-step serial gate-keeping multiple comparisons procedure.

All randomized patients who took at least 1 dose of study drug were included in safety analyses (safety population). Efficacy analyses were based on an intent-to-treat population, which included all patients in the safety population who had at least 1 post-randomization efficacy assessment (note: there was only 1 randomized patient who was not included in this intent-to-treat population).

Supplementary References

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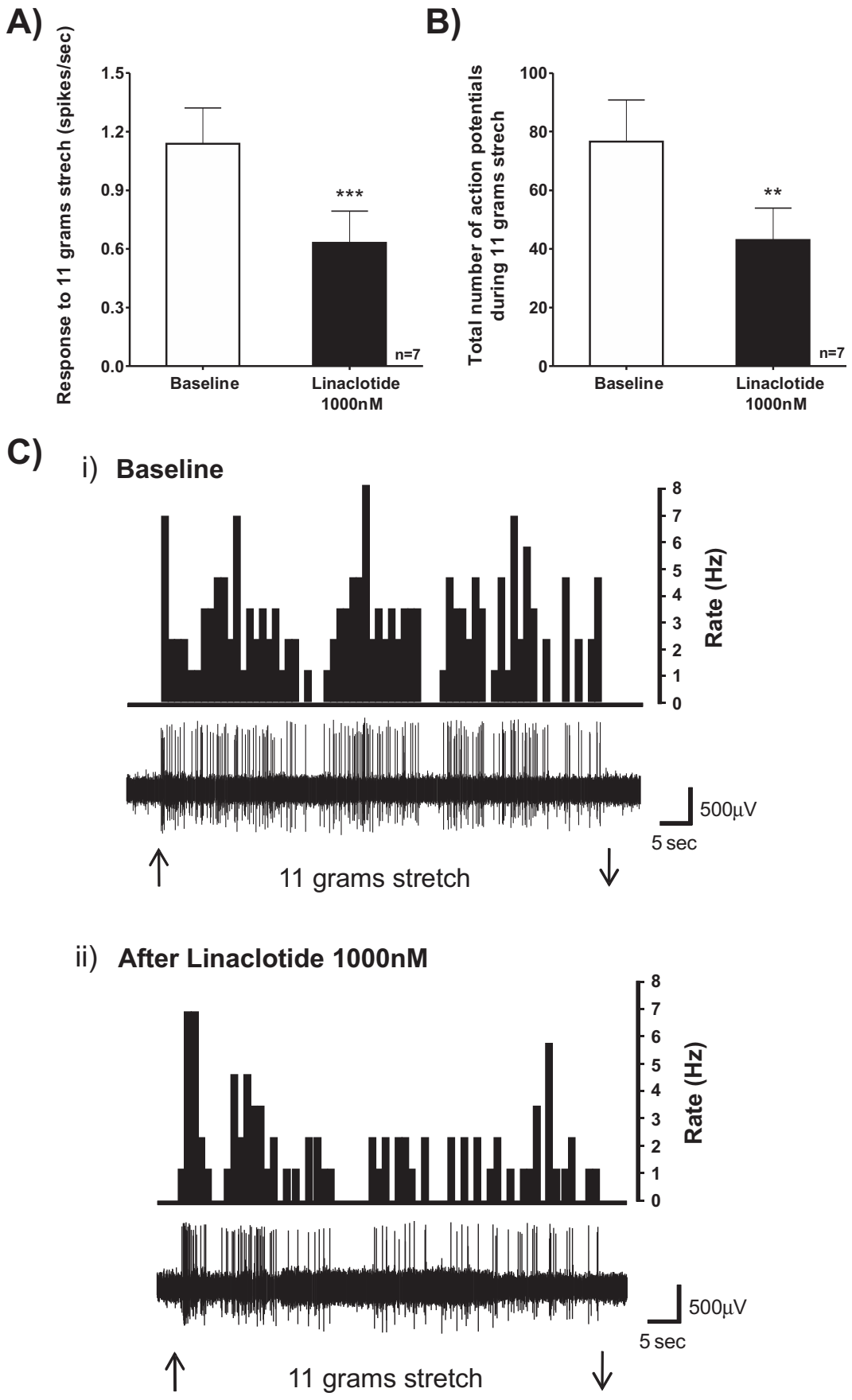
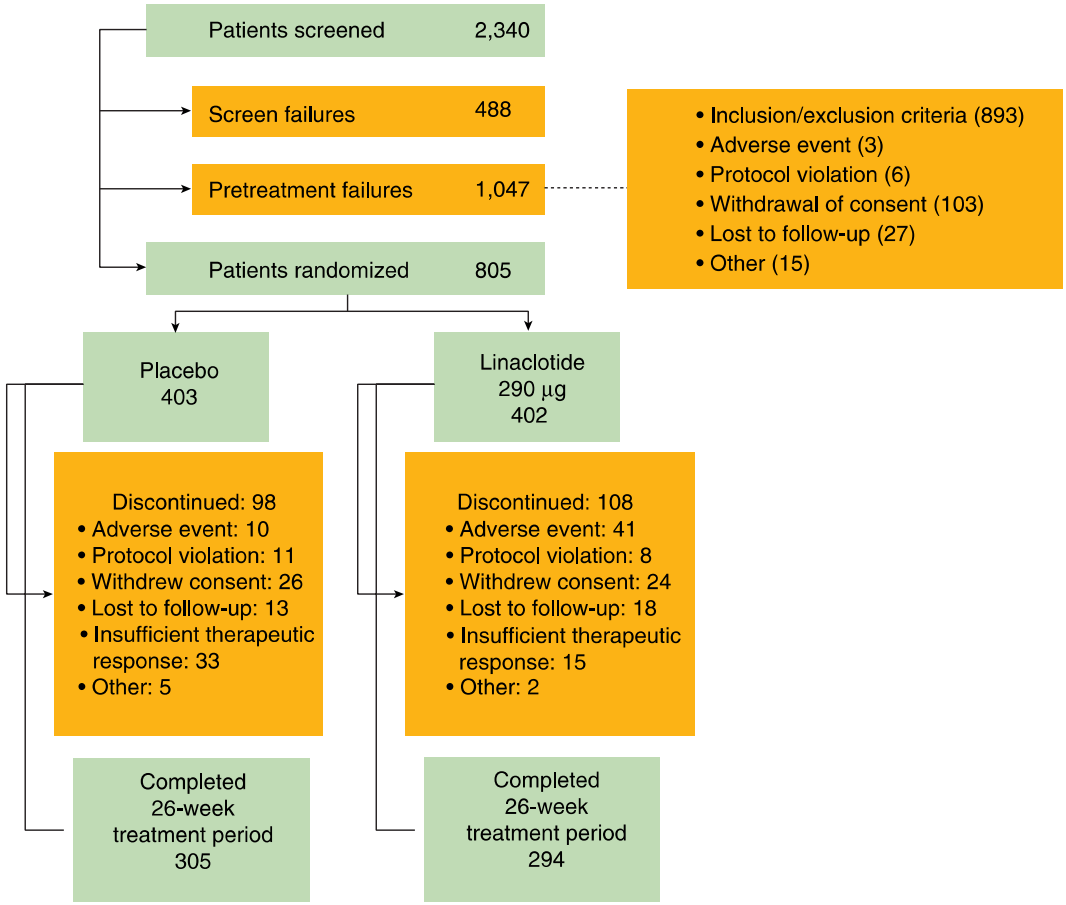
Linaclotide			C	C	E	Y	C	C	N	P	A	C	T	G	C	Y
Uroguanylin	N	D	D	C	E	L	C	V	N	V	A	C	T	G	C	L
Guanylin	P	G	T	C	E	I	C	A	Y	A	A	C	T	G	C	

Supplementary Figure 1. Primary structures of linaclotide, human uroguanylin, and human guanylin. Identical amino acids are shaded by boxes. In this study, human uroguanylin was applied to colonic nociceptors during in vitro recordings.


A)
Response to 11 grams strech (spikes/sec)
1.5
1.2
0.9
0.6
0.3
0.0
***
n=7
Baseline
Linaclotide 1000nM
B)
Total number of action potentials during 11 grams strech
100
80
60
40
20
0
**
n=7
Baseline
Linaclotide 1000nM
C)
i) Baseline
Rate (Hz)
8
7
6
5
4
3
2
1
0
500µV
5 sec
11 grams stretch
ii) After Linaclotide 1000nM
Rate (Hz)
8
7
6
5
4
3
2
1
0
500µV
5 sec
11 grams stretch

Supplementary Figure 2. Linaclotide inhibits colonic nociceptor responses to high-intensity stretch. Application of linaclotide (1000 nM) to the mucosal epithelium subsequently reduces nociceptor responsiveness to noxious circular stretch (11 g) when expressed as (*A*) spikes/s (***$P < .001$, paired *t* test, n = 7) or (*B*) number of spikes/response (**$P < .01$, paired *t* test, n = 7). (*C*) (*i*) Single-unit extracellular recording showing action potential discharge of a healthy mouse colonic nociceptor in response to 11 g circular stretch at baseline. (*ii*) Nociceptor responsiveness is reduced after 5 minutes application of linaclotide (1000 nM). *Top panel* shows the frequency of action potential firing. *Arrows* indicate application and removal of the 11-g circular stretch.



Supplementary Figure 3. Patient flow through the phase III clinical trial. From Chey et al,[15] adapted with permission.

Table 1. Treatment Groups

Treatment	Apical chamber (pH 7.0) Volume 0.3 mL	Basolateral chamber (pH 7.4) Volume 1 mL
Control	Vehicle only	Vehicle only
Linaclotide	1 μM linaclotide	Vehicle only
Linaclotide + probenecid	1 μM linaclotide + 0.5 mM probenecid	0.5 mM probenecid
Linaclotide + probenecid	1 μM linaclotide + 2 mM probenecid	2 mM probenecid

Table 2. Liquid Chromatography Mass Spectrometry Method for Quantitative Determination of Cyclic GMP

HPLC	Waters Acquity UPLC					
Column	Hypersil Gold C18, 2.1 × 50 mm, 1.9 μm					
Guard column	Hypersil Gold, 2.1 × 10 mm, 1.9 μm					
Flow rate	400 μL/min					
Column temperature	Room temperature					
Autosampler temperature	6°C					
Injection volume	10 μL					
Mobile phases	A = 0.1% formic acid in 98/2 water/acetonitrile					
	B = 0.1% formic acid in 2/98 water/acetonitrile					
Gradient	Time, *min*		A, %		B, %	
	0		100		0	
	0.5		60		40	
	1.1		60		40	
	1.75		5		95	
	2.5		5		9	
	2.6		100		0	
Mass spectrograph	Thermo quantum					
Ion mode	Electrospray ionization, positive mode					
Scan type	Multiple reaction monitoring					
Compound: cyclic GMP	Transition	Dwell time, *ms*	Collision energy, *V*	Tube lens	Retention time, *min*	LLOQ, *nM*
	346 > 152	100	28	139	0.8	1

ESI, Electrospray ionisation; HPLC, High performance liquid chromatography; LLOQ, Lower Limit of Quantification.