JNM
J Neurogastroenterol Motil, Vol. 22 No. 2 April, 2016
pISSN: 2093-0879 eISSN: 2093-0887
http://dx.doi.org/10.5056/jnm15082
Journal of Neurogastroenterology and Motility

Original Article

Changes in Enteric Neurons of Small Intestine in a Rat Model of Irritable Bowel Syndrome with Diarrhea

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Background/Aims
Physical and/or emotional stresses are important factors in the exacerbation of symptoms in irritable bowel syndrome (IBS). Several lines of evidence support that a major impact of stress on the gastrointestinal tract occurs via the enteric nervous system. We aimed to evaluate histological changes in the submucosal plexus (SMP) and myenteric plexus (MP) of the distal ileum in concert with the intestinal motor function in a rat model of IBS with diarrhea.

Methods
The rat model was induced by heterotypic chronic and acute stress (CAS). The intestinal transit was measured by administering powdered carbon by gastric gavage. Double immunohistochemical fluorescence staining with whole-mount preparations of SMP and MP of enteric nervous system was used to assess changes in expression of choline acetyltransferase, vasoactive intestinal peptide, or nitric oxide synthase in relation to the pan neuronal marker, anti-Hu.

Results
The intestinal transit ratio increased significantly from control values of 50.8% to 60.6% in the CAS group. The numbers of enteric ganglia and neurons in the SMP were increased in the CAS group. The proportions of choline acetyltransferase- and vasoactive intestinal peptide-immunoreactive neurons in the SMP were increased (82.1 ± 4.3% vs. 76.0 ± 5.0%, $P = 0.021$; 40.5 ± 5.9% vs 28.9 ± 3.7%, $P = 0.001$), while nitric oxide synthase-immunoreactive neurons in the MP were decreased compared with controls (23.3 ± 4.5% vs 32.4 ± 4.5%, $P = 0.002$).

Conclusions
These morphological changes in enteric neurons to CAS might contribute to the dysfunction in motility and secretion in IBS with diarrhea.
(J Neurogastroenterol Motil 2016;22:310-320)

Key Words
Diarrhea; Enteric nervous system; Gastrointestinal motility; Irritable bowel syndrome

Received: May 11, 2015 Revised: October 19, 2015 Accepted: November 8, 2015

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Introduction

Irritable bowel syndrome (IBS) is among the most common conditions diagnosed in clinical practice. It is classified as a functional gastrointestinal disorder characterized by abdominal pain or discomfort and alterations in bowel habits, each of which can be exacerbated by stress. The etiology of IBS is complex and appears to be multi-factorial, including altered gastrointestinal (GI) motility, visceral hypersensitivity, heredity, inflammation, and psycho-social factors.[1,2] Nevertheless, the etiology of IBS remains unknown.

The enteric nervous system (ENS), which is regarded as a "brain-in-the-gut,"[3] consists of 2 major divisions, the submucosal plexus (SMP) and the myenteric plexus (MP). The SMP controls absorptive and secretory functions of the mucosal epithelium, intramural blood flow, and neuroimmune interactions, while the MP programs motility for specific digestive states. Normal functioning of the ENS is required for ordinary GI function. Malfunction of the ENS is recognized in disorders such as neuropathic pseudo-obstruction and autoimmune enteric neuropathy, for example Hirschsprung's disease.[4] Most preclinical, morphological, and functional studies of the ENS have focused on the normal bowel. Only a small number of published studies are centered on pathological conditions such as IBS or inflammatory bowel disease. Conclusions from the few reported studies are generally that the ENS shows a high degree of plasticity for adaptation to disturbances, such as inflammation and stress.[5] Although IBS is regarded as a functional disorder, there is evidence to suggest that an ENS autoimmune degenerative neuropathy might underlie IBS in humans.[6] One study found histopathological abnormalities in the ENS of the proximal jejunum in patients with severe IBS, in which nine of 10 patients had infiltration of lymphocytes in and around the enteric ganglia, while 7 had immune-associated ganglion cell degeneration.[7]

Our laboratory has developed and tested a novel IBS with diarrhea (IBS-D) rat model in which an acute stress is superimposed on rats experiencing heterotypic chronic and acute stress (CAS).[8-10] The CAS model is characterized by increased expulsion of fecal pellets, visceral hypersensitivity, increased colonic epithelial basal ion secretion, and decreased epithelial barrier function, which mimic the characteristics of IBS-D. We also measured gastric emptying of CAS rats using gastrogavage in a previous study, and it was delayed significantly in CAS rats.[11] These changes in model rats have already been validated, proving to be reproducible and more distinct than those of traditional acute restraint stress or chronic stress models.[8-10] It has also been shown that the increased colonic motility and visceral hypersensitivity of the CAS model are associated with altered c-fos, an activated tissue cell marker, which was increased in the central nervous system (CNS; frontal lobe, hippocampus, and cornu dorsale) but normal in the colon. The brain-gut interaction in the CAS model showed that psychological stress might promote activation of the CNS and induce hyperexcitability of the colon indirectly.[8-10] The role of the CNS in IBS models has already been established. Recent reports have shown that corticotropin-releasing factor (CRF)-CRF_1 receptor signaling in the central amygdala induces visceral hypersensitivity accompanied by enhanced noradrenaline and dopamine levels at this site.[12] Activation of glutamate receptors in the rostral ventromedial medulla was shown to facilitate visceral hyperalgesia in zymosan-treated rats. Glutamate, gamma-aminobutyric acid (GABA), and epigenetic mechanisms also play roles in the CNS, regulating the pathophysiology of IBS, as has been reviewed by Moloney.[13] We aimed to evaluate intestinal motor function in CAS rats in concert with morphological changes in SMP and MP in the distal ileum of the model. The results from these studies have been published in abstract form.[14]

Materials and Methods

Animal Models

The experiments were performed on adult male Sprague-Dawley rats (Vital River, Beijing) weighing 160-180 g, which were housed in individual cages in a standardized environment at 20-24°C, 55-60% relative humidity and a 12 hours/12 hours light-dark cycle. The rats received food and water *ad libitum* and were acclimated for 3 days before the experiments were started. The animal care and experimental protocols were approved by the Peking Union Medical College Hospital Laboratory Animal Care and Use Committee.

Following acclimation, the animals were randomly assigned to 1 of 2 groups: a CAS group and a control group. The CAS group was exposed to seven different stressors: (1) water deprivation for 24 hours, (2) food deprivation for 24 hours, (3) painful tail pinch for 1 minute, (4) 5 minutes exposure to a 45°C environment, (5) swimming in 4°C water for 3 minutes, (6) day and night inversion for 12 hours/12 hours, and (7) horizontal vibration (120 rpm) for 45 minutes.[8-10] All stress protocols were applied at random every 7 days for 3 weeks, and no specific stressor was repeated on 2 consecutive days. On day 28, 1 week of rest was followed by acute restraint stress with wrapping of the shoulders, upper forelimbs and

thoracic trunk for 1 hour. The rats were weighed on day 0 (before exposure to any stress) and on day 28 (after completion of the final acute restrain stress protocol). Daily changes in weight over the 28-day period were recorded as an indicator of the animal's general health status.

Intestinal Transit

After fasting for 24 hours, the rats received powdered carbon *via* gastric gavage and 45 min later were euthanized rapidly by cervical dislocation followed by removal of the small and large intestine. The length of bowel from the gastroduodenal junction to the anus, and the length of small intestine that contained the carbon marker 45 minutes after placement in the stomach were measured. The intestinal transit rate was calculated by the length of intestine containing the marker/total bowel length × 100%.

Immunohistochemistry

Rats were anesthetized with sodium pentobarbital anesthesia (30-50 mg/kg) and euthanized by exsanguination from bilateral cervical vessels. Whole-mount preparations of the SMP and MP were obtained by microdissection from the distal ileum, maximally stretched and pinned-out on Sylgard resin, followed by fixation in 2% formalin containing 0.2% picric acid for 24 hours. Double immunohistochemical fluorescence staining was used to investigate the distribution and proportions of neurons in the SMP and MP in stressed animals and controls. These methods were essentially the same as described previously.[15] The preparations were washed in three changes of Krebs solution for 10 minutes. The composition of the Krebs solution was (in mM) 120.9 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 14.4 $NaHCO_3$, 2.5 $CaCl_2$, and 11.5 glucose. Rinsing in dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), 0.1% NaBH4, and PBS each for 3 × 10 minutes in that order was followed by incubation in 10% normal donkey serum containing 0.3% Triton X-100 and 0.1% sodium azide for 2 hours. For neuronal staining, the tissues were placed in mouse anti-Hu antiserum (1:50) overnight. Anti-Hu served as a pan neuronal marker for enteric ganglion cells. After 3 × 10 minutes thorough washes in PBS, the tissues were incubated in secondary antibodies conjugated with fluorescine isothiocyanate (FITC) or indocarbocyanine (Cy3) for 30 minutes in the dark. The tissues were examined with a fluorescence microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan) after 3 × 10 minutes washing with PBS to ensure quality of labeling. Primary antibodies for choline acetyltransferase (ChAT), vasoactive intestinal peptide (VIP) and nitric oxide synthase (NOS) were used to label ENS neurons according to specific chemical markers as follows: (1) ChAT for cholinergic musculomotor neurons, cholinergic interneurons and cholinergic secretomotor/vasodilator neurons; (2) VIP for inhibitory musculomotor neurons and non-cholinergic secretomotor/vasodilator neurons; and (3) NOS for inhibitory musculomotor neurons. After a thorough rinse, the preparations were cover slipped and examined under the fluorescence microscope. Primary and secondary antibodies, dilutions and sources are listed in Table 1. Immunohistochemical controls were incubation with secondary antibody alone. All images were acquired using a digital camera, saved on disk and analyzed with imaging software (NIS-Elements F 3.0, Tokyo, Japan).

Table 1. Antibodies Used for Immunohistochemistry Experiments

Antigen	Host	Code	Dilution	Source
HuC/HuD	Mouse	A-21271	1:50	Mol Probes
ChAT	Goat	AB144P	1:100	Chemicon
VIP	Rabbit	T-4246	1:100	Bachem
VIP	Rabbit	SC-20727	1:100	Santa Cruz
NOS	Sheep	AB1529	1:500	Chemicon
Mouse IgG	Donkey FITC	715-095-150	1:100	Jackson
Mouse IgG	Donkey Cy3	715-165-150	1:1000	Jackson
Rabbit IgG	Goat FITC	ZF-0311	1:100	Zhongshan GoldenBridge
Rabbit IgG	Goat TRITC	ZF-0316	1:200	Zhongshan GoldenBridge
Goat IgG	Rabbit FITC	ZF-0314	1:100	Zhongshan GoldenBridge
Goat IgG	Rabbit TRITC	ZF-0317	1:200	Zhongshan GoldenBridge
Sheep IgG	Donkey FITC	713-095-147	1:100	Jackson
Sheep IgG	Donkey Cy3	713-165-147	1:1000	Jackson

Anti-Hu, anti-human neuronal protein HuC/HuD; ChAT, choline acetyltrasferase; VIP, vasoactive intestinal peptide; NOS, nitro oxide synthase; IgG, immunoglobulin G; FITC, fluorescine isothiocyanate; Cy3, indocarbocyanine; Mol Probes, Molecular Probes.

Ganglion Cell Counts

Ganglion cells were stained with antibodies that identified expression of immunoreactivity for specific chemical markers and for the pan-neuronal marker, anti-Hu. The immunoreactive (IR) neurons were assessed in randomly chosen non-overlapping fields-of-view at a magnification of ×200 throughout the preparations. Counts of labeled cells were assessed in 10 fields in the SMP preparation from each rat and 15 identical fields from the MP preparations to assure that at least 500 cells in the SMP/MP were counted for each rat. Numbers of ganglia and positively-stained ganglion cells for other specific antibodies in each field were counted in a single blinded manner and are expressed as a percent of the total number of anti-Hu labeled neurons. The gross structure of the MP was latticed in such a way that counts of individual ganglia could not be completed satisfactorily, and therefore the numbers of MP ganglia are not included in the data.

Statistical Methods

All results are expressed as mean ± standard error, n refers to the numbers of rats examined. The statistical significance of differences between the control and CAS groups was determined using the independent sample *t* test by means of SPSS 11.0 (SPSS Inc, Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

Results

Body Weight

Body weights for CAS and control groups were not significantly different (148 ± 9 g vs 150 ± 6 g, $P = 0.545$; n = 8 in each group) on day 0. Weight gain for the CAS group was reduced relative to controls over the 28-day period. Mean body weight after 28 days for the CAS group was 240 ±12 g, compared with 262 ± 17 g for the controls ($P = 0.011$).

Intestinal Transit

Transit of the carbon marker 45 minutes after gavage was restricted to the small intestine in both the stressed rats and the controls. The intestinal transit rate was accelerated in the CAS group relative to the control group. Intestinal transit rate for the CAS group was 60.6 ± 5.3% and for the control group was 50.8 ± 9.9% ($P = 0.038$; n = 7 in each group).

Ganglion Cell Counts

The number of anti-Hu-IR neurons in the ileal SMP was significantly increased in the CAS rats compared with the controls ($P = 0.029$) and a similar pattern was found for the total number of enteric ganglia in the ileal SMP ($P = 0.017$; n = 8 in each group) (Table 2).

Secretomotor Neurons in the Submucosal Plexus

Most neurons in the ileal SMP were ChAT-IR positive with staining restricted to the cytoplasm, and the total number and proportion of ChAT-IR neurons in the SMP were higher in the CAS rats compared with the control group ($P = 0.006$; $P = 0.021$, respectively; n = 8 in each group; Fig. 1). The total number and proportion of VIP-IR neurons in the SMP were also increased in the CAS rats compared with the control group ($P = 0.002$; $P = 0.001$, respectively; n = 7 in each group; Fig. 2 and Table 3). There were no differences in the number and proportion of NOS-IR neurons in the SMP of CAS rats compared with controls ($P > 0.05$ for both; n = 7 in each group).

Table 2. Number of Neurons and Ganglia in the Ileal Submucosal Plexus and Myenteric Plexus in Chronic and Acute Stress Rats and Control Rats

Variable	SMP			MP		
	CAS	controls	*P*	CAS	controls	*P*
Neurons	68.5 ± 6.0 (5480/80)	62.1 ± 4.4 (4972/80)	0.029	48.0 ± 3.7 (5040/105)	45.5 ± 5.4 (4781/105)	0.338
Ganglia	22.9 ± 2.3 (1834/80)	19.9 ± 2.2 (1594/80)	0.017	-	-	-

SMP, submucosal plexus; MP, myenteric plexus; CAS, chronic and acute stress.
Values are shown as mean ± SE, while the sum of neurons or ganglia divided by total fields are shown in parentheses below. Total numbers of neurons and ganglia in the ileal SMP were significantly higher in the CAS rats than in controls (n = 8). No difference was found between the 2 groups in the numbers of MP neurons ($P > 0.05$, n = 7). The numbers of MP ganglia were not included because they were not able to be counted. n refers to the numbers of rats examined in each group.

Inhibitory Musculomotor Neurons in the Myenteric Plexus

There was no difference between the numbers of MP neurons in the 2 groups ($P > 0.05$; n = 7 in each group; Table 2). No differences were found in the numbers and proportions of ChAT-IR neurons in the MP between the CAS rats and controls ($P > 0.05$ for both; n = 7 in each group; Table 4). Only a small number of neurons in the MP were VIP-IR positive and no difference was found between the CAS and control groups. In contrast, the numbers and percentage of NOS-IR neurons in the MP were significantly decreased in the CAS rats ($P = 0.001$; $P = 0.002$, respectively; n = 7 in each group; Fig. 3 and Table 4).

Discussion

The IBS-D rat model induced by CAS is a brain-gut interaction model which mimics some clinical and pathophysiologic characteristics of IBS-D,[10] including increased motility of the distal colon and rectum, visceral hypersensitivity, a potential increase of colonic epithelial secretion, as well as increased expression of c-fos in the CNS.[8,9] Combining the results of delayed gastric emptying found in our previous study with the increased GI transit in this study, we conclude that increased transit occurs in the intestine but not in the stomach.[11] So, motor abnormality of this model is not only present in the distal colon and rectum, but also in the small intestine, which indicates that the enhanced small intestinal motility

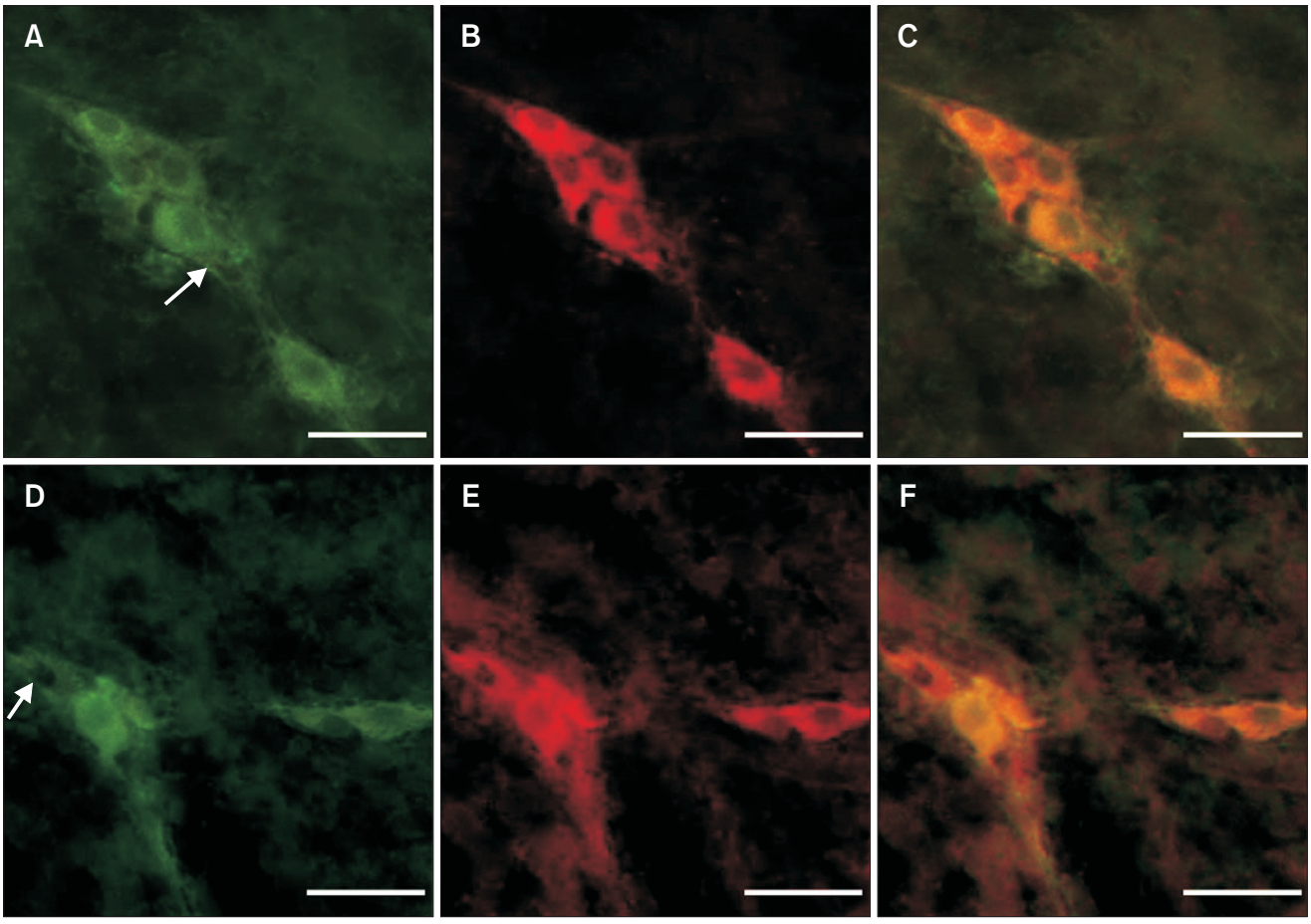

Figure 1. Enteric nervous ganglia and neurons in whole mount preparations of rat ileal submucosal plexus (SMP). Choline acetyltransferase-immunoreactive (ChAT-IR) neurons were increased in the chronic stress (CAS) rats (A-C) compared with the control group (D-F). The arrows in the photos point to the ChAT-IR negative neurons. (A, D) ChAT-IR neurons in the SMP ganglion, (B, E) Anti-Hu-IR labeled all neurons in the SMP, and (C, F) Merged picture of Anti-Hu/ChAT-IR. Scale bar = 50 μm

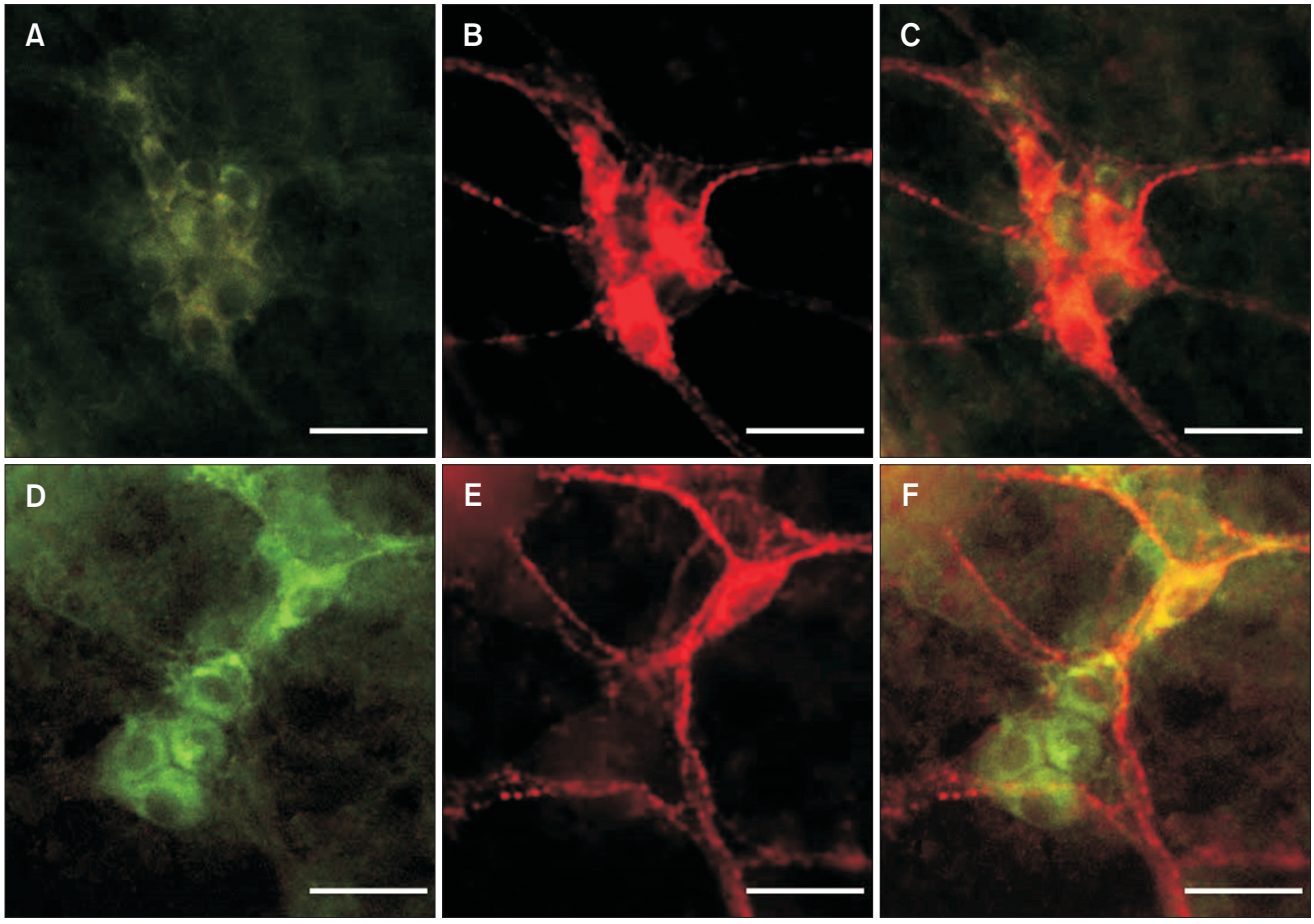

Figure 2. Enteric nervous ganglia and neurons in whole mount preparations of rat ileal submucosal plexus (SMP). Choline acetyltransferase-immunoreactive (VIP-IR) neurons were increased in the chronic stress (CAS) rats (A-C) compared with the control group (D-F). (A, D) Anti-Hu-IR labeled all neurons in the SMP, (B, E) VIP-IR neurons in the SMP ganglion, and (C, F) Merged picture of Anti-Hu/VIP-IR. Scale bar = 50 μm

Table 3. Specific Neurons of the Ileal Submucosal Plexus in Chronic and Acute Stress Rats and Control Rats

Chemical marker	Neuron numbers			Chemical code/Anti-Hu		
	CAS	controls	*P*	CAS	controls	*P*
ChAT	56.3 ± 6.2 (4505/80)	47.2 ± 3.3 (3779/80)	0.006	82.1 ± 4.3% (4505/5480)	76.0 ± 5.0% (3779/4972)	0.021
VIP	28.2 ± 6.2 (1974/70)	17.9 ± 3.3 (1250/70)	0.002	40.5 ± 5.9% (1974/4843)	28.9 ± 3.7% (1250/4312)	0.001
NOS	10.2 ± 1.4 (712/70)	11.6 ± 3.8 (813/70)	0.363	14.5 ± 2.3% (712/4927)	18.8 ± 6.7% (813/4337)	0.135

Anti-Hu, anti-human neuronal protein HuC/HuD; CAS, chronic and acute stress; ChAT, choline acetyltransferase; VIP, vasoactive intestinal peptide; NOS, nitric oxide synthase.

All values are shown as mean ± SE. In the "Neuron numbers" column, the total neuron numbers of the specific chemical marker per field are shown in parentheses below. In the "Chemical marker" column, the total number of neurons with the specific chemical marker and anti-Hu are shown in parentheses below. The number and proportion of ChAT-IR in the ileal SMP were higher in the CAS rats compared with controls ($P < 0.05$, n = 8). The same trend was found in VIP-IR neurons ($P < 0.05$, n = 7). No differences were found in NOS-IR neurons between the two groups ($P > 0.05$, n = 7). n refers to the numbers of rats examined in each group.

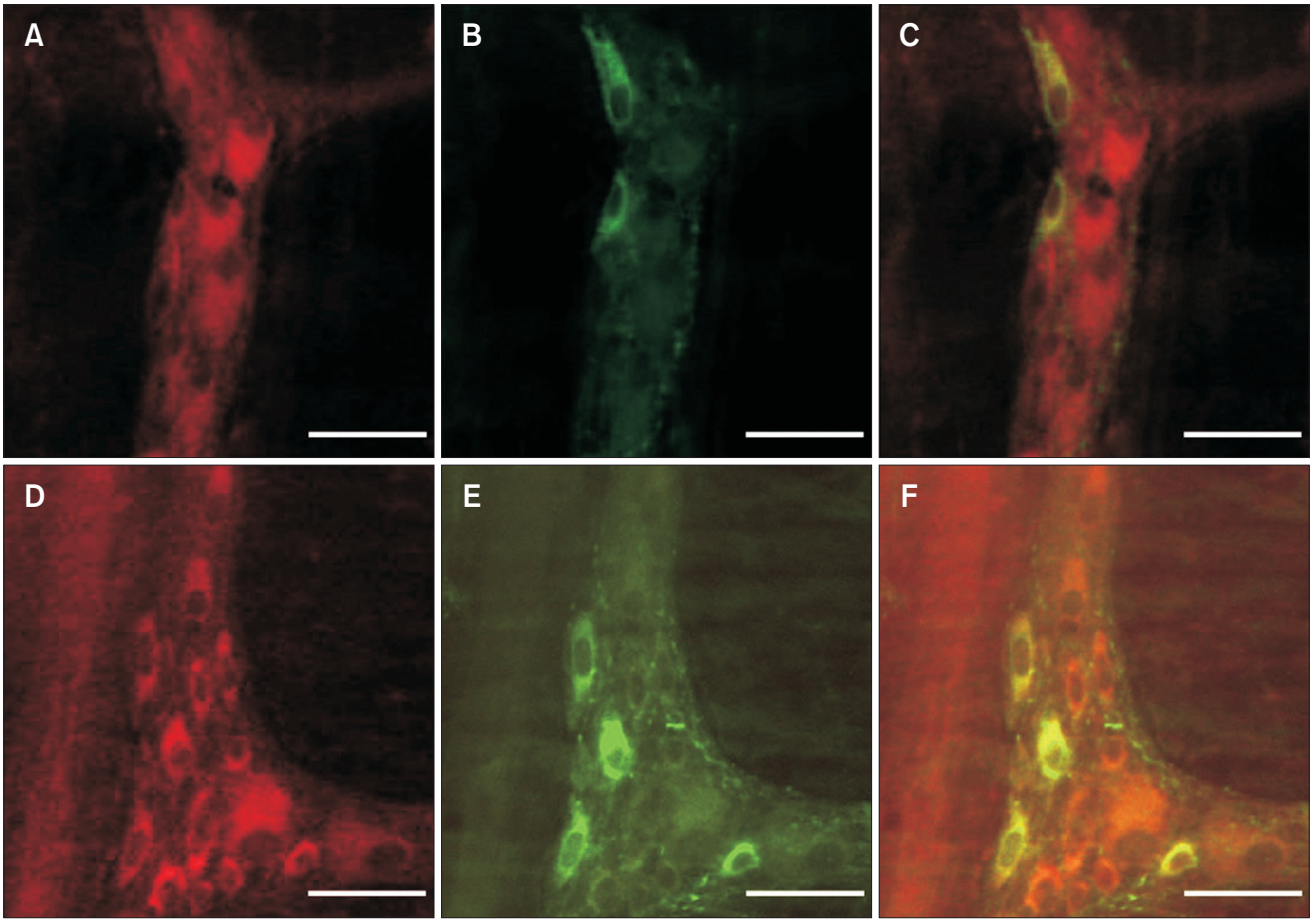

Figure 3. Enteric nervous ganglia and neurons in whole mount preparations of rat ileal myenteric plexus (MP). Nitric oxide synthase-immunoreactive (NOS-IR) neurons were decreased in CAS rats (A-C) compared with the control group (D-F). (A, D) Anti-Hu-IR labeled all neurons in the MP, (B, E) NOS-IR neurons in the MP ganglion, and (C, F) Merged picture of Anti-Hu/NOS-IR. Scale bar = 50 μm.

Table 4. Specific Neurons of the Ileal Myenteric Plexus in CAS and Control Rats

Chemical marker	Neuron numbers			Chemical code/Anti-Hu		
	CAS	controls	*P*	CAS	controls	*P*
ChAT	37.9 ± 3.8 (3976/105)	34.2 ± 4.6 (3589/105)	0.131	78.8 ± 3.3% (3976/5040)	75.0 ± 3.4% (3589/4781)	0.057
VIP	0.9 ± 0.2 (100/105)	0.8 ± 0.2 (86/105)	0.246	2.3 ± 0.6% (100/4324)	1.9 ± 0.6% (86/4565)	0.218
NOS	9.9 ± 2.0 (1039/105)	15.0 ± 2.4 (1579/105)	0.001	23.3 ± 4.5% (1039/4450)	32.4 ± 4.5% (1579/4878)	0.002

Anti-Hu, anti-human neuronal protein HuC/HuD; CAS, chronic and acute stress; ChAT, choline acetyltransferase; VIP, vasoactive intestinal peptide; NOS, nitric oxide synthase.
All values are shown as mean ± SE. In the "Neuron numbers" column, the total numbers of neurons with a specific chemical marker per field are shown in parentheses below. In the "Chemical marker" column, the total number of neurons with a specific chemical marker and anti-Hu are shown in parentheses below. Both number and proportion of NOS-IR neurons in the ileal SMP decreased in the CAS rats ($P < 0.05$, n = 7). No differences were found in ChAT-IR and VIP-IR neurons between the 2 groups. n refers to the numbers of rats examined in each group.

might contribute to the diarrhea in IBS-D rats. These changes are considered to be functional disorders with no pathological changes in the GI wall and brain.

Rats exposed to the CAS protocol responded in a canonical manner, as has been observed for other stressed animals. Most kinds of stress, such as water avoidance, acoustic stimulation, wrap restraint and swimming, suppress gastric emptying and simultaneously increase secretion and motility in the colon of model animals.[16] The stress also stimulated colonic motor activity in human volunteers and altered gut functions in IBS patients in which abnormal propagation existed in the duodenum, jejunum and colon.[17,18]

We focused the present study on enteric neurons because of the known effects of stress on intestinal motility and mucosal secretion and the current finding of accelerated transit in the small intestine of the CAS rats, especially for the musculomotor neurons and secretomotor neurons which are the primary motor neurons in the ENS. Our research has shown that these changes of neurons are not restricted to the ileum but also in the colon.[19]

Secretomotor neurons which release acetylcholine and/or VIP as neurotransmitters are ENS excitatory motor neurons in the SMP innervating the GI secretion. We used immunoreactivity for VIP and ChAT as chemical markers for identification of secretomotor neurons in the SMP. The increase in the total number of neurons found in the SMP in CAS rats vs controls appears to reflect stress-evoked expansion of numbers of secretomotor neurons, as suggested by the elevated numbers of neurons that expressed the secretomotor markers, ChAT and VIP. This suggests that the well-documented neurogenic secretory diarrhea and compromised mucosal barrier function associated with stress in animal models might result from an expanded population of secretomotor neurons. It has already been reported that VIP-IR nerves increase both in the mucosa and SMP of the colon in rats with chemical colitis.[20] In another IBS model established by chronic stress stimulation, VIP contents also increased in serum and in colon tissue.[21] This increase was consistent with the reported increased of VIP levels in ileocecal, sigmoid colonic, rectal tissue, and in plasma of IBS patients.[22,23] These similar changes were also found in SMP neurons and ganglia in colon of CAS rats, which showed that the proportions of both VIP-IR and NOS-IR neurons in colonic SMP were increased,[19] the results were not reported in this paper. Considering the above data together, the increase of ChAT-IR and VIP-IR neurons in ileal SMP in IBS-D rats might promote intestinal secretion, which aggravates diarrhea.

Musculomotor neurons, which are found in the MP, innervate the longitudinal and circular muscle coats of the small intestine. Ach and substance P are the principal excitatory neurotransmitters while VIP, pituitary adenylate cyclase activating peptide, and NO are inhibitory transmitters.[18] We used ChAT as an immunochemical marker for identification of excitatory musculomotor neurons and NOS and VIP as markers for inhibitory musculomotor neurons.

In this CAS model, we found that the total number of neurons in the MP did not change relative to control rats. In contrast, the number of NOS-IR neurons was decreased while the number of cholinergic neurons was unchanged. This suggests that the predominant effect of stress on musculomotor neurons is on the inhibitory musculomotor neurons that release nitric oxide (NO) as an inhibitory transmitter at neuromuscular junctions. The VIP-ergic component of the inhibitory musculomotor population appeared to be unaltered by CAS. In our study decreased numbers of inhibitory musculomotor neurons (and therefore a weaker inhibitory brake on the unitary-type autogenic smooth muscle) might be related to the enhanced propulsive motor behavior found in the CAS model, because inhibition has a major role in the organization of effective propulsive motility.[5,24]

Gut motility and secretion are controlled by the ENS to facilitate real-time digestion and absorption of nutrients. Earlier results reported strong evidence of the relationship between intestinal motility and secretion.[25] It has been confirmed that mechanical distension stimulates submucosal neurons to increase chloride secretion.[26] Therefore neural abnormalities might be the underlying factors in GI disorders. Altered stool patterns are characteristics of IBS patients which may be related to accelerated or reduced intestinal transit and impaired secretion. Early in 1978, Oddson et al[27] reported that bile acids could exaggerate the transmural potential difference (PD) response which primarily reflected electrogenic chloride secretion in IBS patients. Since then only a few studies have focused on changes in the secretory function of IBS patients. Larsson et al confirmed that the increased propagation speed of migrating motor complex phase III was accompanied by elevated maximal PD, and that the PD decline after migrating motor complex phase III was significantly prolonged in IBS patients.[28] Previous data from our laboratory verified that CAS rats show an increase in baseline short-circuit current and increased fecal output.[9] In this study we found IBS-D model rats had less inhibitory neurons in the MP accompanied by fast transit rate and more excitatory secretomotor neurons in the SMP of small intestine segment, which indicates that the morphological changes of the ENS represent a pathophysiologically integrated modification of the intestinal motility and secretion in responds to stresses. The limit of this study was that the SMP promoting secretion was not detected simultaneously.

There are currently many animal models that mimic IBS, including psychological stress models such as restraint stress, water avoidance stress, cold stress, and swimming- induced fatigue; inflammatory stimulation models such as pathogen infection or chemical stimulation; early life events models such as maternal separation; and gene knockout models. Morphological shifts in ENS nerve plexuses and changes of neurotransmitters have been observed in models of experimental colitis, which might contribute to the abnormality in motility. In IBS-D model of intracolonic instillation of acetic acid, the total number of neurons in the colon SMP decreased, while serum and colon NO production increased, compared with controls.[29,30] In IBS with constipation rats induced by stomach irritation with cold water, the number of acetyltransferase immunoreactive (AchE-IR) neurons in the colonic SMP decreased while the nicotinamide dinucleotide phosphate-diaphorase (NADPH-d) positive neurons in the colonic MP increased compared with controls.[29,31] The BioBreeding rat is used as a spontaneous animal model for type 1 diabetes, and its diabetes-prone (BBDP) strains remain normoglycaemic for life. Normoglycaemic BBDP rats showed intestinal dysmotility accompanied by decreased nNOS mRNA expression and decreased nitrergic nerves,as assessed by immunohistochemistry.[32] The plasma level of VIP was not significantly changed in a rat model of repetitive water avoidance stress, but was increased in rats with trinitrobenzene sulfonic (TNBS) acid-induced colitis, accompanied by upregulated VIP mRNA in the colon muscularis externae.[33] Larauche et al[34] developed an IBS-D model by using intraperitoneal injection of selective CRF_1 peptide agonist, which showed stimulation of colonic propulsive motor function linked with myenteric activation. This is consistent with another study showing that peripherally injected CRF ligands stimulate colonic function through colonic cholinergic and nitrergic myenteric neurons.[35] Because of the unclear pathophysiology and heterogeneous manifestations of IBS, different animal models may reflect different aspects of the disease, so the results obtained may be inconsistent.

Until recently, most studies of pathological changes of the ENS in IBS have been performed in animal models because of difficulty in obtaining full-thickness specimens. Previous reports revealed low to heavy grade infiltration of lymphocytes in MP, while the SMP was rarely affected,[7,36,37] with the peri/intraganglionic lymphocytes being CD3+ T cells with CD8+ predominance (cytotoxic T cells).[7] Neuronal degeneration was also observed in IBS patients.[7] Further research is needed with larger samples to prove the relationship between these morphological changes of the ENS, gut function and clinical symptoms. The expansion of the secretomotor population in the SMP and neuronal NOS-IR in the MP would likewise be suggestive of stress-related neurogenesis in the ENS. Neurogenesis has already been found in the adult brain where the new neurons integrate into functioning microcircuitry.[38] Comparable results occuring in the ENS has begun to accumulate.[39-41]

We presume that the responsive changes in enteric neurons to CAS stress might contribute to the dysfunction in motility and secretion in IBS-D, and to the symptom of diarrhea. Understanding the pathophysiological mechanisms of IBS from the viewpoint of integrated controlling functions of the ENS could be helpful for future development effective drugs for functional GI disorders.

Financial support: This study was supported by Project of the National Key Technologies R&D Program in the 11th Five Year Plan period (2007BAI04B01), National High-tech R&D Program ("863" Program, 2010AA023007), and Program of international S&T cooperation (2014DFA31850), China.

Conflicts of interest: None.

Author contributions: Shan Li and Guijun Fei, collected the data and wrote the manuscript; Xiucai Fang, designed the study and critically revised the manuscript; Xilin Yang, collected the data; Xiaohong Sun, Jiaming Qian, and Meiyun Ke, consulted for the designation and IBS modeling; and Jackie D Wood, provided anti-Hu antibody and critically revised the manuscript.

References

1. Al-Khatib K, Lin HC. Immune activation and gut microbes in irritable bowel syndrome. Gut Liver 2009;3:14-19.
2. Arebi N, Gurmany S, Bullas D, Hobson A, Stagg A, Kamm M. The psychoneuroimmunology of irritable bowel syndrome--an exploration of interactions between psychological, neurological and immunological observations. Aliment Pharmacol Ther 2008;28:830-840.
3. Wood JD. Intergrative functions of the enteric nervous system. In: Johnson LR. Physiology of the gastrointestinal tract. Volume 1. 4th ed. New York: Academic Press 2006:665-684.
4. Lake JI, Heuckeroth RO. Enteric nervous system development: migration, differentiation, and disease. Am J Physiol Gastrointest Liver Physiol 2013;305:G1-G24.
5. Wood JD. Enteric nervous system: the brain-in-the-gut. New Jersey: Morgan &Claypool Life Sciences 2011:1-157.
6. Wood JD, Liu S, Drossman DA, Ringel Y, Whitehead WE. Anti-enteric neuronal antibodies and the irritable bowel syndrome. J Neurogastroenterol Motil 2012:18:78-85.
7. Törnblom H, Lindberg G, Nyberg B, Veress B. Full-thickness biopsy of the jejunum reveals inflammation and enteric neuropathy in irritable

bowel syndrome. Gastroenterology 2002;123:1972-1979.
8. Zhang R, Zou N, Li J, et al. Elevated expression of c-fos in central nervous system correlates with visceral hypersensitivity in irritable bowel syndrome (IBS): a new target for IBS treatment. Int J Colorectal Dis 2011;26:1035-1044.
9. Zou N, Lv H, Li J, et al. Changes in brain G proteins and colonic sympathetic neural signaling in chronic-acute combined stress rat model of irritable bowel syndrome (IBS). Transl Res 2008;152:283-289.
10. Lv H, Qian JM, Jin GL, Zhou DF, Shen YC. The establishment of an animal model of gut-brain interaction in irritable bowel syndrome for the evaluation of visceral sensation, motility and psychological behavior. Zhonghua Nei Ke Za Zhi 2009;48:1035-1039.
11. Li S, Yang XL, Fang XC, Fei GJ. Do gastric emptying and gastric myenteric plexus neurons change in rats with IBS-D? J Dig Dis 2009;10:A22.
12. Taché Y. Corticotrophin-releasing factor 1 activation in the central amygdale and visceral hyperalgesia. Neurogastroenterol Motil 2015;27:1-6.
13. Moloney RD, O'Mahony SM, Dinan TG, Cryan JF. Stress-induced visceral pain: toward animal models of irritable-bowel syndrome and associated comorbidities. Front Psychiatry 2015;6:15.
14. Li S, Fei GJ, Yang XL, Qian JM, Wood JD, Fang XC. Changes in morphological characteristics of neurons in the enteric nervous system of the small intestine in a rat model for irritable bowel syndrome with diarrhea (IBS-D). Gastroenterology 2010;138(suppl 1):S766.
15. Fang X, Liu S, Wang XY, et al. Neurogastroenterology of tegaserod (HTF 919) in the submucosal division of the guinea-pig and human enteric nervous system. Neurogastroenterol Motil 2008;20:80-93.
16. Taché Y, Martinez V, Million M, Wang L. Stress and the gastrointestinal tract III. Stress-related alterations of gut motor function: role of brain corticotropin-releasing factor receptors. Am J Physiol Gastrointest Liver Physiol 2001;280:G173-G177.
17. Simrén M, Castedal M, Svedlund J, Abrahamsson H, Björnsson E. Abnormal propagation pattern of duodenal pressure waves in the irritable bowel syndrome (IBS). Dig Dis Sci 2000;45:2151-2161.
18. Clemens CH, Samsom M, Van Berge Henegouwen GP, Smout AJ. Abnormalities of left colonic motility in ambulant nonconstipated patients with irritable bowel syndrome. Dig Dis Sci 2003;48:74-82.
19. Li S, Fei GJ, Fang XC, Yang XL, Wang ZF. The morphological changes of colonic submucosal plexus in rat with irritable bowel syndrome with diarrhea (IBS-D). Zhonghua Xiao Hua Za Zhi 2010:30:250-253.
20. Kishimoto S, Kobayashi H, Shimizu S, et al. Changes of colonic vasoactive intestinal peptide and cholinergic activity in rats with chemical colitis. Dig Dis Sci 1992;37:1729-1737.
21. Wang YJ, Wang DS, Guan HQ, et al. Effects of eye-acupuncture therapy on serum and colonic SP and VIP contents in rats with irritable bowel syndrome. Zhen Ci Yan Jiu 2010;35:8-11, 26.
22. Palsson OS, Morteau O, Bozymski EM, et al. Elevated vasoactive intestinal peptide concentrations in patients with irritable bowel syndrome. Dig Dis Sci 2004;49:1236-1243.
23. Chen XM, Zhang YH, Mao JL, Zhang SS, Lu LG. Changes of substance P, vasoactive intestinal peptide and mast cell in colonic mucosa of patients with irritable bowel syndrome. Chin J Gastroenterol 2008;13:228-230.
24. Wood JD. Enteric nervous system: reflexes, pattern generators and motility. Curr Opin Gastroenterol 2008;24:149-158.
25. Mellander A, Järbur K, Sjövall H. Pressure and frequency dependent linkage between motility and epithelial secretion in human proximal small intestine. Gut 2000;46:376-384.
26. Weber E, Neunlist M, Schemann M, Frieling T. Neural components of distension-evoked secretory responses in the guinea-pig distal colon. J Physiol 2001;536(Pt 3):741-751.
27. Oddson E, Rask-Madsen J, Krag E. A secretory epithelium of the small intestine with increased sensitivity to bile acids in irritable bowel syndrome associated with diarrhoea. Scand J Gastroenterol 1978;13:408-416.
28. Larsson MH, Simrén M, Thomas EA, Bornstein JC, Lindström E, Sjövall H. Elevated motility-related transmucosal potential difference in the upper small intestine in the irritable bowel syndrome. Neurogastroenterol Motil 2007;19:812-820.
29. Xu JR, Luo JY, Shang L, Kong WM. The plasticity of the enteric submucosal plexus in the irritable bowel syndrome rats. Chin J Dig 2009;27:107-110.
30. Paragomi P, Rahimian R, Kazemi MH, et al. Antinociceptive and antidiarrheal effects of pioglitazone in a rat model of diarrhoea-predominant irritable bowel syndrome: role of nitric oxide. Clin Exp Pharmacol Physiol 2014;41:118-126.
31. Xu JR, Luo JY, Shang L, Kong WM. Role of inhibitory neurotransmitter of myoenteric plexus in carcinogenesis of irritable bowel syndrome with different subtypes. World Chin J Digestol 2005;13:2332-2338.
32. Masaoka T, Vanuytsel T, Kindt C, et al. A spontaneous animal model of intestinal dysmotility evoked by inflammatory nitrergic dysfunction. PLoS One 2014;9:e95879.
33. Li Q, Winston JH, Sarna SK. Developmental origins of colon smooth muscle dysfunction in IBS-like rats. Am J Physiol Gastrointest Liver Physiol 2013;305:G503-G512.
34. Larauche M, Gourcerol G, Wang L, et al. Cortagine, a CRF_1 agonist, induces stress like alterations of colonic function and visceral hypersensitivity in rodents primarily through peripheral pathways. Am J Physiol Gastrointest Liver Physiol 2009;297:G215-G227.
35. Yuan PQ, Million M, Wu SV, Rivier J, Taché Y. Peripheral corticotropin releasing factor (CRF) and a novel CRF_1 receptor agonist, stressin1-A activate CRF_1 receptor expressing cholinergic and nitrergic myenteric neurons selectively in the colon of conscious rats. Neurogastroenterol Motil 2007;19:923-936.
36. De Giorgio R, Stanghellini V, Barbara G, et al. Primary enteric neuropathies underlying gastrointestinal motor dysfunction. Scand J Gastroenterol 2000;35:114-122.
37. Veress B, Nyberg B, Törnblom H, Lindberg G. Intestinal lymphocytic epithelioganglionitis: a unique combination of inflammation in bowel dysmotility: a histopathological and immunohistochemical analysis of 28 cases. Histopathology 2009;54:539-549.
38. Zhao C, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. Cell 2008;132:645-660.
39. Liu MT, Kuan YH, Wang J, Hen R, Gershon MD. 5-HT_4 receptor-mediated neuroprotection and neurogenesis in the enteric nervous system

of adult mice. J Neurosci 2009;29:9683-9699.
40. Wood JD. Enteric nervous system neuropathy: repair and restoration. Curr Opin Gastroenterol 2011;27:106-111.
41. Takaki M, Goto K, Kawahara I. The 5-hydroxytryptamine 4 receptor agonist-induced actions and enteric neurogenesis in the gut. J Neurogastroenterol Motil 2014;20:17-30.