Urocortin I is present in the enteric nervous system and exerts an excitatory effect via cholinergic and serotonergic pathways in the rat colon

Takazumi Kimura,1,2 Tomofumi Amano,1 Hirotsugu Uehara,1 Hajime Ariga,1 Tsuyoshi Ishida,3 Akira Torii,2 Hisao Tajiri,2 Kei Matsueda,4 and Shigeru Yamato1

Divisions of 1Gastroenterology and 2Pathology, National Center of Neurology and Psychiatry, Kohnodai Hospital, Chiba, Japan, 3Division of Gastroenterology and Hepatology, Department of Internal Medicine, Jikei University School of Medicine, Tokyo, Japan, and 4Saigata Hospital, Niigata, Japan

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Kimura T, Amano T, Uehara H, Ariga H, Ishida T, Torii A, Tajiri H, Matsueda K, Yamato S. Urocortin I is present in the enteric nervous system and exerts an excitatory effect via cholinergic and serotonergic pathways in the rat colon. Am J Physiol Gastrointest Liver Physiol 293: G903–G910, 2007. First published August 23, 2007; doi:10.1152/ajpgi.00066.2007.—Corticotropin-releasing factor (CRF) and urocortin I (UcnI) have been shown to accelerate colonic transit after central nervous system (CNS) or peripheral administration, but the mechanism of their peripheral effect on colonic motor function has not been fully investigated. Furthermore, the localization of UcnI in the enteric nervous system (ENS) of the colon is unknown. We investigated the effect of CRF and UcnI on colonic motor function and examined the localization of CRF, UcnI, CRF receptors, choline acetyltransferase (ChAT), and 5-HT. Isometric tension of rat colonic muscle strips was measured. The effect of CRF, UcnI on phasic contractions, and electrical field stimulation (EFS)-induced off-contractions were examined. The effects of UcnI on both types of contraction were also studied in the presence of antalarmin, astressin2-B, tetrodotoxin (TTX), atropine, and 5-HT antagonists. The localizations of CRF, UcnI, CRF receptors, ChAT, and 5-HT in the colon were investigated by immunohistochemistry. CRF and UcnI increased both contractions dose dependently. UcnI exerted a more potent effect than CRF. Antalarmin, TTX, atropine, and 5-HT antagonists abolished the contractile effects of UcnI and CRF and UcnI were observed in the neuronal cells of the myenteric plexus. UcnI and ChAT, as well as UcnI and 5-HT, were colocalized in some of the neuronal cells of the myenteric plexus. This study demonstrated that CRF and UcnI act on the ENS and increase colonic contractility by enhancing cholinergic and serotonergic neurotransmission. These peptides are present in myenteric neurons. CRF and, perhaps, to a greater extent, UcnI appear to act as neuromodulators in the ENS of the rat colon.

urocortin; CRF; colon; enteric nervous system

corticotropic-releasing factor (CRF) is a stress-related peptide that regulates the secretion of ACTH from the pituitary gland (28). Urocortin I (UcnI) is a recently identified peptide that has 45% homology with CRF (9, 39). UcnI was originally identified as urocortin (9, 39), and urocortin II and urocortin III were later identified on the basis of their affinities for the different CRF receptors (13, 14, 17). UcnI has been shown to bind to both CRF type 1 (CRF1) and type 2 (CRF2) receptors with much higher affinity than does CRF itself (9, 13, 39). CRF and UcnI have been observed predominantly in the central nervous system (CNS) (28, 36), but they are also found in peripheral tissues (2), including lymphocytes (3), skin (33), and gastric (6) or colonic (25) mucosa. Furthermore, CRF or UcnI have various physiological effects in addition to their central actions; for example, CRF and UcnI have potent immunomodulatory effects on the local immune system and inflammation (3, 6, 32). UcnI has effects on cardiovascular functions (27) and acts as a local growth factor (1).

Psychological stress induces gastrointestinal motor dysfunction, which is considered to be mediated, at least in part, by endogenous CRF in the brain. Intracerebroventricular injection and peripheral administration of CRF each cause acceleration of colonic transit (19, 22, 23). CNS and peripheral administration of UcnI have each also been shown to accelerate colonic transit (19, 22, 23). In these studies, it was reported that CRF or UcnI increased fecal expulsion in rats or mice and that these effects were mediated by CRF1 receptors. Peripheral administration of CRF or UcnI is considered to act primarily on the CNS, stimulating colonic contractility through efferent neuronal pathways (20, 38). However, the precise mechanisms by which CRF or UcnI modulate colonic motor function, especially by peripheral mechanisms, have not been fully investigated.

Recent studies have shown that both the CRF1 and CRF2 receptors are localized in myenteric neurons and nerve fibers in the rat small intestine (7, 29), and CRF immunoreactivity (IR) has been found in the ENS of the guinea pig small intestine and colon, although CRF-IR was very scarce in the ENS (18). Another study has demonstrated the presence of Ucn (UcnI) mRNA in the submucosal and myenteric plexus of the rat gastrointestinal tract (12), and more recently, UcnI immunoreactivity has been demonstrated in the stomach of the rat (30). The localization of UcnI peptide in the ENS of the colon has not been investigated by immunohistochemistry, and no systematic studies have investigated the distribution of CRF, UcnI, and CRF receptors in the ENS of the colon.

The aims of this study were 1) to explore the effect of CRF and UcnI on colonic motor function and to investigate the mechanism of their peripheral effect in isolated rat colonic muscle and 2) to examine by immunohistochemistry the localization of CRF, UcnI, and CRF receptors and to clarify whether UcnI is present in the ENS and its localization in relation to these other markers.

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MATERIALS AND METHODS

Functional studies. Experiments were performed on a total of 39 male Sprague-Dawley rats weighing 200–250 g. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and this protocol was approved by National Center of Neurology and Psychiatry Animal Research Committees. Each animal was killed by cervical dislocation, and the distal colon was removed promptly. The colon was opened along the mesenteric border, and colonic muscle strips about 2 x 10 mm were cut circumferentially. The muscle strips were suspended in an organ bath containing 4 ml of Krebs solution (118.07 mM NaCl, 4.69 mM KCl, 1.01 mM NaH2PO4, 25 mM NaHCO3, 2.52 mM CaCl2, 0.57 mM MgSO4·7H2O, and 11.1 mM glucose) bubbled with a gas mixture of 95% O2-5% CO2. Bath temperature was maintained at 37 ± 0.5°C. One end of the colonic muscle strip was attached via a silk thread to a force-displacement transducer to monitor isometric tension. Muscle strips were equilibrated at an applied tension of 1 g for 1 h. Electrical field stimulation (EFS) was applied via an electrode attached to a stimulator (Nihon Kohden, Tokyo, Japan). Stimulus parameters used were square wave pulses of 0.5 ms, 10 Hz, 30-s train, and 10 V. Muscle strips showed spontaneous phasic contractions. EFS caused inhibition of phasic contractions during stimulation and a large contraction just after cessation of stimulation (off-contraction).

Control responses were obtained first. The effects of CRF and UcnI (10^-8 M to 10^-5 M), and UcnII (10^-6 M) on phasic contractions and EFS-induced off-contraction were examined. These effects were expressed as a percentage of the control. Both responses became stable ~10 min after administration of CRF and UcnI. EFS-induced off-contractions were examined at least twice at any given dose, and the mean amplitude was taken as one observation. To study the mechanism of action of CRF and UcnI, we examined their effects in the presence of selective CRF1 receptor antagonist antalarmin (10^-3 M), selective CRF2 receptor antagonist astressin-B (10^-5 M), tetrodotoxin (TTX) (10^-6 M), atropine (10^-6 M), hexamethonium (10^-6 M), the 5-HT3 antagonist ondansetron (10^-6 M), the 5-HT1a antagonist SB204070 (10^-6 M), and nitro-L-arginine methyl ester (l-NAME) (10^-4 M). Selective CRF receptor antagonist antalarmin and selective CRF2 receptor antagonist astressin-B were used in supramaximal doses (8, 26).

N indicates n muscle strips in n different animals. Quantitative data are expressed as means ± SE. Statistical analyses were performed by Mann-Whitney U-tests and by ANOVA for multiple comparisons. Differences were considered significant at P < 0.05. CRF, UcnI, and UcnII were purchased from PEPTIDE (Osaka, Japan), and antalarmin, astressin-B, TTX, atropine, hexamethonium, ondansetron, SB204070, and l-NAME were purchased from Sigma (St. Louis, MO).

Immunohistochemistry. The localizations of CRF, UcnI, the CRF1 and CRF2 receptors, 5-HT, and choline acetyltransferase (ChAT) in the rat distal colon were investigated by immunohistochemistry using the following primary antibodies: anti-CRF and anti-UcnI (Rabbit; Yanaihara Institute, Fujinomiya, Japan), anti-CRF1 and -CRF2 receptors (Goat; Santa Cruz Biotechnology, Santa Cruz, CA), anti-5-HT (Goat; ImmunoStar, Hudson, WI) and anti-ChAT (Goat; CHEMICON International, Temecula, CA). Immunohistochemical procedures were performed as described previously (15). The frozen tissues were sectioned onto silane-coated glass slides at a thickness of 6 μm and fixed with 4% paraformaldehyde for 10 min. The preparations were then placed in 2% bovine serum albumin for 10 min, followed by incubation with the primary antibody (diluted in 10 mM PBS) for 24 h at room temperature in a moist chamber for double immunostaining. Optimal primary antibody dilutions were 1:1,000 for anti-CRF and anti-UcnI, 1:100 for anti-CRF1 receptor and anti-CRF2 receptor, and 1:200 for anti-5-HT and anti-ChAT. Immunoreactivity for CRF was detected with Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (1:200; Molecular Probes, Leiden, Netherlands). Immunoreactivities for CRF1 and CRF2 receptors, 5-HT, and ChAT were detected with Alexa Fluor 594-conjugated donkey anti-goat secondary antibody (1:200; Molecular Probes). All antisera were diluted with 2% BSA in 10 mM PBS. Secondary antibody incubations were performed for 1 h at room temperature. Nuclei in all sections were labeled with 4,6-diaminophenylindole (DAPI) (Vector Laboratories, Burlingame, CA).

UcnI immunoreactivity was also observed in whole mount preparations. Opened distal colon was pinned on the silicon sheets, and the mucosa and submucosa were removed under a stereomicroscope. The isolated tissue was stretched, then fixed with 4% paraformaldehyde at 4°C for 1 h. The preparations were incubated with primary anti-UcnI antibody and anti-PGP9.5 (1:200; abcam, Cambridge, UK) at 4°C overnight. Thereafter, the preparations were rinsed in PBS and incubated with Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody and Alexa Fluor 594-conjugated donkey anti-mouse secondary antibody. As a negative control for UcnI, preabsorbed antiserum and/or 10 mM PBS were used instead of the primary antibodies. For absorption tests of immunoreactivity, an antibody-antigen mixture containing an optimally diluted antiserum was incubated overnight at room temperature. After centrifugation, the resulting supernatants were used as preabsorbed antibodies (15). Rat midbrain tissue was used as a positive control for UcnI.

All preparations were examined with a light microscope (Nikon Eclipse, Japan) or with a confocal laser microscope (Zeiss LSM5, Germany). For quantitative analyses, a total of 250 ganglia taken from five different animals were analyzed.

RESULTS

Influence of CRF and UcnI on spontaneous phasic contractions and EFS-induced off-contraction. Circular muscle strips developed spontaneous phasic contractions at a frequency ranging from 9 to 13 cycles/min. EFS induced two kinds of responses: an inhibition of phasic contractions during stimulation (on-relaxation), followed by a large contraction just after cessation of stimulation (off-contraction). CRF and UcnI caused significant increases in both phasic contractions and EFS-induced off-contraction in a dose-dependent manner (N = 10, 20, 31, June 25, 2017).
CRF increased the amplitude of phasic contractions to 206 ± 10.1% of the control at a dose of 10^-6 M [N = 8, 95% confidence interval (CI) 159–354, P = 0.036], and UcnI at the same dose increased the amplitude to 332 ± 13.2% of the control (N = 8, 95% CI 274–371, P = 0.002). Fig. 1 shows the dose-response curves for CRF and UcnI on phasic contractions (maximal response was taken as 100%). The ED50 values for CRF and UcnI were 8.21 × 10^-7 and 1.21 × 10^-7, respectively. Thus UcnI was approximately seven times more potent than CRF. After application of CRF and UcnI, the amplitudes of EFS-induced off-contractions showed similar increases to those of phasic contractions (Table 1). Fig. 2, shows typical tracings of phasic contractions (A and B) and EFS-induced off-contractions (C) before and after administration of UcnI. Because the effects of UcnI on both responses were more potent than those of CRF, we focused on UcnI to examine its mechanism of action in the following studies.

Influence of UcnII on spontaneous phasic contractions and EFS-induced off-contraction. UcnII (10^-6 M) caused no increase in the amplitude of phasic contractions or EFS-induced off-contraction (N = 4). The amplitude of phasic contractions was 102 ± 1.5%, and that of EFS-induced off-contraction was 102 ± 1.9%, of the control after administration of UcnII.

Effect of antalarmin, astressin2-B, and TTX on UcnI-induced responses. Antalarmin (10^-5 M), a selective antagonist of the CRF1 receptor, and astressin2-B, a selective antagonist of the CRF2 receptor, caused no change in the amplitude of phasic contractions and EFS-induced off-contraction. Antalarmin completely abolished the effects of UcnI on the amplitude of both phasic contractions and EFS-induced off-contraction, whereas astressin2-B did not modify the effects of UcnI on both types of contractions (N = 6) (Fig. 3, A and B).

TTX (10^-6 M) caused an ~10% increase in the amplitude of phasic contractions. In the presence of TTX, UcnI did not increase the amplitude of phasic contractions (N = 6) (Fig. 3A). TTX completely abolished EFS-induced on-relaxation or off-contraction. Neither of the EFS-induced responses were observed when UcnI was added in the presence of TTX.

Effect of atropine and hexamethonium on UcnI-induced responses. Atropine (10^-6 M) decreased the amplitude of EFS-induced off-contraction to 90%. In the presence of atropine, the effects of UcnI on both phasic contractions and EFS-induced off-contraction were completely inhibited. The amplitudes of phasic contractions and EFS-induced off-contraction were 96 ± 11.1% and 103 ± 8.9% of the control, respectively, after administration of UcnI in the presence of atropine (N = 4) (Fig. 4, A and B).

Hexamethonium (10^-6 M) caused no change in the amplitude of either phasic contractions or EFS-induced off-contraction. In the presence of hexamethonium, UcnI caused a significant increase in the amplitude of phasic contractions or EFS-induced off-contraction (N = 4).

Table 1. Effects of CRF and UcnI on EFS-induced off-contraction

<table>
<thead>
<tr>
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<th>Control</th>
<th>10^-7 M</th>
<th>3 × 10^-7 M</th>
<th>10^-6 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRF</td>
<td>100</td>
<td>103 ± 0.9</td>
<td>108 ± 1.7</td>
<td>110 ± 1.7*</td>
</tr>
<tr>
<td>UcnI</td>
<td>100</td>
<td>118 ± 2.5*</td>
<td>133 ± 2.0†</td>
<td>155 ± 3.2†</td>
</tr>
</tbody>
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Amplitude of EFS-induced-off contraction (control was taken as 100%). CRF, corticotropin-releasing factor; UcnI, urocortin I; EFS, electrical field stimulation. *P < 0.05, †P < 0.01.
significant increase in the amplitude of both phasic contractions and EFS-induced off-contraction. The amplitudes of phasic contractions and EFS-induced off-contraction were 192 ± 19.5% (N = 4, 95% CI 132–272, \( P = 0.013 \)) and 127 ± 22.1% (N = 4, 95% CI 114–142, \( P = 0.019 \)) of the control, respectively, after administration of UcnI in the presence of hexamethonium (Fig. 4, A and B).

Effect of a 5-HT\(_3\) antagonist and a 5-HT\(_4\) antagonist on UcnI-induced responses. The 5-HT\(_3\) antagonist ondansetron (10\(^{-9}\) M) caused no change in the amplitudes of phasic contractions. The effects of UcnI on both types of contraction were inhibited by ondansetron (N = 4) (Fig. 5, A and B).

Fig. 4. The effects of atropine and hexamethonium (HEX) on UcnI-induced responses. Atropine (10\(^{-6}\) M) inhibited the effect of UcnI (10\(^{-6}\) M) on phasic contractions (A) or the EFS-induced off-contraction (B) (N = 4), whereas HEX (10\(^{-6}\) M) did not inhibit the contractile effect of UcnI (10\(^{-6}\) M) (N = 4) \( *P < 0.05 \).

Fig. 5. The effects of ondansetron (ODN) and SB204070 on UcnI-induced responses. Ondansetron (10\(^{-9}\) M) and SB204070 (10\(^{-6}\) M) inhibited the effects of UcnI (10\(^{-6}\) M) on phasic contractions (A) and the EFS-induced off-contraction (B) (N = 4). \( *P < 0.05 \).

Fig. 6. The effects of nitro-L-arginine methyl ester (L-NAME) on UcnI-induced responses. L-NAME (10\(^{-4}\) M) did not inhibit the effect of UcnI on phasic contractions (A) and the EFS-induced off-contraction (B) (N = 5). \( *P < 0.05 \).
The 5-HT₄ antagonist SB204070 (10⁻⁶ M) slightly increased the amplitude of phasic contractions and EFS-induced off-contraction. The effects of UcnI on both types of contraction were inhibited by SB204070 (N = 4) (Fig. 5, A and B).

Effect of L-NAME on UcnI-induced responses. L-NAME (10⁻⁴ M) increased the amplitude of phasic contractions and EFS-induced off-contraction. These contractions were further and significantly enhanced by administration of UcnI in the

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Fig. 7. Immunohistochemical staining for UcnI (A–D), CRF₁ receptor (A) and CRF₂ receptor (B), choline acetyltransferase (ChAT) (C), and 5-HT (D) in the rat distal colon. UcnI-IR (green) was found in neuronal cell bodies within the myenteric plexus. UcnI immunoreactivity (UcnI-IR) was distributed throughout the cell cytoplasm in myenteric neurons, whereas immunoreactivity for the CRF₁ (A2) and CRF₂ (B2) receptors (red) were present in the neuronal fibers around the UcnI positive cells (A2-3, B2-3). ChAT (C) and 5-HT (D) (red) were also present in neuronal cell bodies in the myenteric plexuses, as was the case for UcnI. UcnI and ChAT, and UcnI and 5-HT were colocalized (yellow) in some of the neuronal cell bodies in the myenteric plexus (C3, D3, arrowhead). Nuclei in all sections were labeled blue. Scale bars, 20 μm.
positive neurons. (Fig. 7, 2,437) ChAT-positive neurons, and neurons showing UcnI and UcnI colocalization counted for 79.0% (1,925/neuronal cell bodies in the myenteric plexus. Neurons showing and 5-HT were observed to be colocalized in some of the limited to the cell cytoplasm. UcnI and ChAT as well as UcnI was also present in neuronal cell bodies in the myenteric plexus. Scale bars, 100 μm.

**Immunohistochemistry.** CRF-IR was present in neuronal cells of the myenteric and submucosal plexuses in the rat distal colon. However, CRF-IR-positive cells were sparse, representing 7.8% (282/3,602) of total neuronal cells in the myenteric plexus. CRF-IR-positive cells were present in the myenteric (Fig. 7, A–D) and submucosal plexuses. CRF-IR cells were much more abundant than CRF-IR cells. Among total neuronal cells, 80.8% (2,914/3,602) in the myenteric plexus and 71.0% (454/640) in the submucosal plexus were UcnI-IR-positive. UcnI-IR was distributed throughout the cell cytoplasm in the myenteric and submucosal neurons, but was not present within the nuclei.

Immunoreactivity for CRF1 (Fig. 7, A2 and A3) and CRF2 (Fig. 7, B2 and B3) receptors was present in the neuronal fibers around the UcnI positive cell bodies in the myenteric plexus (Fig. 7, A2 and B2). These receptors were also found in the neuronal fibers in the circular muscle layers.

Immunoreactivity for ChAT and 5-HT (Fig. 7, C2 and D2) was also present in neuronal cell bodies in the myenteric plexuses. As found for UcnI-IR, these immunoreactivities were limited to the cell cytoplasm. UcnI and ChAT as well as UcnI and 5-HT were observed to be colocalized in some of the neuronal cell bodies in the myenteric plexus. Neurons showing UcnI and ChAT colocalization counted for 79.0% (1,925/2,437) ChAT-positive neurons, and neurons showing UcnI and 5-HT colocalization counted for 64.7% (1,384/2,140) 5-HT-positive neurons. (Fig. 7, C3 and D3, arrowhead).

In whole mount preparations, UcnI was localized in interganglionic nerve fibers as well as ganglionic neuronal cells in the myenteric plexus(Fig. 8).

Fig. 8. Whole mount immunohistochemical staining of myenteric nerve (anti-PGP9.5 antibody, red) and UcnI (green). UcnI was localized in interganglionic nerve fibers (yellow) as well as ganglionic neuronal cells in the myenteric plexus. Scale bars, 100 μm.
tivity and ChAT immunoreactivity, and UcnI immunoreactivity and 5-HT immunoreactivity, were found simultaneously in some of these cells. These findings further support our contention that UcnI acts on the ENS and enhances cholinergic and serotonergic neurotransmission.

We have shown that exogenous UcnI enhances cholinergic and serotonergic neurotransmission via CRF$_1$ receptor and that UcnI exists in the ENS of the colon, but it is unknown whether UcnI can modulate colonic motility under physiological conditions. CRF is known to be a stress-related peptide, but it is unclear whether peripheral CRF or UcnI are induced by psychological stress. Further investigation is needed to clarify the peripheral functions of CRF and UcnI and to determine their interactions with CNS and other neurotransmitters or substances in the ENS.

We found that myenteric and submucosal UcnI was much more abundant than CRF; similar findings have been reported for other peripheral organs or tissues. In immune cells, heart, and gastric mucosa, UcnI has been demonstrated to be much more abundant than CRF (2, 3, 6). As UcnI has a higher affinity for the CRF$_1$ receptor than does CRF itself, the effect of UcnI on colonic contractility was much larger than that of CRF. This, together with the higher abundance of UcnI in the ENS, suggests that UcnI might have a more important physiological role than CRF in the ENS.

Irritable bowel syndrome (IBS) is one of the most common diseases in gastroenterology. One aspect of the pathophysiology of IBS is increased responsiveness of the gut to various stimuli (10). CRF is induced in the CNS by chronic stress and has been shown to enhance colonic motility and visceral sensitivity; therefore, CRF is considered to play a key role in IBS (35). As this study demonstrated that CRF and UcnI act not only in the CNS but also at the peripheral level, CRF or UcnI might have other roles in IBS. Inflammation has been postulated to be involved in the pathophysiology of IBS in addition to motility disorders and visceral hypersensitivity (10). In patients with IBS, infiltration of inflammatory cells into the myenteric plexus is seen in full thickness biopsy samples (37), and mast cell degranulation occurs near the intestinal nerves (4). Postinfectious IBS is widely recognized as a disease entity (34). Relationships between inflammation and gastrointestinal motility or visceral hypersensitivity have been the subject of much recent attention. UcnI has been found in inflammatory cells such as macrophages and lymphocytes in the colonic mucosa (25), and UcnI-containing cells are increased in the colonic mucosa of patients with ulcerative colitis (31). Moreover, it is known that human mast cells synthesize and secrete CRF and urocortin (32). As mucosal inflammation induces UcnI expression in inflammatory cells, and this study has demonstrated that CRF and UcnI enhance contractility of the colon, CRF and UcnI may act as intermediaries between inflammation and GI dysmotility, thus playing an important role in the development of IBS.

In summary, this study has demonstrated that CRF and UcnI have peripheral effects on the ENS of the rat distal colon and that they increase colonic contractility by enhancing cholinergic and serotonergic neurotransmission. Furthermore, UcnI is present in myenteric neurons at a much higher abundance than CRF. Both CRF and, perhaps more importantly, UcnI appear to act as neuromodulators in the ENS. These observations, together with the known ability of inflammatory cells to produce UcnI, suggest that UcnI may act as an intermediary between inflammation and GI dysmotility.

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