neurogastroenterology & motility

Neurogastroenterol Motil (2009) **21,** 1222–e113 doi: 10.1111/j.1365-2982.2009.01335.x

5-HT_3 receptors mediate the time-dependent vagal afferent modulation of nociception during chronic food allergen-sensitized visceral hyperalgesia in rats

S. CHEN,*[1] J. LI,†[1] L. ZHANG,* X. DONG,* W. GAO,* J. MO,* H. CHEN,† S. XIAO* & Y. LI‡

*Department of Gastroenterology, Shanghai Institute of Digestive Disease, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China

†Department of Pharmacology, Institute of Medical Sciences, Shanghai Jiaotong University School of Medicine, Shanghai, China

‡Gastroenterology Research Unit, Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA

Abstract *Converging lines of evidence demonstrate a vagally mediated antinociceptive pathway in animals undergoing acute visceral insults, the contribution of this system to visceral pain following chronic noxious stimuli is unknown. 5-HT_3 receptor (5-HT_3Rs) on spinal afferents are crucially involved in nociceptive processing, the role of 5-HT_3Rs on vagal afferents is unclear. The aim of the present study was to determine the contribution of vagal afferents to visceral nociception in rats undergoing chronic luminal allergen stimulation and whether it involves vagal 5-HT_3Rs. Sensitized rats received chicken egg albumin (EA, 1 mg mL^{-1}) in drinking water for 2 weeks (day 1–14). Visceromotor response (VMR) to colorectal distension [colorectal distension (CRD), 60 mmHg] and the levels of mRNA encoding 5-HT_3R (including 3A and 3B subunits) in the nodose ganglia (NG) were evaluated on day 2, 4, 8 and 15. Chronic EA challenge induced gradually increased visceral nociception, with a peak on day 15. Subdiaphragmatic vagotomy or functional deafferentation with capsaicin abolished this time-dependent manner, inducing hyperalgesia from day 2, lasting to day 15. Intraluminal infusion of a 5-HT_3R antagonist (granisetron), whether alone or infused after local mucosa anaesthetic with 1% lidocaine, mimicked the effects of vagotomy. The mRNA levels for 5-HT_{3B} or 5-HT_{3A} subunit in the NG showed an opposite time-course to that of visceral pain, which increased from day 2, then decreased gradually to levels lower than those of controls. Our results demonstrate a time-dependent vagal afferent modulation of chronic allergen-sensitized visceral hyperalgesia, which may involve a 5-HT_3R pathway.*

Keywords *5-HT_3 receptors, food allergen, vagal afferents, visceral hyperalgesia.*

INTRODUCTION

Irritable bowel syndrome (IBS) is a common gastrointestinal (GI) disorder characterized by recurrent abdominal pain and discomfort associated with altered bowel habits, with visceral hypersensitivity or hyperalgesia as a pathophysiological hallmark. Several interacting mechanisms, including disturbance of brain–gut interaction, psychosocial factors, inflammation and food antigen-evoked allergic response in the gut, have been stated to underlie the mechanisms for visceral hyperalgesia.[1–3]

Vagal afferents are extensively distributed in the gut and involved in not only regulation of GI secretion and motility, but also signalling or modulation of visceral perception associated with IBS.[4,5] Experimental studies have suggested a vagal modulation of visceral pain in rats subjected to acute stressful or mechanical insults.[6–8] However, the contribution of this vagally mediated antinociceptive pathway to visceral sensation during chronic noxious stimuli has not been established. Clinical data demonstrate vagal dysfunction in a subset of IBS patients, and that IBS symptoms such as abdominal pain or discomfort correlate with reduced vagal tone,[9–11] indicating that the functional state of

Address for correspondence
Dr Shengliang Chen, Department of Gastroenterology, Shanghai Institute of Digestive Disease, Renji Hospital, Shanghai Jiaotong University School of Medicine, 145 Middle Shandong Road, Shanghai 200001, China.
Tel.: +86 21 63200784; fax: +86 21 63266027;
e-mail: chenslmd@yahoo.com.cn
[1]Both authors contributed equally to this article.
Received: 16 December 2008
Accepted for publication: 16 April 2009

the vagus nerve under chronic pathologic stimuli might be different from that in response to acute insults.

Luminal chemical or mechanical stimuli evokes 5-hydroxytryptamine (5-HT) release from mucosal enterochromaffin cells and immune cells.[12,13] Through activating the specific receptor subtypes on the extrinsic or intrinsic neurons, 5-HT is crucially involved in motor and secretory reflexes in the gut, and the regulation of nociceptive inputs to brain.[14] It is well documented that 5-HT$_3$ receptors (5-HT$_3$Rs) on spinal afferents mediate visceral nociceptive processing,[6] and 5-HT$_3$R antagonists can effectively alleviate abdominal pain in male and female IBS patients with diarrhoea.[15,16] 5-HT$_3$Rs are also expressed on vagal afferents innervating the gut.[17] Their possible role in visceral nociception has not been fully elucidated.

Exposure of sensitized intestine to chicken egg albumin (EA), which can evoke a mast cell and IgE-dependent mucosal immunologic response,[18] has been frequently used to experimentally induce visceral hypersensitivity.[19,20] In the present study, using the chronic EA-challenged sensitized rats, we aimed to determine the contribution of vagal afferent pathway to chronic food allergen-induced visceral hyperalgesia and whether it involves vagal 5-HT$_3$Rs.

MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley rats (275–300 g) were maintained on a normal light–dark cycle, housed in pairs or singly after surgery and given access to food and water *ad libitum*. All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and animal care and use guidelines of Shanghai Jiaotong University School of Medicine.

Surgical procedures

Implantation of electrodes Rats were anesthetized with 45 mg kg^{-1} sodium pentobarbital (Sigma, St Louis, MO, USA) administered intraperitoneally (i.p.). The electrodes (Teflon®-coated stainless steel wire, AstraZeneca, Mölndal, Sweden) were stitched into the external oblique musculature, just superior to the inguinal ligament. The lead wires were then tunnelled subcutaneously and exteriorized at the back of the neck. During the experiment, the electrodes were connected via a shielded cable to a chart recorder (PowerLab /8SP, ADInstruments, Bella Vista, NSW, Australia) to monitor the number of abdominal muscle contractions. Following surgery, rats were allowed to recover for 5–7 days.

Subdiaphragmatic vagotomy This procedure was performed according to the previous descriptions.[8] Under sodium pentobarbital anaesthesia, a midline incision was performed to open the abdomen. The distal 3 cm of the oesophagus were stripped of the external muscle coat, this results in a subdiaphragmatic vagotomy. In addition, to ensure the completeness of vagotomy, the left gastric artery was isolated and stripped of all connective tissue and nerves, which includes the accessory branch of the vagus. Some rats underwent a sham operation (opening of the abdomen and manipulation of the stomach and oesophagus). After surgery, rats were allowed to recover for 7–10 days.

Vagal afferent denervation (perivagal capsaicin treatment) This procedure is described in detail elsewhere.[6,8] Rats were anesthetized with sodium pentobarbital, the cervical vagi were exposed and the vagal trunks freed from the surrounding tissue. Cotton wool soaked in capsaicin (1 mg mL^{-1}, Sigma) was placed around the nerve trunk for 30 min after the area was thoroughly rinsed with physiological saline (saline). Animals in which the vagi were treated with vehicle (10% Tween 80 in olive oil) served as vehicle controls. Rats were used in experiments 10–14 days after treatment.

Experimental protocol

Assessment of visceromotor response (VMR) to colorectal distension (CRD) The visceral stimulus employed was CRD using a well-established and validated method for the evaluation and quantification of visceral pain responses.[21] After a fasting period of 18–24 h, rats were lightly anaesthetized with ketamine (150 mg kg^{-1}, i.p., Sigma) in combination with ether. A flexible latex balloon (6 cm) was inserted into the descending colon and rectum such that its end was 1 cm proximal to the anus, with a catheter fixed at the tail. CRD was produced by rapidly injecting saline into the colonic balloon. Pressure was regulated with a distention control device and monitored using a pressure transducer.[7,19] Once recovered from anaesthesia, the VMR to CRD was quantified by measuring the number of abdominal muscle contractions, which were recorded 20 s before (baseline), 20 s during, and 20 s after termination of CRD. Spike bursts higher than 0.3 mV were regarded as significant and therefore used to estimate the pain response. It was found that the pressure of 60 mmHg elicited obvious and stable VMR in our preliminary studies. So similar to the previous study,[8] we adopted this pressure in our VMR study.

Chronic EA challenge The sensitization and chronic allergen challenge to the rats were performed according to the previous descriptions.[18–20] The rats were sensitized by i.p. injection of 1 mL saline containing 10 μg EA (Sigma) as the allergen and 10 mg aluminium hydroxide as the adjuvant. Seven days later, sensitized rats, which had serum IgE titres of ≥1 : 64, were challenged by adding 1 mg mL^{-1} EA to their drinking water and allowed rat chow *ad libitum* for 2 weeks. Control groups included non-sensitized rats receiving water (Control), sensitized unchallenged and non-sensitized challenged rats. Control and non-sensitized challenged groups were sham sensitized with saline. Sterilized drinking water, including that containing EA, was changed daily after feeding devices were washed and sterilized. CRD experiments were performed on day 2, 4, 8 and/or 15.

In the preliminary experiment, we found that sensitized rats receiving water or non-sensitized animals receiving EA exhibited similar visceral pain responses compared with those of controls, indicating that the sensitization procedure we took or dietary EA challenge has no remarkable influence on pain behaviour. So, in the following CRD experiments, we used the non-sensitized rats receiving water as the control group.

At the end of the study, histological examinations at light microscope demonstrated no overt signs of inflammation in the intestinal mucosa (data not shown).

Measurement of mucosal mast cell numbers and 5-HT content In another set of experiment, some animals in the four groups were sacrificed by intravenous injection of sodium pentobarbital on day 2, 4, 8 or 15, a segment of proximal jejunum was removed for determination of mast cell numbers or 5-HT content. For mast cell number measurement, jejunal tissue was fixed and stained with 0.5% toluidine blue for 30 min. Mast cells were counted in 10 crypt-villus units for each animal at light microscope. For 5-HT content determination, the mucosa was gently scraped off, collected and weighed, then homogenized in 0.2 mol L^{-1} perchloric acid (10 μL mg^{-1} mucosa) and centrifuged at 10 000 ***g*** for 5 min at 4 °C. The supernatant was filtered through a 0.22-μm filter membrane, neutralized with 1 mol L^{-1} borate buffer (pH 9.25) and centrifuged for 1 min. The 5-HT content was analysed with an enzyme immunoassay kit according to the manufacturer's instructions (Beckman Coulter, Fullerton, CA, USA).

Acute intraluminal infusion of drugs In a set of experiments, the control or sensitized rats receiving chronic EA challenge were anesthetized with ketamine and ether on day 2, 4, 8 or 15. According to the previously reported methods,[13,22] a 20-cm segment of small intestine, including the duodenum and the proximal jejunum, was cannulated at both ends with two polyethylene tubes (outer diameter, 1.22 mm) positioned at 5 cm and 25 cm from the pylorus. The cannulas were fixed in the intestine with sutures and their remaining free ends were exteriorized in the abdominal wall. The distal cannula was kept open to permit drainage, thus avoiding an increase in intraluminal pressure.

The solution of a selective 5-HT_3R antagonist granisetron (250 μmol L^{-1} dissolved in saline, Sigma) or saline was infused at a rate of 0.2 mL min^{-1} for 10 min (similar to the dose used in the previous studies[22]). As the animals recovered from anaesthesia, a local anaesthetic lidocaine (1% solution in saline) was topically applied at the incision sites, and the VMR study was performed 5 min later.

In another set of experiments with EA-challenged sensitized rats ($n = 6$ at each time point), lidocaine solution (1%, 0.3 mL min^{-1} for 10 min, this dosage has been found to effectively blunt the sensitivity of mucosal afferents to luminal EA challenge[13]) was luminally perfused 30 min after the first VMR study (baseline). The second VMR study was conducted after the animals recovered from anaesthesia. Ten minutes later, granisetron solution was infused (0.2 mL min^{-1} for 10 min) and the third VMR study was performed.

Expression of mRNA encoding 5-HT_{3A} and 5-HT_{3B} subunits in the nodose ganglia (NG) during chronic EA challenge On day 2, 4, 8 or 15, the control or sensitized rats receiving chronic EA challenge were sacrificed after VMR studies, the NG samples were exposed, resected and immediately snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Two micrograms of total RNA was reverse transcribed with random primers and multiscribe reverse transcriptase contained in the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time reverse transcription-polymerase chain reaction (RT-PCR) with specific fluorescence-labelled probe was used to quantify mRNA expression of 5-HT_{3A} and 5-HT_{3B} subunits. PCR reactions were performed in a total volume of 50 μL containing cDNA samples, TaqMan Gene Expression Assays Target Mix (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems). The primer sequences were as follows: for 5-HT_{3A}: 5'ATAGCCCTCTTCCACCACCAA3' (forward) and 5' CACATATCCCACCCGCAACC3' (reverse); and for 5-HT_{3B}: 5' GCTCCATGTGCTGAGGACAC3' (forward) and 5' AGCCACTCCACCTCCTTCTG3' (reverse). Amplification was performed under the following conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Fluorescence in each well was measured using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Quadruplicate reactions were performed for each cDNA sample and cycle threshold (Ct) data were obtained. The fold changes in 5-HT_{3A} or 5-HT_{3B} mRNA transcript (target gene) relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, reference gene) were determined by ΔCt ($Ct_{Target} - Ct_{GAPDH}$). The relative amount of subunit mRNA transcript in EA-treated sensitized rats to the corresponding controls on day 2, 4, 8 or 15 was determined by $2^{-\Delta\Delta Ct}$ calculation, where $\Delta\Delta Ct = (Ct_{Target} - Ct_{GAPDH})_{EA} - (Ct_{Target} - Ct_{GAPDH})_{control}$.[23]

Data analysis and statistics

The VMR to CRD was evaluated with the number of abdominal muscle contractions per 5 s during the distension period. Data were expressed as mean ± SE throughout the manuscript. Differences between the VMR in different treatment groups were analyzed using repeated measures two-way ANOVA followed by Bonferroni posttest comparisons. A difference of $P < 0.05$ was considered significant.

RESULTS

Effect of chronic EA challenge on visceral nociception in the sensitized rats

Two weeks of EA challenge resulted in time-dependent changes in visceral pain response to CRD (60 mmHg) in the sensitized rats. The VMR to CRD on day 2 and 4 showed no significant difference from those of controls. On day 8, VMR increased to 42.0 ± 3.6, and it was 35.1 ± 3.1 in the control group. A week later, the response reached to 52.0 ± 3.5, which was significantly higher than that of controls (36.4 ± 3.3, $P < 0.01$) (Fig. 1). Compared with the control group, the sensitized rats receiving water or non-sensitized animals receiving EA exhibited slightly (but not significantly) increased visceral pain responses.

Mast cell numbers and 5-HT content in the jejunal mucosa in the sensitized rats during chronic EA challenge

During chronic EA challenge, the number of mast cells and 5-HT content in the jejunal mucosa in sensitized rats receiving EA challenge was significantly higher than those of control, sensitized animals receiving water and non-sensitized animals receiving EA ($P < 0.05$ or 0.01). Compared with the control group, no remarkable difference was found in mucosal mast cell numbers and 5-HT content in sensitized rats receiving water or non-sensitized animals receiving EA (Fig. 2).

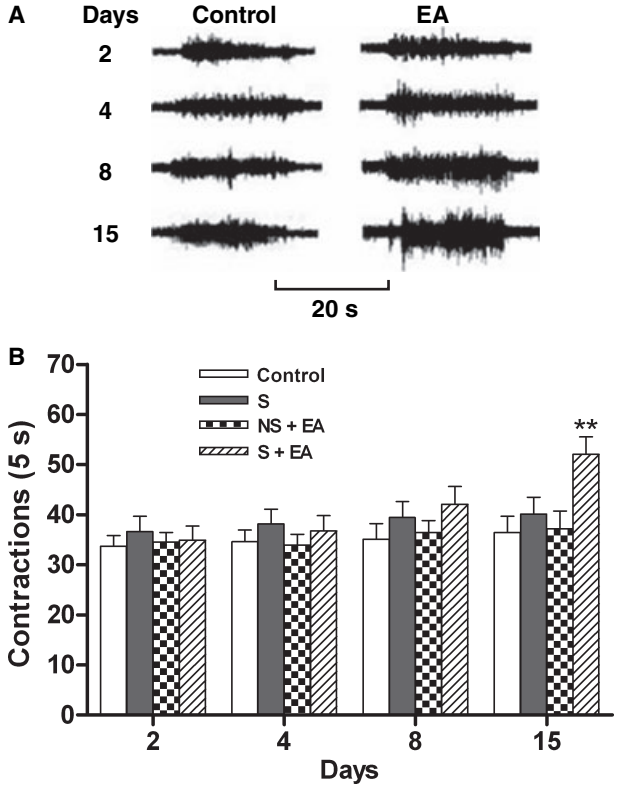

Figure 1 Visceral pain response to colorectal distension (CRD) during chronic egg albumin (EA) challenge in sensitized rats. The rats were sensitized by i.p. injection of 1 mL saline containing 10 μg EA, control and non-sensitized challenged (NS + EA) groups were sham sensitized with saline. EA (1 mg mL^{-1}) in drinking water was given to sensitized rats (S + EA) for 2 weeks, control and sensitized unchallenged (S) groups received water. CRD experiments were performed on day 2, 4, 8 and 15. Data are expressed as the mean ± SE of the number of abdominal contractions per 5 s during distension (60 mmHg). n = 7 for each group. $^{**}P < 0.01$ *vs* control.

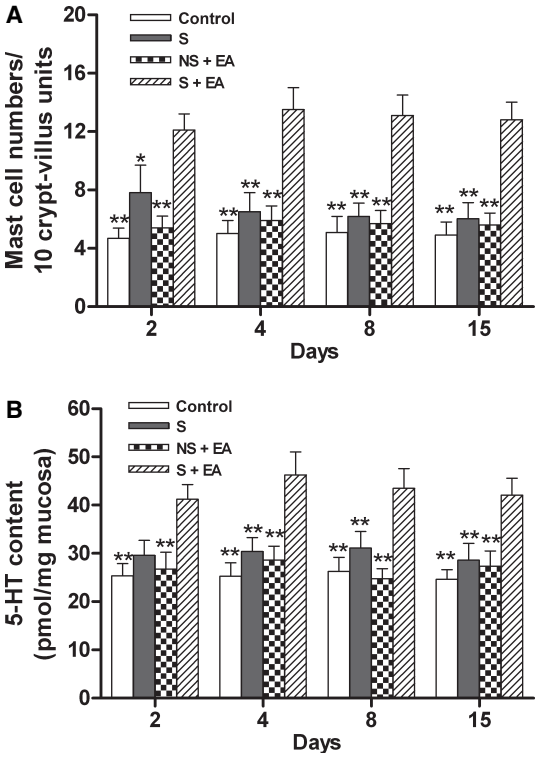

Figure 2 Mast cell numbers (A) and 5-HT content (B) in the jejunal mucosa during chronic egg albumin (EA, 1 mg mL^{-1} in drinking water for 2 weeks) challenge in sensitized rats. Control, non-sensitized unchallenged group; S, sensitized unchallenged group; NS + EA, non-sensitized challenged group; S + EA, sensitized challenged group. Mast cell numbers were obtained from 10 crypt-villus units for each animal. 5-HT content was expressed as a function of wet weight of mucosa (in milligrams). Data are expressed as the mean + SE. n = 5 at each time point for each group. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* S ± EA group.

Effects of subdiaphragmatic vagotomy or functional vagal afferent denervation on visceral nociception in the sensitized rats during chronic EA challenge

In the following experiment, we assessed the possible role of the subdiaphragmatic vagus on visceral sensation during chronic EA exposure. Compared with sham vagotomy, subdiaphragmatic vagotomy led to significantly increased VMR to CRD in EA-challenged sensitized rats on day 2 and 4 ($P < 0.01$). No remarkable changes in VMR were found on day 8 and 15. In addition, the VMR in vagotomized control group on day 2, 4, 8 or 15 was increased compared with that of sham control group ($P < 0.05$) (Fig. 3A).

To further evaluate the possible involvement of the vagal afferents, sensitized rats were subjected to perivagal capsaicin treatment to functionally obliterate vagal afferents. Similar to the effects of subdiaphragmatic vagotomy, capsaicin-induced functional vagotomy produced significantly increased VMR to CRD in EA-challenged sensitized rats on day 2 and 4 compared with vehicle-treated sensitized rats receiving EA ($P < 0.01$). No remarkable changes in VMR were found on day 8 and 15. The VMR in capsaicin-treated control group on day 2, 4, 8 and 15 was also markedly increased compared with that of vehicle-treated controls ($P < 0.05$) (Fig. 3B).

Effect of intraluminal infusion of granisetron on visceral nociception in the sensitized rats during chronic EA challenge

In the following experiments, we evaluated the influence of acute intraluminal infusion of a selective 5-HT$_3$R antagonist granisetron on VMR to CRD on day 2, 4, 8 or 15. Application of granisetron (250 μmol L^{-1}) on day 2 or 4 led to enhanced VMR in sensitized rats on EA challenge compared with those of saline-treated sensitized rats receiving EA ($P < 0.01$). On day 8 or 15,

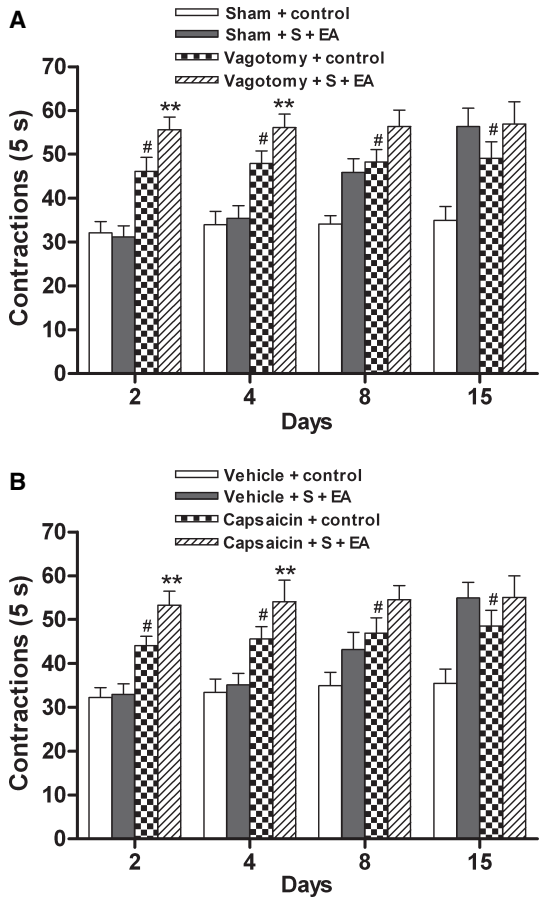

Figure 3 Effect of subdiaphragmatic vagotomy (A) or perivagal capsaicin treatment (B) on visceral pain response to colorectal distension (CRD) during chronic egg albumin (EA, 1 mg mL^{-1} in drinking water for 2 weeks) challenge in sensitized rats. S + EA, sensitized challenged group. CRD experiments were performed on day 2, 4, 8 and 15. Data are expressed as the mean ± SE of the number of abdominal contractions per 5 s during distension (60 mmHg). $n = 7$ for each group. $^{**}P < 0.01$ *vs* sham + S + EA or vehicle + S + EA, $P < 0.05$ *vs* sham + control or vehicle + control.

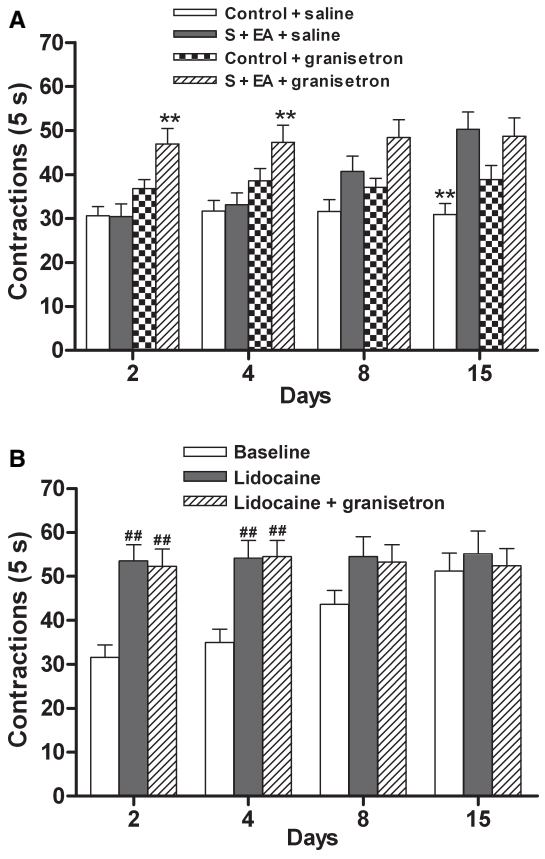

Figure 4 Effect of granisetron on visceral pain response to colorectal distension (CRD) during chronic egg albumin (EA, 1 mg mL^{-1} in drinking water for 2 weeks) challenge in sensitized rats. CRD experiments were performed in control or EA-challenged sensitized rats (S + EA) receiving acute luminal administration of granisetron (250 μmol L^{-1}) or saline (A) or EA-challenged sensitized rats receiving local mucosa anaesthetic (1% lidocaine) and the following granisetron infusion (B) on day 2, 4, 8 or 15. Data are expressed as the mean ± SE of the number of abdominal contractions per 5 s during distension (60 mmHg). $n = 6$ at each time point for each group. $^{**}P < 0.01$ *vs* S + EA + saline; $^{\#\#}P < 0.01$ *vs* baseline.

VMR in these two groups was similar. The VMR in granisetron-treated control group had a tendency to increase, but with no significant difference from that of saline-treated control rats (Fig. 4A).

In EA-treated sensitized rats, local mucosa anaesthetic (1% lidocaine) resulted in significantly increased VMR to CRD on day 2 or 4 compared with baseline ($P < 0.01$). The following infusion of granisetron failed to elicit further changes in VMR to CRD (Fig. 4B).

The levels of mRNA encoding 5-HT_{3A} and 5-HT_{3B} subunits in the NG in the sensitized rats during chronic EA challenge

Vagal afferents have cell bodies in the NG,[4,5] in which receptors can be synthesized and transported to the peripheral axon.[4,24] It has been found that native 5-HT_3Rs exist as pentameric complexes, usually comprising of 3A and 3B subunits,[25] and NG neurons normally express heteromeric 5-HT_{3AB} receptors.[26]To further explore whether the 5-HT_{3A} or 5-HT_{3B} levels on vagal afferents are associated with alterations in visceral sensation, we observed the expression of 5-HT_{3A} or 5-HT_{3B} mRNA in the NG during chronic EA challenge. The levels of mRNA encoding 5-HT_{3B} subunit in the NG displayed an opposite time-course to that of VMR to CRD, which were markedly increased from day 2, then decreased gradually to levels lower than those of controls. Expression of 3A subunit underwent a similar time-course except that the down-regulation of mRNA levels occurred from day 8 (Fig. 5).

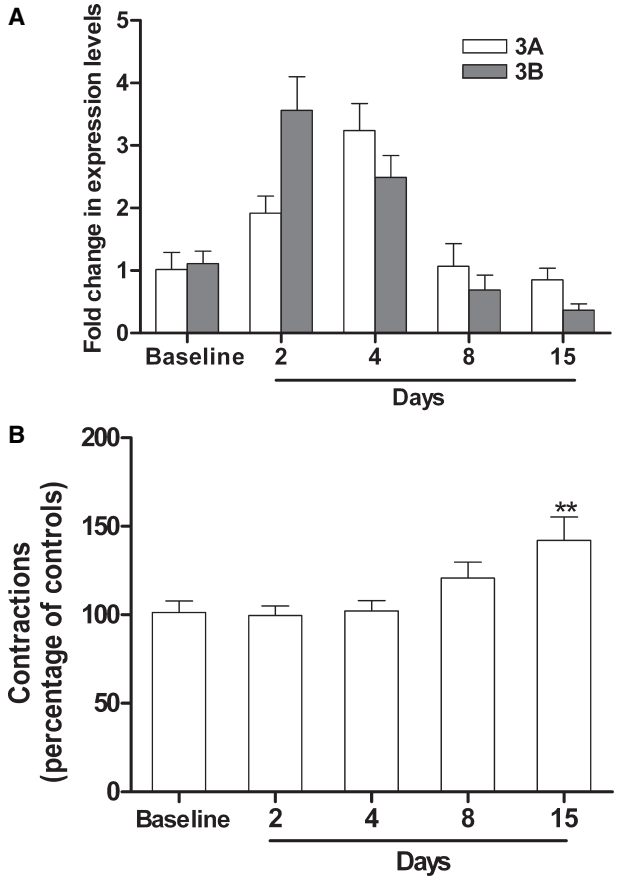

Figure 5 Expression of mRNA encoding 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits in the nodose ganglia (NG) (A) and visceromotor response (VMR) to colorectal distension (CRD) (B) during chronic egg albumin (EA, 1 mg mL^{-1} in drinking water for 2 weeks) challenge in sensitized rats. Six rats per group were sacrificed after VMR studies on day 2, 4, 8 or 15. The relative amount of 5-HT$_{3A}$ or 5-HT$_{3B}$ mRNA transcript in EA-treated sensitized rats (S + EA) to the controls was determined by $2^{-\Delta\Delta Ct}$ calculation. The VMR to CRD in S + EA rats was expressed as the percentage of controls. Data are expressed as the mean ± SE. $^{**}P < 0.01$ *vs* controls.

We also performed measurements in rats that have not been exposed to CRD, and found that CRD stimulation did not elicit obvious changes in mRNA expression of the subunits in the NG (data not shown).

DISCUSSION

The results of the present study demonstrate a time-dependent vagal modulation of chronic food allergen-sensitized visceral hyperalgesia. It is likely that this pathway is mediated via activation of 5-HT$_3$Rs, since blockade of them elicited similar responses to those of vagotomy in vagus-intact rats. Furthermore, the functional state of this vagally mediated pathway seems to have a link with the expression profile of 5-HT$_3$R subunits on vagal afferents.

Vagal afferents are extensively distributed in the gut except for the distal colon and rectum. Aside from spinal afferents, sensory information from the gut is conveyed in part by vagal afferents projecting to the brain stem, where they make synaptic connections with the second order neurons involved in the descending control of spinal nociceptive transmission. Converging lines of evidence suggest that vagal afferents mediate inhibitory modulation of visceral pain in response to acute noxious stimuli.[6–8] In the current study, chronic luminal EA stimulation in the sensitized rats led to gradual increases in visceral pain response, with a peak on day 15. Surgical vagotomy or vagal afferent denervation by capsaicin abolished this time-dependent manner, inducing visceral hyperalgesia from day 2, lasting to day 15. These results suggest that vagal afferents may underlie the time-course of visceral nociception, and, similar to the previous observations,[6–8] they trigger endogenous antinociception at the early stage of allergen stimulation.

Although vagal afferents are not involved in conveying primary information in the distal colon and rectum, the functional changes of vagal afferents resulting from delivering the drug in the intestine may influence pain response to CRD. To determine a possible role of 5-HT$_3$Rs on mucosal afferents, we assessed the effects of acute luminal infusion of a selective 5-HT$_3$R antagonist (granisetron) on VMR to CRD. Similar to the results of vagotomy, luminal application of granisetron on day 2 or 4 led to markedly enhanced pain responses, indicating the involvement of endogenous 5-HT in visceral hyperalgesia via a 5-HT$_3$R pathway in this model. This is supported by our results showing increased mucosal 5-HT content (mainly derived from mast cells) and the observations demonstrating 5-HT$_3$Rs involvement in response to luminal EA challenge.[13] Local mucosa anaesthesia with 1% lidocaine also resulted in significantly increased visceral pain on day 2 or 4, which was unaffected by the following infusion of granisetron. These results suggest an analgesic effect mediated by 5-HT$_3$Rs on mucosal afferents at the early stage of EA stimulation.

The action of luminally applied granisetron is unlikely exerted through blockade of 5-HT$_3$Rs on spinal afferents, because evidence has demonstrated that 5-HT$_3$Rs on spinal afferents facilitate visceral nociception.[6] Anatomically, spinal afferents terminate in the serosa and mesenteric attachments, often in association with blood vessels; peripheral vagal afferents terminate close to the mucosa epithelium, where they are in close contact with the chemicals absorbed from the lumen or mediators released from mast

cells.[27,28] Furthermore, pharmacological functional studies suggest that 5-HT_3Rs predominantly reside on mucosal afferents rather than mechanosensitive fibres in the muscle and serosa.[4,29] So we presume that, at the early stage of EA challenge, the visceral hypersensitive condition elicited by granisetron was mainly attributed to blockade of 5-HT_3Rs on vagal mucosal afferents.

To further explore whether the expression of vagal 5-HT_{3A} or 5-HT_{3B} subunits are associated with the time-dependent visceral hyperalgesia, we observed the expression profile of mRNA encoding these subunits in the NG during chronic EA challenge. The mRNA levels of 5-HT_3R subunits in the NG, especially those of 3B, showed an opposite time-course to that of visceral pain, which increased from day 2 and decreased gradually to levels lower than those of controls. Evidence has shown that 3A subunit forms functional 5-HT_3Rs and 3B subunit specifically potentialize the pharmacological potency of 3A.[30–32] However, sustained activation of 3A subunit may give rise to desensitization of 5-HT_3Rs and excitotoxic cell death of sensory afferent neurons.[33]

Our observations support a hypothesis that, at the early stage of allergen challenge, elevated level of 5-HT (mainly released from mucosal mast cells) may elicit facilitated vagally triggered descending antinociception via activation of 5-HT_3Rs and up-regulation of their subunits expression on vagal afferents innervating the gut. However, sustained exposure to excessive 5-HT might lead to desensitization of vagal 5-HT_3Rs or neuronal injury of vagal afferents resulting from excitotoxicity. These changes may contribute to the impairment of vagal modulation of nociception, resulting in visceral hyperalgesia. The concurrent down-regulation of the mRNA levels of 5-HT_3R subunits may be resultant from receptor desensitization or neuronal cell death.[33] Our hypothesis might be of value to clarify the mechanisms underlying the observed vagal dysfunction in IBS. Of course, we cannot rule out the possible role of other bioactive mediators released from mast cells. The NG contains afferents from other organs except for the gut, whether the changes in 5-HT_3R subunit mRNA levels in the NG result from expression plasticity of 5-HT_3Rs on the subpopulations of afferents from the gut needs further validation. In addition, dietary EA may change gastric emptying, luminal osmolarity and lead to elevated GI hormones. These changes may affect nociceptive responses.

IBS-related visceral hyperalgesia involves hypersensitive spinal mechanisms, the functional state of spinal afferents and involvement of spinal 5-HT_3Rs during chronic EA exposure need to be clarified. Although we have no answer in the current study, we found that 5-HT_3R subunits expression on spinal afferents did not experience a typical time-dependent change in this model (S. Chen, L. Zhang, X. Dong, J. Mo and S. Xiao, unpublished observations).

In summary, our study demonstrates a time-dependent vagal modulation of chronic allergen-sensitized visceral hyperalgesia, which may involve a vagal 5-HT_3R pathway. The functional state of this pathway seems to be associated with plastic changes of 5-HT_3R subunits expression on vagal afferents, confirming a link between molecular data and functional sequelae.

ACKNOWLEDGMENTS

This study was sponsored by grant from Shanghai Leading Academic Discipline Project (No. Y0205) and Corporation Project for Renji Hospital and Basic Medical College of Shanghai Jiaotong University (No. PY07009).

DISCLOSURES

Competing interests: the authors have no competing interests.

REFERENCES

1 Drossman DA. The functional gastrointestinal disorders and the Rome III process. *Gastroenterology* 2006; **130**: 1377–90.
2 Anand P, Aziz Q, Willert R, van Oudenhove L. Peripheral and central mechanisms of visceral sensitization in man. *Neurogastroenterol Motil* 2007; **19**: 29–46.
3 Zar S, Kumar D, Benson MJ. Review article: food hypersensitivity and irritable bowel syndrome. *Aliment Pharmacol Ther* 2001; **15**: 439–49.
4 Andrews PL, Sanger GJ. Abdominal vagal afferent neurones: an important target for the treatment of gastrointestinal dysfunction. *Curr Opin Pharmacol* 2002; **2**: 650–6.
5 Grundy D. Neuroanatomy of visceral nociception: vagal and splanchnic afferent. *Gut* 2002; **51**: i2–5.
6 Bradesi S, Lao L, McLean PG *et al.* Dual role of 5-HT_3 receptors in a rat model of delayed stress-induced visceral hyperalgesia. *Pain* 2007; **130**: 56–65.
7 Chen SL, Wu XY, Cao ZJ *et al.* Subdiaphragmatic vagal afferent nerves modulate visceral pain. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G1441–9.
8 Gschossmann JM, Mayer EA, Miller JC, Raybould HE. Subdiaphragmatic vagal afferent innervation in activation of an opioidergic antinociceptive system in response to colorectal distension in rats. *Neurogastroenterol Motil* 2002; **14**: 403–8.
9 Smart HL, Atkinson M. Abnormal vagal function in irritable bowel syndrome. *Lancet* 1987; **ii**: 475–8.
10 Elsenbruch S, Orr WC. Diarrhea- and constipation-predominant IBS patients

differ in postprandial autonomic and cortisol responses. *Am J Gastroenterol* 2001; **96**: 460–6.
11 Spaziani R, Bayati A, Redmond K *et al.* Vagal dysfunction in irritable bowel syndrome assessed by rectal distension and baroreceptor sensitivity. *Neurogastroenterol Motil* 2008; **20**: 336–42.
12 Spiller R. Serotonin and GI clinical disorders. *Neuropharmacology* 2008; **55**: 1072–80.
13 Jiang W, Kreis ME, Eastwood C, Kirkup AJ, Humphrey PP, Grundy D. 5-HT$_3$ and histamine H$_1$ receptors mediate afferent nerve sensitivity to intestinal anaphylaxis in rats. *Gastroenterology* 2000; **119**: 1267–75.
14 Greenwood-van Meerveld B. Importance of 5-hydroxytryptamine receptors on intestinal afferents in the regulation of visceral sensitivity. *Neurogastroenterol Motil* 2007; **19**: 13–8.
15 Andresen V, Montori VM, Keller J, West CP, Layer P, Camilleri M. Effects of 5-hydroxytryptamine (serotonin) type 3 antagonists on symptom relief and constipation in nonconstipated irritable bowel syndrome: a systematic review and meta-analysis of randomized controlled trials. *Clin Gastroenterol Hepatol* 2008; **6**: 545–55.
16 Matsueda K, Harasawa S, Hongo M, Hiwatashi N, Sasaki D. A phase II trial of the novel serotonin type 3 receptor antagonist ramosetron in Japanese male and female patients with diarrhea-predominant irritable bowel syndrome. *Digestion* 2008; **77**: 225–35.
17 Kidd EJ, Laporte AM, Langlois X *et al.* 5-HT$_3$ receptors in the rat central nervous system are mainly located on nerve fibres and terminals. *Brain Res* 1993; **612**: 289–98.
18 Curtis GH, Patrick MK, Catto-Smith AG, Gall DG. Intestinal anaphylaxis in the rat. Effect of chronic allergen exposure. *Gastroenterology* 1990; **98**: 1558–66.
19 Cao Z, Wu X, Chen S *et al.* Anterior cingulate cortex modulates visceral pain as measured by visceromotor responses in viscerally hypersensitive rats. *Gastroenterology* 2008; **134**: 535–43.
20 Gao J, Wu X, Owyang C, Li Y. Enhanced responses of the anterior cingulate cortex neurones to colonic distension in viscerally hypersensitive rats. *J Physiol* 2006; **570**: 169–83.
21 Ness TJ, Gebhart GF. Colorectal distension as a noxious visceral stimulus: physiological and pharmacological characterization of pseudoaffective reflexes in the rat. *Brain Res* 1988; **450**: 153–69.
22 Zhu JX, Zhu XY, Owyang C, Li Y. Intestinal serotonin acts as a paracrine substance to mediate vagal signal transmission evoked by luminal factors in the rat. *J Physiol* 2001; **530**: 431–42.
23 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* 2001; **25**: 402–8.
24 Lacolley P, Owen JR, Sandock K *et al.* 5-HT activates vagal afferent cell bodies *in vivo*: role of 5-HT$_2$ and 5-HT$_3$ receptors. *Neuroscience* 2006; **143**: 273–87.
25 Thompson AJ. Lummis SC. 5-HT$_3$ receptors. *Curr Pharm Des* 2006; **12**: 3615–30.
26 Morales M, Wang SD. Differential composition of 5-hydroxytryptamine3 receptors synthesized in the rat CNS and peripheral nervous system. *J Neurosci* 2002; **22**: 6732–41.
27 Williams RM, Berthoud HR, Stead RH. Vagal afferent nerve fibres contact mast cells in rat small intestinal mucosa. *Neuroimmunomodulation* 1997; **4**: 266–70.
28 Aerssens J, Hillsley K, Peeters PJ *et al.* Alterations in the brain-gut axis underlying visceral chemosensitivity in *Nippostrongylus brasiliensis*-infected mice. *Gastroenterology* 2007; **132**: 1375–87.
29 Hillsley K, Kirkup AJ, Grundy D. Direct and indirect actions of 5-hydroxytryptamine on the discharge of mesenteric afferent fibres innervating the rat jejunum. *J Physiol* 1998; **506**: 551–61.
30 Davies PA, Pistis M, Hanna MC *et al.* The 5-HT$_{3B}$ subunit is a major determinant of serotonin-receptor function. *Nature* 1999; **397**: 359–63.
31 Dubin AE, Huvar R, D'Andrea MR *et al.* The pharmacological and functional characteristics of the serotonin 5-HT$_{3A}$ receptor are specifically modified by a 5-HT$_{3B}$ receptor subunit. *J Biol Chem* 1999; **274**: 30799–810.
32 Hu XQ, Peoples RW. The 5-HT$_{3B}$ subunit confers spontaneous channel opening and altered ligand properties of the 5-HT$_3$ receptor. *J Biol Chem* 2008; **283**: 6826–31.
33 Bhattacharya A, Dang H, Zhu QM *et al.* Uropathic observations in mice expressing a constitutively active point mutation in the 5-HT$_{3A}$ receptor subunit. *J Neurosci* 2004; **24**: 5537–48.

