NEURAL REGENERATION RESEARCH
Volume 7, Issue 27, September 2012
www.nrronline.org

doi:10.3969/j.issn.1673-5374.2012.27.008 [http://www.crter.org/nrr-2012-qkquanwen.html]
Xu XL, Li Q, Zhou L, Ru LQ. Neurochemical mechanism of the gastrointestinal interdigestive migrating motor complex in rats with acute inflammatory stomach ache. Neural Regen Res. 2012;7(27):2136-2143.

Neurochemical mechanism of the gastrointestinal interdigestive migrating motor complex in rats with acute inflammatory stomach ache

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Abstract
The normal gastrointestinal interdigestive migrating motor complex cycle was interrupted, and paroxysmal contraction appeared after formaldehyde-induced stomach ache. Activities of nitric oxide synthase, acetylcholinesterase and vasoactive intestinal peptide neurons were significantly reduced, whereas activities of calcitonin gene-related peptide neurons were significantly increased in the pyloric sphincter muscular layer, myenteric nerve plexus and submucous nerve plexus. Electroacupuncture at *Zusanli* (ST36) suppressed paroxysmal contraction in rats with formaldehyde-induced stomach ache, and neurons in the enteric nervous system were normal. These results indicated that nitrergic neurons, cholinergic neurons, vasoactive intestinal peptide neurons and calcitonin gene-related peptide neurons in the enteric nervous system may be involved in changes to the gastrointestinal interdigestive migrating motor complex following stomach ache, and that electroacupuncture can regulate this process.

Key Words
pyloric sphincter; inflammatory pain; interdigestive migrating motor complex; enteric nervous system; electroacupuncture; neural regeneration

Research Highlights
Nitrergic, cholinergic, vasoactive intestinal peptidergic and calcitonin gene-related peptide neurons participate in changes to the gastrointestinal interdigestive migrating motor complex following chemical inflammatory pain. Electroacupuncture has a regulatory effect on these changes.

Abbreviations
NOS, nitric oxide synthase; AChE, acetylcholinesterase; VIP, vasoactive intestinal peptide; CGRP, calcitonin gene-related peptide; MMC, migrating motor complex

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Received: 2012-05-11
Accepted: 2012-07-10
(N20111102002/WLM)

INTRODUCTION

The migrating motor complex (MMC) in the interdigestive phase is a main form of fasting gastrointestinal motility. MMC can be promoted by gastrointestinal myoelectric activity that is initiated by interstitial cells of Cajal between the gastrointestinal longitudinal muscle and circular smooth muscle[1]. MMC is regulated by interneurons of the myenteric plexus in the enteric nervous system[2-5]. A previous study has confirmed that a small amount of

formaldehyde-induced gastric mucosal lesion can induce c-fos expression in the nociceptive information conduction pathway[6]. When the digestive tract was affected by a nociceptive stimulus, pain was induced following activation of the enteric nervous system, peripheral sympathetic nerve, parasympathetic nerve and non-adrenergic non-cholinergic nerve, resulting in changes in intestinal motility and enteric nervous system function[7]. However, there is a lack of evidence linking function to cell morphology.

The pyloric sphincter can regulate and control gastrointestinal motility. Electroacupuncture can regulate gastrointestinal mobility disorder[8]. This study sought to observe gastrointestinal MMC changes, as well as changes in nitrergic, cholinergic, vasoactive intestinal peptide, and calcitonin gene-related peptide neurons in the pyloric sphincter following exposure to acute gastric mucosal lesions, particularly focusing on changes to function and morphology. In addition, we aimed to investigate the regulatory effects of electroacupuncture.

RESULTS

Quantitative analysis of experimental animals

A total of 72 rats were assigned to a normal control group (saline by intragastric administration), model group (formaldehyde by intragastric administration), electroacupuncture control group (electroacupuncture stimulus), and electroacupuncture treatment group (formaldehyde + electroacupuncture). A total of 72 rats were included in the final analysis.

Effects of formaldehyde-induced stomach ache on pain threshold in rats

After intragastric administration of formaldehyde, rats experienced restlessness, rapid breathing, embracing of the abdomen, back arching, and less activity. Electrical measurement methods displayed that the pain threshold of normal rats (7.63 ± 1.85 mA) was diminished at 30 minutes following formaldehyde administration, reached a minimum (3.86 ± 0.8 mA) at 1 hour, which lasted for 2 hours. From then on, the pain threshold gradually recovered. Thermal measurement methods obtained similar results as electrical measurement methods (Table 1). Pain was relieved at 1–2 hours following formaldehyde administration.

MMC characteristics in the rat gastric antrum and duodenum

Gastrointestinal motility detection displayed that the MMC cycle in the rat gastric antrum was 17.52 ± 5.82 minutes, with significant time phase characteristics. In phase I, there was no contraction wave, and time duration was 5.41 ± 1.07 minutes. In phase II, there was an intermittent contraction wave, and the time duration was 7.13 ± 2.81 minutes, with a contraction frequency of 1.64 ± 0.41 times/minute and a contraction amplitude of 119.59 ± 15.83 g. In phase III, there were intense successive contractions, and the time duration was 3.18 ± 0.75 minutes, with a contraction frequency of 3.80 ± 0.40 times/minute and a contraction amplitude of 201.45 ± 61.38 g. Phase IV was the transition period of phase III and phase I, and the characteristics were identical to that in phase II; however the time duration was 1.80 ± 0.40 minutes, with a contraction frequency of 1.36 ± 0.33 times/minute and a contraction amplitude of 28.77 ± 23.84 g. The MMC cycle in the rat gastric antrum could migrate to duodenum.

The MMC cycle in the small intestine was 19.36 ± 5.21 minutes. In phase I, there was no contraction wave, and time duration was 6.20 ± 0.89 minutes. In phase II, there was an intermittent contraction wave, and the time duration was 7.88 ± 1.93 minutes, with a contraction frequency of 1.83 ± 0.42 times/minute and a contraction amplitude of 39.59 ± 5.63 g. In phase III, there were intense successive contractions, and the time duration was 3.76 ± 0.75 minutes, with a contraction frequency of 38.86 ± 1.40 times/minute and a contraction amplitude of 67.34 ± 6.14 g. Phase IV was the transition period of phase III and phase I, and the characteristics were identical to that in phase II; however, the time duration was 1.52 ± 0.40 minutes (Figure 1).

Table 1 Changes in pain thresholds in rats with formaldehyde-induced acute inflammatory pain

Time of intragastric administration (minute)	Pain threshold by electrical measurement method (mA)	Pain threshold by thermal measurement method (second)
0	7.63±1.85	0.52±0.07
15	6.47±1.98	0.51±0.02
30	5.31±1.70[a]	0.53±0.01
45	5.16±1.50[a]	0.43±0.01[a]
60	3.86±0.89[a]	0.33±0.05[a]
75	3.89±0.86[a]	0.34±0.06[a]
90	4.58±1.04[a]	0.33±0.04[a]
105	5.23±1.50[a]	0.38±0.06[a]
120	5.43±1.90[a]	0.41±0.08[a]
135	6.30±1.80	0.48±0.02
150	6.90±0.20	0.51±0.01

Results are expressed as mean ± SEM. Twenty-four rats for each method; measured three times at each time point. [a]$P < 0.05$, *vs.* 0 minute intragastric administration group (normal control) (one-way analysis of variance).

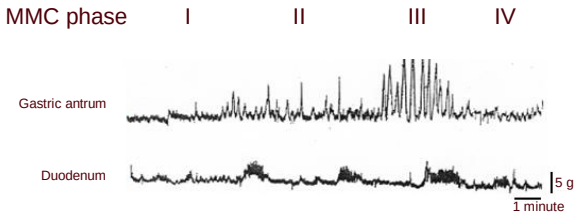

Figure 1 Interdigestive migrating motor complex (MMC) from normal conscious control rats.

The rat gastric MMC cycle shows significant phase characteristics; phase I with no contraction wave; phase II with intermittent contraction wave; phase III appears to have a strong continuous contraction; phase IV is a transition period. The intestinal MMC cycle amplitude is relatively small.

Effects of formaldehyde on the gastric antrum and duodenum MMC, and the regulatory effects of electroacupuncture

Formaldehyde was given after three normal MMCs were recorded. We recorded an interrupted gastric MMC cycle, paroxysmal contraction, and increased wave amplitude, at a contraction frequency of 4.64 ± 0.72 times/minute and a contraction amplitude of 95.93 ± 40.64 g. The duodenum MMC disappeared, and an incomplete tetanus wave appeared. Electroacupuncture at *Zusanli* (ST36) suppressed the paroxysmal contraction wave induced by formaldehyde, showing sustained low-amplitude activity. The paroxysmal contraction wave appeared at 1 hour following needle withdrawal (Figure 2).

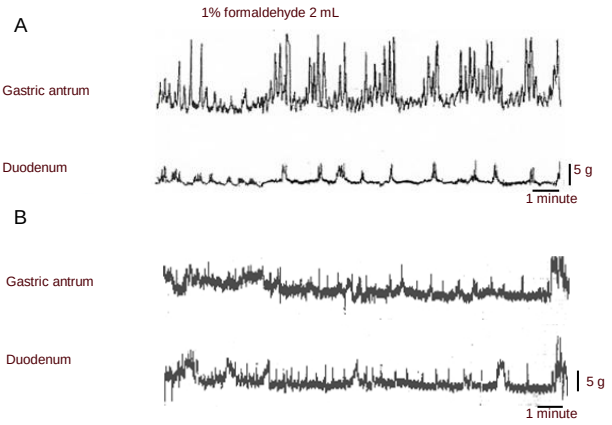

Figure 2 Effect of formaldehyde and treatment with electroacupuncture on the interdigestive migrating motor complex (MMC) in conscious rats.

(A) Effect of formaldehyde on the gastric antrum and duodenum MMC; the MMC cycles were interrupted, loss of phase nature.

(B) Treatment of electroacupuncture on the MMC. Electroacupuncture suppressed the paroxysmal contraction wave, showing persistence of low-amplitude activity.

Distribution of nitric oxide synthase (NOS)-, acetylcholinesterase (AChE)-, vasoactive intestinal peptide (VIP)- and calcitonin gene-related peptide (CGRP)-positive neurons in the rat pyloric sphincter, and electroacupuncture intervention

Histological detection revealed that the sphincter was surrounded by circular muscle bundles where there were four to seven NOS-positive nerve plexuses and one to five NOS-positive perikarya in each plexus. In thickened circular muscle bundles, there were abundant NOS-positive perikarya, 5–12 perikarya in each plexus, and strong enzymatic activity (+++). Abundant NOS-positive fibers and moderate enzymatic activity (++) was visible in the muscular layer, but only a few NOS-positive fibers were found in the lamina propria and mucosa. AChE-positive ganglia (or nerve plexuses) of unequal size and number were observed in the muscularis propria. AChE-positive perikarya were observed, and enzymatic activity was uneven and mainly moderate (++). In the thickened muscular layer, we found five to six plexuses and each plexus had at least 10 cells with a high enzymatic activity (++ – +++). The distribution of AChE-positive fibers was similar to that of NOS. In the sphincter, the number of VIP-immunoreactive nerve fibers was more than CGRP-immunoreactive fibers. In the thickened site of the sphincter, VIP-immunoreactive nerve fibers formed a dense fasciculate structure, whereas CGRP-immunoreactive fibers were scattered (Figures 3–6).

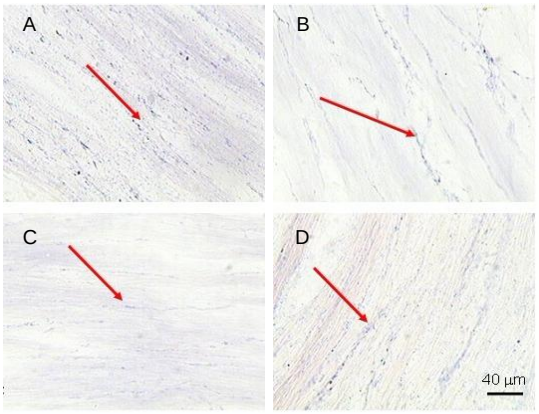

Figure 3 Effect of electroacupuncture on nitric oxide synthase (NOS)-positive neurons in rats with gastric inflammatory pain (NADPH staining, light microscope; scale bar: 40 μm). Arrows exhibit positive neurons.

Compared with the normal control group (A) and electroacupuncture control group (C), the number of NOS-positive perikarya and enzymatic activity were lower in the submucosal plexus and myenteric nerve plexus of the sphincter in the model group (B). Above-mentioned data in the electroacupuncture treatment group (D) were near to the normal control group.

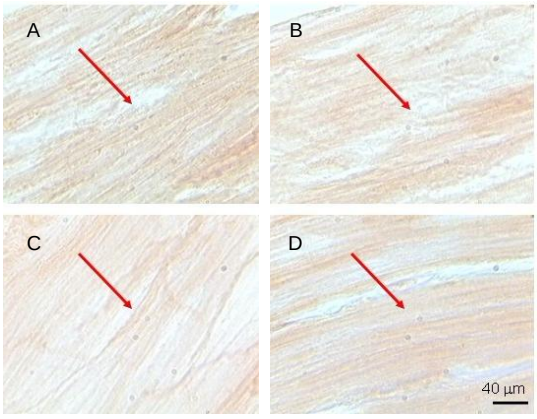

Figure 4 Effect of electroacupuncture on acetylcholinesterase (AChE)-positive neurons in rats with gastric inflammatory pain (NADPH staining, light microscope; scale bar: 40 μm). Arrows exhibit positive neurons.

Compared with the normal control group (A) and electroacupuncture control group (C), the number of AChE-positive perikarya and enzymatic activity were lower in the submucosal plexus and myenteric nerve plexus of the sphincter in the model group (B). Above-mentioned data in the electroacupuncture treatment group (D) were near to the normal control group.

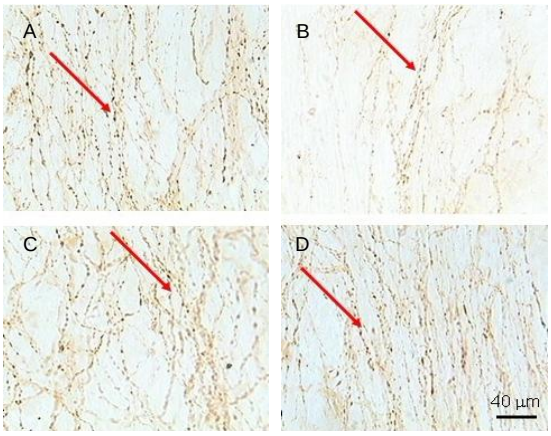

Figure 5 Effect of electroacupuncture on vasoactive intestinal peptide (VIP)-positive neurons in rats with gastric inflammatory pain (NADPH staining, light microscope; scale bar: 40 μm). Arrows exhibit positive neurons.

The number of VIP-positive nerve fibers was significantly reduced in the model group (B), with the presence of low distribution density, weak immunoreactivity, lack of fine reticular fiber structure, and no positive fibers in the lamina propria. The number of positive nerve fibers was increased in the electroacupuncture treatment group (D), and close to the normal control group (A) and electroacupuncture control group (C).

Statistical results displayed that viabilities of NOS-, AChE-positive and VIP-immunoreactive neurons were decreased ($P < 0.01$), but the number of CGRP-immunoreactive nerve fibers was increased ($P < 0.05$) following formaldehyde-induced inflammatory pain. After electroacupuncture at *Zusanli*, the viabilities of NOS-positive, AChE-positive, VIP-immunoreactive and CGRP-immunoreactive neurons were normal in the rat pyloric sphincter (Table 2).

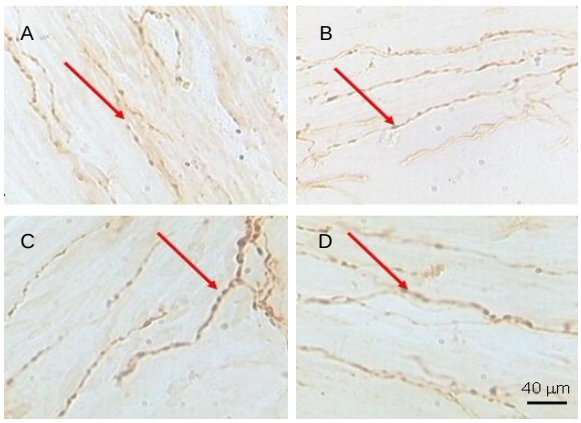

Figure 6 Effect of electroacupuncture on calcitonin gene-related peptide (CGRP)-positive neurons in rats with gastric inflammatory pain (NADPH staining, light microscope; scale bar: 40 μm). Arrows exhibit positive neurons.

CGRP-positive crude fibers were visible in the sphincter of the normal control group (A) and electroacupuncture control group (C). The number of CGRP-positive nerve fibers was obviously increased in the model group (B), showing strong immunoreactivity. The number of positive nerve fibers was decreased in the electroacupuncture treatment group (D).

Table 2 Changes in nitric oxide synthase (NOS) (absorbance), acetylcholinesterase (AChE) (absorbance), vasoactive intestinal peptide (VIP) (absorbance), calcitonin gene-related peptide (CGRP) activities (absorbance) in the pyloric sphincter of formaldehyde-treated rats

Group	NOS	AChE
Normal control	0.094±0.029	0.083±0.011
Model	0.045±0.011[b]	0.042±0.010[b]
Electroacupuncture control	0.091±0.016	0.079±0.022
Electroacupuncture treatment	0.102±0.021[b]	0.089±0.024[b]

Group	VIP	CGRP
Normal control	0.163±0.038	0.101±0.014
Model	0.104±0.014[b]	0.156±0.013[a]
Electroacupuncture control	0.141±0.008	0.116±0.015
Electroacupuncture treatment	0.142±0.010[b]	0.108±0.013[a]

Results are expressed as mean ± SEM. Ten rats in each group. [a]$P < 0.05$, [b]$P < 0.01$, *vs*. normal control group (one-way analysis of variance).

DISCUSSION

This study established a model of visceral nociception

using direct intragastric administration of formaldehyde. In order to avoid the effects of anesthetics on pain threshold and neurotransmitter release, we did not use anesthetics. To identify the effective time course of the animal model, we measured the change in visceral pain threshold using electric/thermal pain measurement methods. In accordance with the theory of somatic-visceral pain afference gathering in the spinal dorsal horn[9], we observed the effects of stomach ache using the measurement method of somatic pain, which can reflect the change in pain threshold. Results showed that the pain threshold decreased at 1 hour following formaldehyde-induced acute injury to the gastric mucosa, and the level of pain changed greatly at 1–2 hours. Therefore, this study observed the correlation of gastrointestinal motility changes and neurochemical changes at 1–2 hours after gastric mucosal injury.

The gastrointestinal MMC removes food debris, increases motor coordination, stimulates pancreatic juices and bile secretion and clears the intestines. The MMC is strongly associated with gastrointestinal diseases. Gastroparesis and functional dyspepsia can prolong the MMC cycle and induce contraction deletion of phase III of the MMC cycle. This study recorded the typical MMC activity of the rat stomach and intestine when fasting, and showed stable results and characteristic phases. After formaldehyde administration, a diffuse inflammatory reaction (bleeding point) appeared in the gastric mucosa, and pain reaction (pain threshold) was decreased. The above-mentioned changes peaked at 1–2 hours. Simultaneously, the normal MMC cycle was interrupted and paroxysmal contraction appeared after formaldehyde-induced stomachache.

Formaldehyde-induced gastritis and pain are correlated with gastrointestinal MMC disorder. However, the mechanism of MMC dysfunction remains unclear. The enteric nervous system is embedded in all layers of the pyloric sphincter and adjacent gastrointestinal wall. Thus, we hypothesized that the MMC is regulated by the nerves and its transmitters or gut hormones. In particular, the enteric nervous system most likely mediates sphincter activity and adjusts the MMC. Results from this study verified that the gastrointestinal MMC was disordered after formaldehyde-induced stomach ache, and simultaneously, innervations were also disordered, showing that NOS, AChE and VIP activities were significantly reduced in the sphincter and gastrointestinal wall, whereas CGRP activity was increased. These results indicate that the changes in neurotransmitters in the enteric nervous system may be due to a neurochemical-mediated MMC disorder.

Cholinergic nerve release of ACh stimulates contraction of the gastrointestinal muscle (including the pyloric sphincter) and promotes gastrointestinal motility. The decrease in AChE activity most likely represents an increase in ACh activity. Thus, it is inferred that sphincter contraction and regulation of the MMC is ACh-mediated.

Nitric oxide, an inhibitory transmitter, is mainly produced or released by myenteric nerve plexus neurons and results in the regulation of gastrointestinal MMC motility. Nitric oxide can induce dilatation of the pyloric sphincter and affect gastric emptying. The decrease in NOS activity observed was most likely due to the increased synthesis or release of nitric oxide by nitroxidergic neurons. The distribution of NOS-positive nerve fibers in the stomach and intestine was obviously different. Distribution of NOS-positive neurons in the sphincter was altered when inflammatory pain appeared. This result suggests that inherent nitroxidergic neuron disorders directly affect the closing and opening function of the sphincter and pathophysiological functions of gastrointestinal motility disorders[8].

VIP acts as a peptidergic neurotransmitter capable of directly inducing gastrointestinal smooth-muscle relaxation, and mediating relaxation of the lower esophageal and internal anal sphincter.

VIP-immunoreactive neurons in the enteric nervous system most likely mediated the closing and opening function of the pyloric sphincter, affected gastric emptying, and the regulated MMC[10]. VIP and NOS expression was significantly diminished in the gastric mucosa after ethanol-induced gastric mucosal injury. VIP can promote nitric oxide release. Therefore, the decrease of VIP damages the gastric mucosa by decreasing nitric oxide release and increasing endothelin-1 levels following ethanol-induced gastric mucosal injury or formaldehyde-induced stomach ache[10].

CGRP-regulated gastrointestinal tract function may be associated with gastrointestinal motion and sensory function. CGRP has complicated effects on gastrointestinal smooth muscle function. Results from the present study demonstrated thick CGRP fibers in the sphincter and abundant positive fiber networks in the thickened sphincter. These results indicate that the relaxation and contraction of the sphincter may be associated with CGRP in the enteric nervous system.

In summary, some neurotransmitters in the enteric nervous system affected the closing and opening function of the sphincter and gastrointestinal motility, directly regulated the MMC, and participated in the neurological pathophysiology of gastrointestinal motility disorders following acute chemical inflammatory pain.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

Experiments were performed at the School of Medicine, Jianghan University, Laboratory of Neurobiology, Tongji Medical College, Huazhong University of Science and Technology, Laboratory of Physiology, Institute of Basic Medical Science, and the Chinese Academy of Medical Science, China in October 2008.

Materials

A total of 64 healthy, clean, Sprague-Dawley rats aged 3–6 months and weighing about 200 g, of both genders, were supplied by the Department of Experimental Zoology, Tongji Medical College, Huazhong University of Science and Technology, China (license No. SYXK (E) 2008-0045). All rats were housed at 20 ± 2°C under subdued light. An additional eight healthy, clean, Sprague-Dawley rats aged 3–6 months and weighing 180–220 g, of both genders were supplied by the Experimental Animal Center, Chinese Academy of Medical Sciences (license No. SCXK (Jing) 2004-0001). All protocols were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[11].

Methods

Measurement of pain threshold in rats with formaldehyde-induced visceral pain

A total of 24 conscious rats were intragastrically administered 2 mL of 1% (v/v) formaldehyde after 12-hour fasting. Fifteen minutes later, pain threshold (the electric current when the animal responded to the stimulus) was measured using the WQ-9E pain threshold detector (Beijing Haidian Electronic Medical Instruments, Beijing, China). Pain threshold (duration of leg-raising) was measured using a thermal pain threshold detector (Fourth Military Medical University, China). A measurement was recorded every 15 minutes for 3 hours. The pain threshold measured before formaldehyde administration was considered as a baseline.

Establishment of animal models of formaldehyde-induced visceral pain and electroacupuncture intervention

A total of 40 rats were equally and randomly assigned to four groups. After fasting for 18–24 hours, the conscious rats were intragastrically administered 2 mL saline in the normal control group. The rats in the model group were intragastrically administered 2 mL of 1% (v/v) formaldehyde[6]. The rats in the electroacupuncture control group were intragastrically administered saline. Electroacupuncture at *Zusanli* was conducted for 50 minutes, with a frequency of 4–16 Hz, using an electroacupuncture apparatus (Shanghai Medical Electronic Apparatus, Shanghai, China). The pulsed voltage ranged from 1 V to 5 V, and was increased by 1 V progressively every 10 minutes[8]. In the electroacupuncture treatment group, electroacupuncture at *Zusanli* was conducted as that in the electroacupuncture control group after formaldehyde treatment. Perfusion and sample collection were performed after experimental procedures in each rat were completed.

Gastrointestinal motility recording

Eight rats provided by the Experimental Animal Center, Chinese Academy of Medical Sciences, were allowed free access to water after 18–24 hours of fasting. The rats were intraperitoneally anesthetized with chloral hydrate (10% (v/v), 300 mg/kg), and a strain gauge was used[12]. The manufacture of the strain gauge (Department of Physiology, Institute of Basic Medical Science, Chinese Academy of Medical Science) is as follows: a 5 mm × 4 mm gauge[12] was consolidated by a copper sheet. A guideline was welded at the output site. The specimen was embedded using silica gel. The strain gauge was separately embedded into the gastric antrum (5 mm from the pylorus) and duodenum (2 cm from the pylorus) subserosa. The guideline was subcutaneously fixed on the neck and back. At 5–7 days following surgery, the strain gauge was connected to the X-Y balance recorder (Sichuan Instrument Factory, Sichuan, China) through an electric bridge[12]. Gastrointestinal motility was recorded when the rats were conscious. Three consecutive normal MMCs were recorded first. 2 mL of 1% (v/v) formaldehyde was given intragastrically, and then the gastrointestinal MMC was recorded dynamically for 1 hour. After perfusion, the materials were collected. In the electroacupuncture treatment group, at least three consecutive normal MMCs were recorded first. 2 mL of 1% (v/v) formaldehyde was given intragastrically, and simultaneously electroacupuncture at *Zusanli* was conducted. Gastrointestinal MMC was recorded dynamically for 1 hour. After perfusion, the materials were collected. Stimulus intensity and duration were the same as above.

Preparation of animal specimen

Rats provided by the Experimental Animal Center,

Chinese Academy of Medical Sciences, from the experimental and control groups were intraperitoneally anesthetized with 10% (v/v) chloral hydrate (300 mg/kg). After the chest was opened, the heart was exposed and perfused with about 100 mL of saline (37°C) through the ascending aorta. The blood was rapidly removed by washing. The heart was fixed with 400 mL of 4% (w/v) Millioning's paraformaldehyde (4°C) buffer (pH 7.2). Stomach-pylorus-duodenum specimens were fixed in the same fixative (enzyme stained preparation for 6 hours; peptide stained preparation for 48 hours), and then sliced into full-thickness sections or cryostat sections after the specimens were immersed in 20% (w/v) sucrose buffer (pH 7.2) at 4°C overnight. Horizontal or longitudinal sections (25 μm-thick) were made for enzyme histochemical staining or immunohistochemical staining.

Enzyme histochemical staining

In accordance with a previous method for histochemical staining of NOS[13], sections were incubated in a mixture of 0.25 mM nitroblue tetrazolium (Sigma, St. Louis, MO, USA), 1 mM nicotinamide-adenine dinucleotide phosphate (reduced form; Sigma), 0.25% (v/v) Triton X-100, and 0.1 M Tris-HCl buffer (pH 7.6) at 37°C for 30 minutes. After washing in PBS (0.01 M, pH 7.3), the sections were dehydrated, permeabilized and mounted.

In accordance with a previous method for histochemical staining of AChE[14], sections were incubated in a mixture of 12.5 mg acetylthiocholine iodide (Shanghai Dongfeng Reagent Factory, Shanghai, China), 0.06 M sodium acetate, 0.1 M acetic acid, 0.1 M sodium citrate, 30 mM copper sulphate, 5 mM potassium ferricyanide, and 4 mM tetraisopropyl pyrophosphoramide at 37°C for 1.5 hours. After washing in PBS, the sections were dehydrated, permeabilized and mounted.

Immunohistochemical staining

Following a PBS wash, sections were incubated in methanol and H_2O_2 at 37°C for 1 hour, in 0.25% (v/v) Triton X-100 at 37°C for 1 hour, and 10% (v/v) calf serum at 37°C for 1 hour. Sections were then incubated with rabbit anti-rat VIP monoclonal antibody (1:4 000; Peninsula Lab, Torrance, CA, USA), rabbit anti-rat CGRP monoclonal antibody (1:6 000; Paesel+Lorei GmbH & Co., Hanau, Germany) at 37°C for 2 hours and 4°C for 48 hours, respectively, and incubated with goat anti-rabbit IgG (1:200; Peninsula Lab) at 37°C for 1 hour, and non-labeled antibody peroxidase-antioxidant enzyme complex (1:400; Peninsula Lab) at 37°C for 1 hour. Sections were developed with 0.05% (w/v) diaminobenzidine-0.01% (v/v) H_2O_2-Tris-HCl buffer for 3–4 minutes. Three washes in PBS were performed between each step, each for 5 minutes. The sections were placed on a slide, dehydrated, permeabilized and mounted.

Imaging analysis

Five sections were randomly selected from each rat and observed under a light microscope (10 × 20 magnification; 3–5 visual fields; Olympus, Tokyo, Japan). Positive results were expressed as "+", with four levels of immunoreactivity. The HPIAS-1000 high definition color pathology image analysis system (Qianping Yingxiang, Tongji Medical College, Huazhong University of Science and Technology, China) was used to measure the viability of positive neurons in the stomach-pyloric sphincter-intestine specimens. The average value was obtained and expressed as an absorbance.

Statistical analysis

All data were expressed as mean ± SEM, and analyzed with SPSS 12.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance was used. A value of $P < 0.05$ was considered statistically significant.

Author contributions: Xiaoli Xu and Lv Zhou participated in the study design and concept. All authors performed experiments. Xiaoli Xu was in charge of data analysis and manuscript writing. Lv Zhou was responsible for manuscript authorization.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee, Tongji Medical College, Huazhong University of Science and Technology, China.

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(Edited by Wang HL, Qi JP/Qiu Y/Wang L)