Restraint stress stimulates colonic motility via central corticotropin-releasing factor and peripheral 5-HT<sub>3</sub> receptors in conscious rats

Yukiomi Nakade, Hiroyuki Fukuda, Masahiro Iwa, Kiyoshi Tsukamoto, Hidenori Yanagi, Takehira Yamamura, Christopher Mantyh, Theodore. N. Pappas, and Toku Takahashi

Department of Surgery, Duke University Medical Center and Durham Veterans Affairs Medical Center, Durham, North Carolina; and Department of Second Surgery, Hyogo College of Medicine, Nishinomiya, Japan

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IRRITABLE BOWEL SYNDROME (IBS) is one of the most common gastrointestinal (GI) conditions encountered by general practitioners. Disorders of colonic transit may contribute to symptoms in constipation- and diarrhea-predominant IBS (35). Indeed, transit and contractile abnormalities have been shown in a subset of patients with IBS. In some patients with IBS, abdominal pain, urgency, and diarrhea are temporally associated with high-amplitude contractions, which originate in the proximal colon and traverse the distal colon at very high-propagation velocities (5).

It has been suggested that the primary alteration of mucosal absorption and secretion may not contribute to the frequent stools in patients with functional diarrhea (3). The accelerated transit of colonic contents in these patients may result in increased stool water due to diminished contact time of the luminal contents with the colonic mucosa (27, 28). The numbers of fasting- and postprandial-propagated contractions of the colon were increased in patients with diarrhea compared with results in healthy subjects (3).

There are distinct functional differences between the proximal and distal colon. As a conduit, the distal colon mainly serves to expel the fecal bolus, displaying a pattern of intense peristaltic contractions and mass action associated with the propulsion of dehydrated feces. In contrast, the proximal colon serves as a reservoir and is considered to be the primary site of storage for feces to absorb water (25, 33). It is conceivable that colonic function may be altered selectively by diseases and that overall assessments of whole colon transit could overlook important regional abnormalities. Colonic transit in the proximal colon is significantly accelerated in diarrhea-predominant IBS patients. This suggests that accelerated transit through the proximal colon is a factor in the pathophysiology of diarrhea-predominant IBS (35). However, the mechanism of the hyperactivity of the proximal colon in these patients still remains unknown.

5-HT<sub>3</sub> receptor antagonists possess a number of interesting pharmacological properties that may make them suitable for treatment of IBS. Besides decreasing colonic sensitivity to distention, these drugs prolong colonic transit and are particularly useful in diarrhea-predominant IBS (29). A 5-HT<sub>3</sub> receptor antagonist, alosetron, delays colonic transit in patients with IBS (2). In clinical trials in patients with IBS, alosetron is effective in relieving abdominal pain and discomfort. Alosetron is most effective in female patients, particularly those with diarrhea-predominant IBS (5). Antagonism of 5-HT<sub>3</sub> receptors should be useful in treatment of functional bowel disease because this can inhibit excitation of extrinsic sensory nerves by 5-HT without interfering with intrinsic enteric reflexes (9). However, the mechanism of beneficial effects of 5-HT<sub>3</sub> antagonists in IBS patients still remains unclear.

Stress is a highly contributing factor to the pathogenesis of IBS. Central corticotropin-releasing factor (CRF) released by stress accelerates colonic transit via stimulation of vagal efferent in rats (11). Increased defecation induced by stress and central CRF is abolished by a systemic treatment with 5-HT<sub>3</sub> receptor antagonists (17, 18). However, it remains unknown whether the stimulatory effect of stress and central CRF is mediated via 5-HT containing neurons and/or enterochromaffin (EC) cells. It is noteworthy that vagal nerve stimulation increases 5-HT release into the lumen in the cat jejunum (37).
It has been shown that 5-HT content and the density of EC cells of the epithelium in the proximal colon are six- to sevenfold higher than those in the distal colon in rats (24). However, the physiological importance of 5-HT-containing cells at the proximal colon remains unknown. Our group (8) has previously shown that released 5-HT from EC cells of the proximal colon stimulates colonic motility via 5-HT3 receptors located on the vagal afferent.

We hypothesize that restraint stress and central CRF stimulate colonic motility and transit via vagal efferent and 5-HT3 receptors of the proximal colon in rats. We studied whether the luminal administration of a 5-HT3 receptor antagonist (ondansetron) affects colonic transit and motility stimulated by restraint stress and central CRF in conscious rats.

METHODS

Colonic transit study. Male Sprague-Dawley rats (250–300 g) were obtained from Charles River Laboratories (Raleigh, NC). Protocols were approved by the Institutional Animal Care and Use Committee of Durham Veterans Affairs Medical Center and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Rats were anesthetized by pentobarbital sodium (45 mg/kg ip). An in-dwelling Silastic cannula was inserted into the cecum and positioned to enter the proximal colon. One week after the surgery, rats were fasted overnight. A nonabsorbable radioactive marker (0.5 μCi; Na51CrO4 in 0.2 ml saline) was administered into the proximal colon, and rats were restrained in hemi-cylindrical, well-ventilated, Plexiglas tubes for 90 min, as previously described (22).

After stress loading was completed, the rats were euthanized by pentobarbital sodium (200 mg ip). The entire colon was surgically removed and divided into 10 equal segments. Each segment was placed into a vial, and the radioactivity was counted by a gamma counter for 1 min. The geometric center (GC) of the distribution of 51Cr within the colon is the center of gravity for the distribution of radiochromium. GC was calculated using the following equation, as previously reported (31):

Geometric center

\[ \text{GC} = \Sigma (\text{fraction of } 51\text{Cr per segment} \times \text{segment number}) \]

For intracisternal injection of CRF, rats were lightly anesthetized with isoflurane (2%) and mounted on a stereotaxic apparatus, as previously reported (22). Five minutes after intracisternal injection of CRF (0.25, 0.5, and 1.0 μg in 5 μl saline) Na51CrO4 was instilled into the proximal colon through the cannula. It has been shown that intracerebroventricular injection of CRF (0.3–1.0 μg) accelerates colonic transit in a dose-dependent manner in rats (36).

To investigate whether the adrenergic receptor is involved in the mechanism of restraint stress-induced colonic transit, guanethidine (5 mg/kg ip) was injected 20 min before the restraint stress loading.

To investigate the possible participation of 5-HT3 receptors located on the colonic mucosa mediating restraint stress- and CRF-induced colonic transit, a 5-HT3 receptor antagonist (ondansetron, 5 × 10−8 M in 1 ml saline) was administered 20 min before restraint stress loading and CRF injection.

To investigate whether a capsaicin-sensitive vagal afferent is involved in restraint stress-induced acceleration of colonic transit, rats were treated perivagally with capsaicin. Perivagal capsaicin treatment has been shown to selectively destroy the activity of the vagal afferent but not vagal efferent (26). Capsaicin (10 mg) was sonicated with 0.1 ml of Tween 80 for 10 min and made up to 1 ml with olive oil and mixed thoroughly. After ketamine and xylazine anesthesia, both cervical vagal trunks were exposed, as previously described (32). A small piece of gauze soaked in capsaicin was placed around the nerve trunk for 30 min. The surrounding area was covered with gauze, which was frequently replaced to minimize the spread of capsaicin to surrounding tissues. Additional capsaicin was applied perivagally every 5 min. The area was thoroughly rinsed with olive oil and then with saline and dried with sterile swabs, and the neck incision was then closed. Experiments were performed 10–14 days after the peripheral capsaicin treatment. Vehicle-treated rats served as controls.

Restraint stress-induced colonic transit was compared between capsaicin-treated rats and vehicle-treated rats.

To investigate whether vagal nerves are involved in restraint stress- and CRF-induced acceleration of colonic transit, rats received truncal vagotomy at the time of colonic cannulation. As previously reported (23), the lower part of the esophagus was exposed, and anterior and posterior branches of the vagal nerves were incised above the hepatic and celiac branches. In sham-operated rats, the vagal trunks were similarly exposed but not cut.

To investigate whether central CRF is involved in mediating restraint stress-induced colonic transit, a nonselective CRF receptor antagonist (10 μg astressin) was intracisternally administered 5 min before the stress loading. To investigate whether central effects of astressin is from leakage from the cerebrospinal fluid into the bloodstream, rats received intraperitoneal injection of astressin (10 μg) 5 min before the stress loading.

Colonic motility study. Rats were anesthetized by pentobarbital sodium (45 mg/kg ip). Four strain gauge transducers were sutured on the serosal surface of the proximal, mid, and distal colon to monitor the circular muscle contraction, as previously reported (31). Wires to the transducers were run under the skin to an opening made in the back of the neck. An in-dwelling Silastic cannula was inserted into the cecum and positioned to enter the proximal colon. The abdominal wall was closed, and rats were allowed to recover for 7 days.

After an overnight fast, wires from the transducers were connected to the recording system. After basal contractions were recorded for 2 h, saline (2 ml) or ondansetron (5 × 10−8 M in 1 ml saline) was administered into the proximal colon 15 min before stress loading. Another group received saline or ondansetron into the proximal colon 15 min before intracisternal injection of CRF (1.0 μg) or saline (5 μl). Rats were lightly anesthetized with isoflurane (2%) and mounted on a stereotaxic apparatus for intracisternal injection of CRF or saline. Rats were allowed to recover from isoflurane- and CRF-induced colonic contractions, which were recorded for 2–3 h.

We studied whether the high-amplitude contractions in the proximal colon induced by stress or CRF migrate aborally to the mid and distal colon. Computer analysis was performed to show the time delay of each peak contraction between each recording site. Propagation velocity was also calculated, as previously described (31).

As previously reported (12), phasic contractions were identified by their frequency in the range of 10–13 cycles/min. The giant contractions were defined as contractions of duration >150% and amplitude >300% of that of phasic contractions at the same recording site. The giant migrating contractions (GMCs) were considered to propagate if GCs traveled over at least three consecutive transducers, as previously reported (12). Each rat was subjected to the restraint stress loading at least 7 days apart.

The area under the curve was calculated with a computer-assisted system (PowerLab/8SP; ADInstruments, Colorado Springs, CO) as a motility index (MI), as previously reported (31). MI was calculated every 15 min before and after restraint stress and intracisternal injection of CRF. MI of 60 min before restraint stress and intracisternal injection of CRF was expressed as 100% (basal MI). MI was similarly calculated every 15 min after restraint stress for 90 min and intracisternal injection of CRF for 60 min, respectively. The stimulated MI after restraint stress and intracisternal injection of CRF was compared with the basal MI and expressed as % increase of MI in each rat.
Luminal release of 5-HT in response to restraint stress and CRF.

To study whether restraint stress stimulates the luminal release of 5-HT, rats received restraint stress for 90 min. After stress loading was completed, rats were euthanized by pentobarbital sodium (200 mg ip).

Restraint stress-induced acceleration of colonic transit is mediated via central CRF1 receptors (15). To study whether central CRF receptors are involved in mediating the luminal release of 5-HT in response to restraint stress, rats received intracisternal injection of CRF1 receptor antagonist NB1-27914 (100 µg). Rats were lightly anesthetized with isoflurane (2%) and mounted on a stereotaxic apparatus for intracisternal injection of NB1-27914. After recovery from isoflurane anesthesia, rats received restraint stress for 90 min. The rats were euthanized by pentobarbital sodium (200 mg ip).

To study whether central CRF stimulates the luminal release of 5-HT, rats received intracisternal injection of CRF (1.0 µg) under isoflurane (2%) anesthesia. The rats were euthanized by pentobarbital sodium (200 mg ip) 60 min after CRF injection.

After death, feces in the proximal colon were removed, weighed, and homogenized in 0.1 N perchloric acid at 4°C and then centrifuged for 30 min at 3,000 rpm at 4°C. The supernatant was filtered with a 0.45-µm centrifuge tube filter (Coster, Corning, NY) for 30 min at 3,000 rpm at 4°C. Ten microliters of sample were injected into HPLC to measure 5-HT content, as previously reported (8).

Materials. Atropine, capsaicin, guanethidine, hexamethonium, CRF, and astressin were purchased from Sigma (St. Louis, MO). Ondansetron was purchased from Smith Klein Glaxo (Research Triangle Park, NC).

Statistical analysis. All results are expressed as means ± SE. Student’s t-test was used for determination of statistical significance between unpaired variable. A multiple group comparison was performed by ANOVA followed by Fisher’s paired least-significant difference method. Values of P < 0.05 were considered statistically significant.

RESULTS

Accelerated colonic transit induced by restraint stress. Restraint stress significantly accelerated colonic transit, compared with nonrestrained rats. Restraint stress-induced acceleration of colonic transit was not affected by guanethidine (Fig. 1A).

Truncal vagotomy and perivagal capsaicin treatment itself did not cause any significant effects on colonic transit. Restraint stress-induced acceleration of colonic transit was significantly reduced by truncal vagotomy and perivagal capsaicin treatment (Fig. 1B).

Effects of intracisternal and intraperitoneal injection of astressin on restraint stress-stimulated colonic transit. Intracisternal injection of astressin (10 µg) by itself did not affect colonic transit in the nonrestrained rats. Intracisternal injection of astressin (10 µg) almost completely abolished restraint stress-induced acceleration of colonic transit (n = 6) (Fig. 2). In contrast, intraperitoneal injection of astressin (10 µg) did not affect restraint stress-induced acceleration of colonic transit (GC of 7.2 ± 0.6, n = 6).

Effects of intracisternal injection of CRF on colonic transit. Intracisternal injection of CRF (0.25–1.0 µg) significantly accelerated colonic transit in a dose-dependent manner (n = 6) (Fig. 3). Intracisternal injection of CRF (1.0 µg)-induced acceleration of colonic transit was significantly reduced by truncal vagotomy (Fig. 4).

Effects of intraluminal administration of ondansetron on colonic transit stimulated by restraint stress and CRF. Luminal application of 5-HT3 antagonist ondansetron into the proximal colon had no significant effect on the colonic transit in nonrestrained rats. Restraint stress- and CRF-induced accelerations of colonic transit were significantly reduced by intraluminal application of ondansetron into the proximal colon (Fig. 5).

Increased colonic motility induced by restraint stress. Restraint stress significantly increased colonic motility (Figs. 6 and 7). In the nonrestrained state, the frequency of giant contractions in the proximal and distal colon was 46.1 ± 7.8 and 21.6 ± 3.6 contractions/h, respectively. Restraint stress significantly increased the frequency of giant contractions to 102.2 ± 12.8 and 53.6 ± 6.7 contractions/h in the proximal and distal colon, respectively. During the restraint stress, the frequency of GMCs was also increased from 5.5 ± 1.2/h to 12.9 ± 2.8 contractions/h (n = 6; P < 0.01 by paired t-test) (Fig. 6). Immediately after stress loading was completed, increased GMC and GC returned to basal levels (Fig. 7).

The calculated MI for 90 min at the proximal colon was increased from 235.1 ± 22.5 to 558.2 ± 65.3 g·min by restraint stress. MI for 90 min at the distal colon was also increased from 274.9 ± 31.3 to 832.6 ± 75.9 g·min by restraint stress (P < 0.05, by paired t-test).
After the intraluminal administration of ondansetron into the proximal colon, the frequency of GMCs was no longer increased during the restraint stress (from 5.3 ± 1.0 to 5.9 ± 1.7 contractions/h; n = 6). Ondansetron almost completely abolished the increase of MI of the proximal colon induced by restraint stress. In the distal colon, ondansetron significantly reduced the increase of MI induced by restraint stress (Figs. 8 and 9).

**Increased colonic motility induced by intracisternal injection of CRF.** Intracisternal injection of CRF (1.0 μg) significantly increased the motility of the proximal and distal colon. Increased motility of the proximal colon induced by CRF was abolished by intraluminal administration of ondansetron. In the distal colon, ondansetron significantly reduced the increase of MI induced by CRF (Fig. 8). The MI of the distal colon stimulated by CRF was significantly reduced by the intraluminal administration of ondansetron (Fig. 9).

**Luminal release of 5-HT in response to restraint stress.** The 5-HT content in the feces of the proximal colon was 694 ± 95 pg/wet wt in controls. Restraint stress significantly increased the 5-HT content in the feces of the proximal colon (1,120 ± 165 pg/wet wt; P < 0.05, n = 7). Intracisternal injection of NB1-27914 significantly reduced the luminal content of 5-HT in response to restraint stress to 854 ± 174 pg/wet wt (P < 0.05, n = 6).

Intracisternal injection of CRF also significantly increased the 5-HT content in the feces of the proximal colon (1,656 ± 215 pg/wet wt), compared with that shown in saline-injected rats (880 ± 101 pg/wet wt; P < 0.01, n = 8).

**DISCUSSION**

CRF neurons are located in the paraventricular nucleus of the hypothalamus, the amygdala, and the locus coeruleus complex (30). The experimental data point to a role of cerebral CRF₁ receptors in the stimulation of colonic transit by restraint stress or centrally injected CRF (30).

CRF₁ receptors are also expressed in the myenteric plexus of the rat colon (6). It is controversial whether stress-induced acceleration of colonic transit is mediated via peripheral CRF receptors (6), in addition to the central CRF receptors (7). Intracerebroventricular injection of CRF₁ receptor antagonists (NB1-27914) dose dependently reduced the defecatory response to water avoidance stress, whereas NB1-27914 injected peripherally did not influence the defecatory response to water avoidance stress (15).

In contrast, others showed that stress-induced acceleration of colonic transit is antagonized by peripheral administration of CRF antagonists (13, 14, 36). Peripheral injection of CRF₁ receptor antagonists reduced the defecation in response to water avoidance stress (16), suggesting that peripheral CRF receptors are involved in mediating stress-induced acceleration of colonic transit.

We showed that restraint stress accelerates colonic transit in conscious rats. Central administration of CRF antagonist (astressin), but not systemic administration of astressin, almost completely abolished restraint stress-induced acceleration of colonic transit. This suggests that stress-induced acceleration of colonic transit is mainly mediated via central CRF receptors.

Our present study shows that accelerated colonic transit induced by restraint stress was not affected by guanethidine, suggesting no involvement of sympathetic pathways. Accelerated colonic transit induced by restraint stress was significantly reduced by truncal vagotomy and perivagal capsaicin treatment. This suggests that stress-induced acceleration of colonic...
transit is mediated via vagal afferent fibers and vagal efferent fibers.

There is considerable evidence suggesting that peripheral release of 5-HT was involved in stress- and central CRF-mediated alterations in GI function (21). Studies in experimental animals showed that CRF released from the central nervous system in response to stress peripherally promotes the release of 5-HT, which stimulates propulsive bowel function through the 5-HT3 receptors (17, 18). Increased defecation induced by stress and central CRF is abolished by a systemic treatment with 5-HT3 receptor antagonists (17, 18). However, it remains unknown whether the stimulatory effect of stress and central CRF is mediated via 5-HT-containing neurons and/or EC cells.

5-HT acts within the enteric nervous system (ENS) as a neurotransmitter and can transmit fast excitatory signals via 5-HT3 receptors (9). 5-HT also acts on 5-HT3 receptors at the endings of vagal sensory axons in the proximal and mid colon. 5-HT3 receptors containing nerves, both intrinsic and extrinsic, are located at the lamina propria (9). The motor activity in response to vagal stimulation involves a different type of motility in a different part of GI tract. It remains unclear which 5-HT released from the ENS or EC cells plays a major role in mediating colonic motility.

It is not well established whether 5-HT is primarily released into the colonic lumen and/or into the colonic wall from EC cells. We have previously showed that released 5-HT from colonic EC cells plays a major role to regulate colonic transit and motility in normal rats. Luminally released 5-HT from EC cells activates mucosal 5-HT3 receptors located on the vagal afferent. The activation of vagal afferents stimulates colonic motility via the vagovagal reflex (8).

Vagal nerve stimulation increases the 5-HT release into the lumen in the cat jejunum (37). This suggests that 5-HT release from EC cells is under the regulation of vagal efferent. Once 5-HT3 receptor antagonists are administered into the lumen of the proximal colon, restraint stress had no more stimulatory effects on colonic transit. This suggests that released 5-HT from EC cells in response to restraint stress may have a major role in mediating the acceleration of colonic transit. As mentioned above, restraint stress-induced acceleration of colonic transit was antagonized by perivagal capsaicin treatment, suggesting that restraint stress primarily stimulates vagal cholinergic fibers innervating the submucosal plexus and stimulates 5-HT release from EC cells. Thus released 5-HT accelerates colonic transit via mucosal 5-HT3 receptors and vagovagal reflex.

Alternatively, it is also possible that restraint stress provokes a motor response in the colon via a vagal efferent. The vagal motor drive to the colon may stimulate 5-HT neurons of ENS, which utilizes 5-HT3 receptors. It is likely, although not proven, that administered ondansetron into the lumen is free to traverse the epithelium and could act on ENS. However, this pathway may be minor for the following reasons. 1) Restraint stress-induced acceleration of colon is antagonized by perivagal capsaicin treatment, suggesting the mediation of vagal afferents. 2) Restraint stress stimu-
lated the luminal release of 5-HT, suggesting the mediation of luminal EC cells of the proximal colon.

The ED$_{50}$ dose of systemic intravenous administration of ondansetron is reported as 354 µg/kg for inhibiting increased defecation stimulated by restraint stress and central CRF in rats (18). In our present study, intraluminal administration of 1.8 µg (1 ml of 5 × 10^-6 M) of ondansetron was effective to antagonize the stimulatory effects of restraint stress and CRF on colonic transit and motility. Therefore, it is unlikely that the inhibitory effect of luminally administered ondansetron is due to the leakage to the systemic circulation.

The vagal nerves innervate to the proximal and mid colon, whereas the pelvic nerves innervate to the distal colon in rats (1, 4). When vagotomy is carried out, the pelvic nerves are still functioning. This may explain why vagotomy does not significantly influence the colonic transit.

Truncal vagotomy attenuates acceleration of colonic transit induced by intracerebroventricular injection of CRF (10). Subdiaphragmatic vagotomy prevented, by 36%, the stimulation of

Fig. 7. Effects of intraluminal administration of saline (A) and ondansetron (B) on restraint stress-stimulated colonic motility. Restraint stress-induced augmentation of colonic motility was significantly reduced by intraluminal application of ondansetron.

Fig. 8. Effects of intraluminal administration of saline (A) and ondansetron (B) on CRF (1.0 µg)-stimulated colonic motility. CRF significantly increased the motility of the proximal and distal colons. Stimulated colonic motility induced by CRF was significantly reduced by the intraluminal administration of ondansetron into the proximal colon.
colonic transit induced by the microinjection of CRF into the paraventricular nucleus (19), suggesting that the stimulatory effect of central CRF on colonic transit is mediated, in part, by the vagal nerves. CRF microinjected into the locus coeruleus/subcoeruleus nucleus increased colonic motility in rats (20). Water avoidance stress activates the sacral parasympathetic nucleus and increases fecal pellet output in rats (16). CRF projections from the Barrington nucleus innervate the sacral parasympathetic preganglionic neurons (pelvic nerves), which are synaptically linked to the distal colon (34). Therefore, it is highly likely that central CRF activates the vagal nerves and the pelvic nerves to stimulate colonic motor function.

We showed that restraint stress and intracisternal injection of CRF significantly increased the motility of the proximal and distal colon. Augmented motor functions of the proximal colon induced by restraint stress and intracisternal injection of CRF were significantly reduced by the intraluminal application of ondansetron into the proximal colon. The stimulated contractions of the distal colon induced by restraint stress and intracisternal injection of CRF migrated distally to the distal colon. The intraluminal application of ondansetron into the proximal colon significantly reduced the GMCs. This suggests that the stimulatory effect of restraint stress and intracisternal injection of CRF on the proximal colonic motility is mediated via 5-HT3 receptors located on the vagal nerves, which innervate the proximal colon.

In addition, restraint stress and intracisternal injection of CRF probably act on the Barrington nucleus, which in turn stimulates the pelvic nerves for inducing the distal colonic motility. The distal colonic motility stimulated by the pelvic nerves may not be antagonized by the intraluminal application of 5-HT3 antagonists into the proximal colon.

The distribution of EC cells is significantly higher than that of the distal colon in rats (24). We showed that restraint stress and central administration of CRF significantly increased the luminal release of 5-HT from cells of the proximal colon. Restraint stress-stimulated luminal release of 5-HT was antagonized by intracisternal injection of CRF1 receptor antagonists.

It is, therefore, suggested that restraint stress stimulates vagal nerves innervating to the proximal colon via central CRF1 receptors, resulting in 5-HT release from the proximal colon. Luminally released 5-HT activates 5-HT3 receptors located at the vagal afferent. Activation of 5-HT3 receptors stimulates colonic motility via vagovagal reflex. Thus the main target of restraint stress may be EC cells of the proximal colon (Fig. 10). This study may contribute to the mechanism of altered colonic motility induced by stress.

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