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Electroacupuncture relieved visceral and referred hindpaw hypersensitivity in colitis rats by inhibiting tyrosine hydroxylase expression in the sixth lumbar dorsal root ganglia

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ABSTRACT

Irritable bowel syndrome patients frequently complain of pain in body regions somatotopically distinct from the gut, suggesting the involvement of an exaggerated signaling process in both visceral and somatic sensory pathways. Increasing evidence has shown that sprouting of tyrosine hydroxylase immunoreactive (TH-IR) fibers toward sensory neurons in dorsal root ganglia maintains and exacerbates the neuropathic and inflammatory pain, as well as colonic inflammation. The aim of the present study was to determine whether electroacupuncture could alleviate the visceral and secondary somatic hyperalgesia in colitis rats by suppressing the TH-IR expression in related dorsal root ganglia. After trinitrobenzene sulfonic acid irritation, rats developed inflammatory tissue damage in the distal colon, which was accompanied by visceral hypersensitivity and secondary hind paw hyperalgesia, as indicated by enhanced visceromotor response to colorectal distension and decreased mechanical and thermal withdrawal latency of the hind paw. Additionally, excessive TH-IR fibers sprouted toward calcitonin gene-related peptide immunoreactive sensory neurons, and TH-IR neurons also increased in the sixth lumbar dorsal root ganglia of colitis rats. Both electroacupuncture and guanethidine attenuated visceral and referred hind paw hyperalgesia by inhibiting the over-expression of TH-IR neurons and fibers in the sixth lumbar dorsal root ganglia. Moreover local inflammatory damage in the distal colon was restored after 7 days of electroacupuncture intervention. These results suggest that electroacupuncture relieved visceral and referred hind paw hypersensitivity in colitis rats by inhibiting TH expression in the sixth lumbar dorsal root ganglia.

1. Introduction

Patients with irritable bowel syndrome (IBS) have a wide variety of somatic sensory disturbances, including back pain, abdominal muscle pain, dyspareunia, and hand/foot hyperalgesia (Mayer and Raybould, 1990; Verne et al., 2001). In the rat model of IBS, secondary somatic hyperalgesia in hind paws was elicited in subsets of IBS rats (Katarzyna et al., 2017; Zhou et al., 2008). Thus, it has been hypothesized that in the spinal dorsal horn, noxious visceral stimulus facilitates the responses of projection neurons to somatic inputs, resulting in referred pain, or hyperalgesia (Ruch, 1947).

With respect to the amplified sensory process, increasing evidence has emphasized the critical role of sympathetic activity in the development and persistence of pathologic pain. Abnormal sympathetic-somatosensory interaction aggravates several types of neuropathic pain, while sympathetic block or α1-adrenergic antagonists are effective in relieving pain symptoms (Xanthos and Coderre, 2008). Of particular interest is that sympathetic fibers sprouting into dorsal root ganglia (DRG) were observed in chronic constriction injury (Wu et al., 2017) and partial sciatic nerve injury models (Brumovsky et al., 2006). Furthermore, localized sympathectomy profoundly reduced mechanical pain behaviors in ligated L5 spinal nerves and L5 DRG inflamed rats(Li et al., 2018), indicating that sympathetic nerves exacerbated pain and inflammation in innervated regions. Additionally, sympathetic tyrosine hydroxylase immunoreactive (TH-IR) fibers have been found to form basket-like structures around colon-innervating DRG neurons following colonic inflammation (Xia Jr et al., 2011) and were associated with colitis-induced chronic visceral hypersensitivity and/or referred pain (Lü et al., 2019). These studies suggest that referred pain or hypersensitivity exhibited in chronic visceral disorders may be partially attributed to the sprouting of TH-IR fibers into the DRG, thus shedding new light on strategies to alleviate chronic visceral pain and referred

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somatic hyperalgesia.

Electroacupuncture (EA) has long been used as an effective intervention to treat chronic visceral pain (Wang et al., 2008) and somatic hyperalgesia (Wang et al., 2018) through peripheral, spinal, and supraspinal mechanisms (Zhang et al., 2014). However, whether EA relieves visceral pain and somatic hyperalgesia by inhibiting sympathetic sprouting in referred DRGs remains unclear. In the present study, firstly we determined the effects of EA at acupoints ST36 and ST37 on the visceral hypersensitivity and referred somatic hyperalgesia in trinitrobenzene sulfonic acid (TNBS)-induced colitis rats. Secondly, the TH-IR sprouting to sensory neurons in DRG, and particularly, the potential impact of EA on this ectopic TH-IR sprouting were observed to elucidate the neural basis of the effects of EA.

2. Materials and methods

2.1.1. Ethical statement

Animal care and all protocols used in this study were approved by the Institutional Animal Welfare and Use Committee of the Institute of Acupuncture-Moxibustion, China Academy of Chinese Medicine (No. 20170313). This investigation adhered to The National Academies *Guide for the Care and Use of Laboratory Animals, 8th edition* (National Research Council (US) Committee, 2011). All effort was made to minimize the potential for animal pain, stress, and distress.

2.1.2. Animals

A total of 96 adult male Sprague-Dawley rats (Beijing Spriff Laboratory Animal Technology Co., Ltd., Beijing, China) weighing 180–200 g were used for this study. Animals were randomly divided into 5 groups based on the experimental protocol: control group (n = 18), vehicle group (n = 18), colitis group (n = 18), EA group (n = 18), and guanethidine group (n = 18). In addition, 6 normal rats were used in a retrograde labeling experiment. Animals were housed in groups of 3 per cage and had free access to standard chow and water. Rats were allowed to acclimate to the housing conditions of constant room temperature (22 ± 2 °C) and humidity (60%) under controlled illumination (on from 06:00 to 18:00 and off from 18:00 to 06:00) for 7 days before starting the experiment.

2.1.3. TNBS-induced colitis

Rats fasted overnight with free access to water before the TNBS administration. Under 2% isoflurane anesthesia (Yipin Pharmaceutical Co., Hebei, China), a mixture of TNBS (100 mg/kg body weight) and 50% ethanol (volume ratio 2:1) was instilled via the rectum into the distal colon lumen (7 cm proximal to the anus) using a plastic feeding tube. An equivalent volume of sterilized saline was administered into control rats, and an equivalent volume of 50% ethanol into vehicle rats. After intracolonic infusion, rats were kept in a vertical position for at least 5 min to avoid leakage of the instilled solutions.

2.1.4. Injection of guanethidine

Guanethidine, a sympathetic blocker, has been used to inhibit sympathetic adrenergic nerve activity by blocking the release of catecholamines (Pertin et al., 2007). Guanethidine was dissolved in 0.9% saline and injected intraperitoneally at a dose of 30 mg/kg into rats on days 6, 7, 13, and 14 after TNBS administration.

2.1.5. Electroacupuncture intervention

Twenty-four hours after TNBS administration, EA intervention was applied at 10 AM each day for 7 or 14 consecutive days in rats anaesthetized with 2% isoflurane. Acupoints ST 36 and ST 37 were chosen since they are commonly used for relieving visceral pain and digestive system disorders in the clinic (Li et al., 2015). In rats, ST 36 is approximately 5 mm inferior to the head of the fibula and posterolateral to the hind-limb knee joint. ST 37 is 5 mm distal to ST 36 and 1 mm lateral to the anterior tibial margin.

Stainless steel acupuncture needles (0.18 mm diameter, 13 mm length; Beijing Zhongyan Taihe Medicine Co., Beijing, China) were swiftly inserted into the bilateral acupoints ST 36 and ST 37 each at a depth of 3 mm. The needles were then connected to an electrical stimulator (HANS-100A, Wuxi Shenping Xintai Medical Technology, Wuxi, China) for EA stimulation with parameters of 2 Hz, 1 mA, lasting for 30 min. Behavior tests and tissue collection were performed 2 h after EA treatment.

2.1.6. Assessment of disease activity index

Body weight, stool consistency, and intestinal bleeding were assessed and scored from 0 to 4 to obtain a disease activity index (DAI), which was used to determine the severity of colitis based on Koetzner (Koetzner et al., 2010). All animals were evaluated on days 7 and 14 post-TNBS.

Normal stools were moderately hard and oval; loose stools were paste-like and the anus did not show sticky residue; diarrhea was manifested by thin stools and sticky residues on the anus. Occult bleeding was tested by a commercial kit (Baso Diagnostics Inc., Zhuhai, China) with two drops of the reagent delivered onto the stool sample, which was placed on a white filter paper to observe the change in color. Presence of occult blood was graded using a score of 0, for no color change; 1, for a very light blue (±) taking more than 30 s to appear; 2, for blue, developing in 30 s or more (+); 3, for an immediate change in color occurring in less than 30 s (+ +); and 4, for gross blood observable on the slide (+ + +).

2.1.7. Measurement of mechanical and thermal pain thresholds

To evaluate somatic hyperalgesia induced by TNBS instillation, mechanical and thermal behavioral tests were measured in all groups on days 7 and 14. Rats were put in individual plastic cages. Each cage was bottomless and rested on a metal mesh as part of an electronic von Frey instrument (Dynamic Plantar Aesthesiometer [DPA] 37,450, Ugo Basile, Gemonio, Italy). Rats were allowed to adapt to the testing environment for 30 min. Mechanical paw withdrawal (PWL) of the plantar surface of the right hind paw was measured using the DPA with a von Frey filament (0.5 mm diameter) with a cutoff set at 50 g. Response time was recorded in seconds and force threshold in g-units. The measurement was repeated thrice with an interval of 5 min between testings. The average values of PWL were then calculated.

Two hours after the mechanical PWL test, the thermal pain threshold was tested. Rats were put into individual compartments with glass bottoms of the Thermal Plantar Tester (37,370, Ugo Basile, Gemonio, Italy), and were allowed to habituate to the environment for 30 min before testing. A movable radiant heat source under the glass surface was focused on the plantar surface of the right hind paw to detect the PWL with 50% active intensity, 10% idle intensity, and 25-second cutoff time in order to avoid tissue damage. Each hind paw was tested thrice with an interval of 5 min between the testing. The average value of withdrawal latency for each hind paw was calculated.

2.1.8. Visceromotor response to colorectal distension

Visceromotor response (VMR) is an evoked electromyography (EMG) reflex of abdominal muscle contraction in response to colorectal distension (CRD) and has been widely used to reflect visceral nociceptive perception (Traub et al., 2008; Zhou et al., 2012). Rats were fasted overnight with free access to water before VMR measurement on days 7 and 14 post-TNBS instillation. Under 2% isoflurane anesthesia, the external oblique abdominal muscles were exposed. Two electrodes made of Teflon-coated platinum wires were inserted into the muscles. Then a 3 cm-long, 1.5 cm max diameter flexible latex balloon was inserted through the rectum into the descending colon with the distal end 1 cm proximal to the external anal sphincter. The balloon was attached to a distension pressure controller with a polyethylene catheter and was

secured by taping the catheter to the tail. Anesthesia was then decreased to 1% isoflurane for EMG recording. CRD was produced by inflating the balloon by air with steped pressures (20, 40, 60, and 80 mmHg) for a 20-second stimulation period with an interval of 5 min between each testing. EMG signals were amplified (5000 ×) and filtered (30–1000 Hz) using an electrophysiologic amplifier (NeuroLog NL900D, DigiTimer North America, Ft. Lauderdale, FL, USA), then transmitted into a data acquisition device (PowerLab 8/35, ADInstruments, Bella Vista, New South Wales, Australia). Each CRD stimulus was repeated 3 times, and the EMG reflex frequencies were averaged and analyzed off-line using Prism 6 software (GraphPad, San Diego, CA, USA).

2.1.9. Perfusion and tissue preparation

Intracardiac perfusion was performed for tissue fixing. On days 7 and 14 after TNBS irritation, rats were deeply anaesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally) and perfused transcardially with 0.9% normal saline, followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer (PB, 4 °C). Colonic tissue at 7 cm proximal to the anus was removed, paraffin sections were prepared, and hematoxylin and eosin (H&E) staining was performed. Histopathologic changes were observed under a microscope (Olympus, IX73P2F, Tokyo, Japan). Colonic damage was evaluated using the colon mucosal damage index (CMDI) (Butzner et al., 1996) and histologic tissue damage index (TDI) (Dieleman et al., 1999).

2.1.10. Retrograde labeling of the colon and ST 36

To determine the overlapping segment of DRG between the site of colon innervation and ST 36 region, a retrograde neuronal tracer, Alexa Fluor® 488 conjugated CTB molecules (CTB-488, green fluorescence, Molecular Probes, Eugene, OR, USA) was injected into the distal colonic wall of 3 normal rats. Alexa Fluor® 555 conjugated CTB molecules (CTB-555, red fluorescence, Molecular Probes, Eugene, OR, USA) was injected into the underlying muscle layer at ST 36 in 3 other normal rats.

Rats were administered with 2% isoflurane anesthesia and the colon was then exposed. 0.05% CTB-488 was injected into 5 sites (4 μL per site) of the muscle wall of the descending colon (5–7 cm proximal to the external anal sphincter) with a 10 μL Hamilton micro syringe (no. 25 needle), to label colonic afferent neurons innervating this area. The abdominal cavity was then rinsed with a large amount of sterile saline. The colon was gently placed back into the abdomen, while the muscles and skin were closed with 4–0 sutures. Additionally, 8 μL 0.05% CTB555 was injected into the underlying muscle layer at ST 36 at a depth of 3 mm in the 3 other rats. Each injection was performed carefully to prevent the dye from spreading to the adjacent organs. Any spillage was absorbed with a cotton swab. Rats were returned to their cages and allowed free access to water.

Three days after tracer injection, rats were deeply anaesthetized and perfused with 4% paraformaldehyde. The T1-S3 DRGs were removed and stored in 4% paraformaldehyde solution for 2 h, and subsequently in 30% sucrose solution for 48 h for cryoprotection. The T1-S3 DRGs with whole ganglia were thaw-mounted on SuperFrost® Plus slides (Thermo Scientific, USA) and observed under a fluorescence microscope (Olympus, IX73P2F). The occurrence of neurons labeled by retrograde neuronal tracers in each segment-matched DRG was recorded to ascertain the overlapping segment of DRG between the colon and ST 36.

2.1.11. Immunohistochemistry of DRG

On days 7 and 14, L5 and L6 DRGs of the rats were removed after intracardiac perfusion and placed in 30% sucrose solution for 48 h for cryoprotection. Then the DRGs were sectioned parasagittally at a thickness of 40 μm to observe the expression of fibers labeled by tyrosine hydroxylase (TH) and neurons labeled by TH as well as calcitonin gene-related peptide (CGRP). Slides were washed once with 0.1 M PB at room temperature for 10 min, and blocked with goat serum at room temperature for 30 min. This was followed by incubation with 1:1000 rabbit anti-TH antibody (AB112; Abcam, Cambridge, UK) and 1:1000 mouse anti- CGRP antibody (AB81887; Abcam) overnight at 4 °C. After being washed with 0.1 M PB thrice, the slides were incubated with 1:500 Alexa Fluor 594-conjugated donkey anti-rabbit antibodies (R37199; Thermo Fisher, Molecular Probes, Eugene, OR, USA)/1:500 Alexa Fluor 488-conjugated donkey anti-mouse (A11055) at room temperature for 2 h, then sealed with glycerine and photographed on a confocal imaging system (FV1200, Olympus). The number of TH-IR and CGRP-IR neurons in one DRG were calculated while the length (in mm) of TH-IR fibers was measured using microimaging software (cellSens Standard 1.11, Olympus). The average number and length were then calculated and compared.

2.1.12. Statistical analysis

Prism 6 software was used to perform statistical analysis. Data were reported as mean (SD). One-way analysis of variance (ANOVA) with multiple comparisons was applied for total differences among the 5 groups (control, vehicle, TNBS, EA, and guanethidine). Two-way ANOVA with multiple comparisons was applied for total differences among the 5 groups at different times. Differences between means at a level of $P < .05$ were considered to be significant.

3. Results

3.1.1. EA alleviated colitis severity and somatic hyperalgesia in colitis rats

Rats developed colitis characterized by decreased movement, loss of coat luster, appetite decline, loss of weight, and loose stools from day 1 to 14 after TNBS administration. Moreover, the disease activity index (DAI), which was calculated by factoring in weight loss, diarrhea, and occult blood or rectal bleeding, was significantly increased at days 7 and 14 in colitis rats (colitis vs. vehicle: 7 d: 2.9 ± 0.4 vs. 0, 14 d: 3.4 ± 0.3 vs. 0, $P < .001$, Fig. 1A). Both EA and guanethidine treatment markedly decreased DAI scores on days 7 and 14 (EA: 7 d: 1.2 ± 0.3, 14d: 0.3 ± 0.4; guanethidine: 7 d: 1.2 ± 0.2, 14 d: 0.2 ± 0.4. $P < .001$ vs. colitis, Fig. 1A), suggesting that EA alleviated the disease severity in TNBS-induced colitis rats. No obvious alterations in behavioral performance and DAI scores were found in control and vehicle groups.

Both mechanical and thermal PWL of colitis rats were dramatically decreased compared with those in vehicle rats on days 7 and 14 (colitis vs. vehicle: mechanical PWL: 7d: 5.7 ± 1.4 s vs. 12.1 ± 1.9 s, 14d: 5.2 ± 1.1 s vs. 12.9 ± 2.9 s; thermal PWL: 7d: 7.9 ± 1.4 s vs. 14.4 ± 2.1 s, 14d: 5.6 ± 1.1 s vs. 14.3 ± 1.8 s; $P < .001$), indicating that referred hypersensitivity occurred after inflammatory injury of the distal colon. EA and guanethidine significantly restored hind paw hypersensitivity by increasing both mechanical (EA: 7d: 11.3 ± 1.4 s, 14d: 12.7 ± 2.9 s; guanethidine: 7d: 10.5 ± 2.8 s, 14d: 10.8 ± 2.6 s; $P < .01$ vs. colitis) and thermal (EA: 7d: 12.1 ± 1.5 s, 14d: 12.9 ± 1.6 s; guanethidine: 7d: 13.4 ± 1.6 s, 14d: 12.2 ± 1.4 s; $P < .001$ vs. colitis) withdrawal latency of colitis rats (Fig. 1B and C).

3.1.2. EA suppressed visceral hypersensitivity in colitis rats

Colorectal sensitivity was tested by recording the VMR in response to CRD (40, 60, and 80 mmHg) on days 7 and 14 after TNBS instillation (Fig. 2A). 40 mmHg CRD barely evoked VMR reflexes in the control and vehicle rats. However, 40 mmHg CRD elicited modest VMR reflex in colitis rats on day 14 (colitis vs. vehicle: 17.6 ± 2.4 Hz vs. 1.0 ± 0.4 Hz; $P < .001$). Both 60 and 80 mmHg CRD evoked robust VMR reflexes in all groups, with particularly increased firing frequency in colitis rats on days 7 and 14 (60 mmHg: 7d: 58.8 ± 12.7 Hz, 14d: 67.6 ± 7.8 Hz; 80 mmHg: 7d: 63.9 ± 13.4 Hz, 14d: 91.1 ± 5.1 Hz; $P < .001$ vs. vehicle), indicating the gradual development of colonic

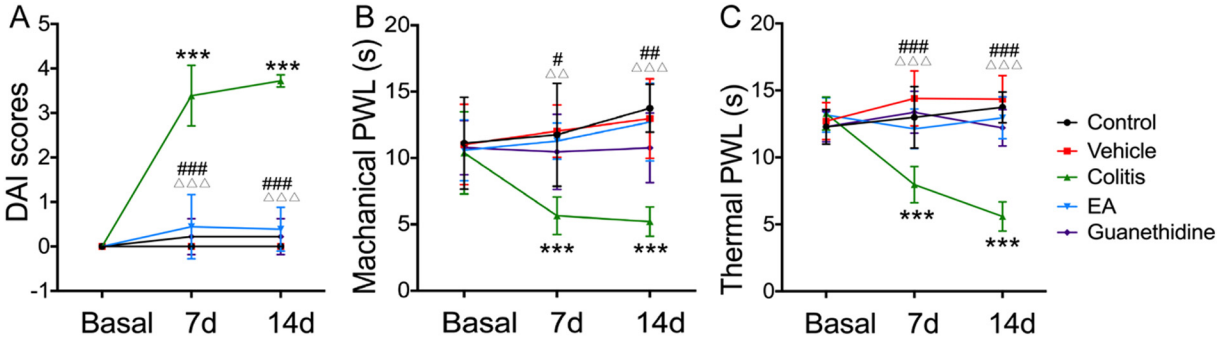

Fig. 1. DAI scores, mechanical and thermal PWLs of each group. After TNBS irritation, DAI scores (A) were significantly increased in colitis rats on days 7 and 14, and alleviated by both EA and guanethidine. Mechanical (B) and thermal (C) PWL were reduced in colitis rats, indicating the development of secondary somatic hyperalgesia following colonic inflammation. Both EA and guanethidine dramatically restored these nociceptive reflexes on days 7 and 14 ($^{***}P < .001$ vs. control; $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ vs. colitis. n = 6 for each group).

hypersensitivity. EA markedly inhibited VMR reflexes to noxious CRD stimuli in colitis rats on days 7 and 14 (7d: 60 mmHg: 18.8 ± 10.1 Hz, 80 mmHg: 26.9 ± 9.5 Hz; 14d: 40 mmHg: 0.8 ± 0.4 Hz, 60 mmHg: 16.3 ± 5.5 Hz, 80 mmHg: 27.5 ± 5.7 Hz. $P < 0.001$ vs. colitis), and similar trends was found in guanethidine group (7d: 60 mmHg: 15.8 ± 4.5 Hz, 80 mmHg: 23.1 ± 7.3 Hz; 14d: 40 mmHg: 0.9 ± 0.3 Hz, 60 mmHg: 15.1 ± 2.8 Hz, 80 mmHg: 22.6 ± 6.5 Hz; $P < .001$ vs. colitis).

3.1.3. EA alleviated colonic histological damage in colitis rats

At days 7 and 14 after TNBS irritation, severe transmural inflammation characterized by goblet cell depletion (blue arrows, Fig. 3A), massive infiltration of polymorpho-nuclear leukocytes (black arrows, Fig. 3A), edema (*, Fig. 3A) and partial destruction or ablation of the mucosal architecture were observed in colon tissues. EA or guanethidine alleviated inflammatory cell infiltration as well as the change in colon tissue architecture (Fig. 3A).

Colitis rats displayed a significant increase in CMDI and TDI scores on days 7 and 14 compared with scores of the vehicle group (CMDI: 7 d: 4.2 ± 1.2 vs. 0, 14 d: 6.2 ± 1.5 vs. 0. $P < .001$; TDI: 7 d: 13.7 ± 0.8 vs. 0, 14 d: 16.2 ± 0.9 vs. 0. $P < .001$). EA and guanethidine gradually decreased CMDI and TDI scores on days 7 and 14 compared with colitis rats (EA: CMDI: 7 d: 1.0 ± 0.6, 14 d: 0.5 ± 0.5; TDI: 7 d: 1.2 ± 0.8, 14 d: 0.5 ± 0.5. Guanethidine: CMDI: 7 d: 0.5 ± 0.5, 14 d: 0.3 ± 0.5; TDI: 7 d: 1.2 ± 0.8, 14 d: 0.7 ± 0.8. $P < .001$) (Fig. 3B).

3.1.4. EA suppressed sympathetic fiber sprouting to the sensory neurons of L6 DRG in colitis rats

The labeled neurons traced by CTB-488 from the muscle wall of the colon were distributed in the segments of T13-L2, L6 (Fig. 4B), and S1 DRGs. The labeled neurons traced by CTB-555 from acupoint ST 36 were found to be expressed in the segments of L4, L5 (Fig. 4C), and L6

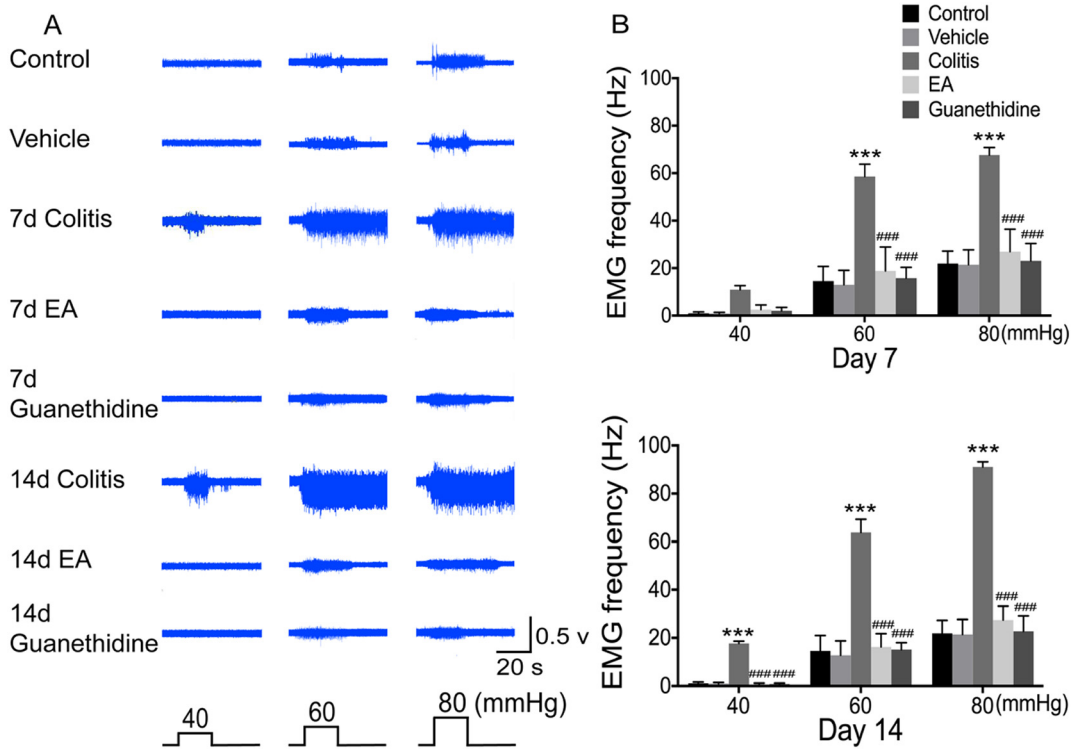

Fig. 2. EA and guanethidine attenuated EMG response evoked by CRD in colitis rats. (A) EMG tracings evoked by 20-second CRD (40, 60, and 80 mmHg) in different groups. (B) CRD to 40 mmHg did not evoke any EMG activity in control and vehicle rats, but elicited robust EMG reflexes, which indicated the gradual development of colonic hypersensitivity in colitis rats. EA and guanethidine suppressed all VMR reflexes under 40 mmHg CRD. 60 and 80 mmHg CRD evoked stronger VMR reflexes in colitis rats, which were attenuated by EA and guanethidine ($^{***}P < .001$ vs. control; $^{\#\#\#}P < .001$ vs. colitis. n = 6 for each group).

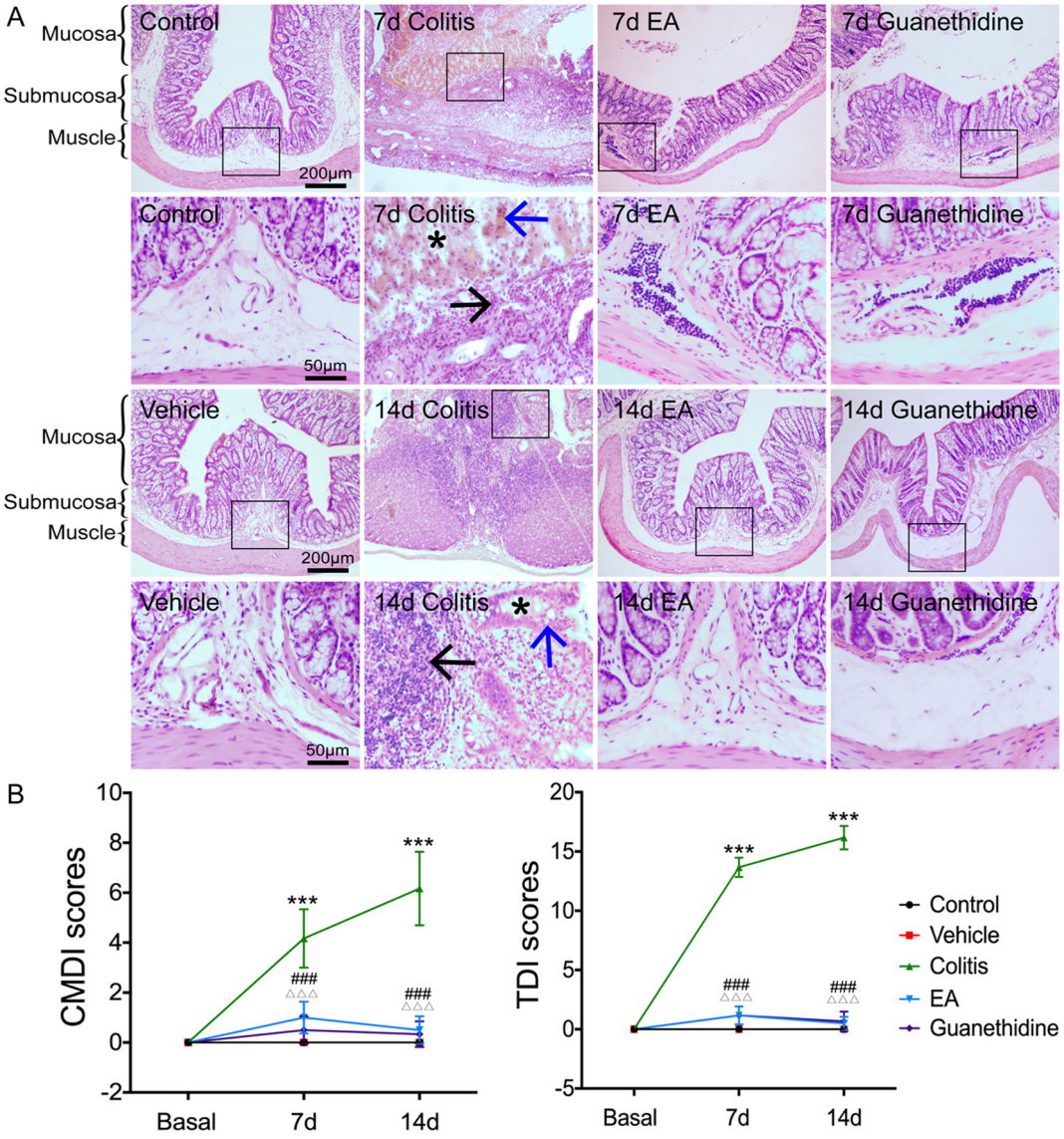

Fig. 3. Histologic injury of colon tissues following TNBS-induced colitis was attenuated by EA and guanethidine. Photomicrographs represent H&E-stained colon tissues. (A) Colon mucosa of rats in each group on days 7 and 14. Control and vehicle groups exhibited normal colon mucosa. Mucosa of colitis rats show severe inflammation with inflammatory cell infiltration (black arrows), goblet cell depletion (blue arrows), edema (*) on days 7 and 14. EA and guanethidine interventions ameliorated mucosal inflammatory cell infiltration on day 7, and continued to improve until day 14. (B) Both CMDI and TDI scores were significantly decreased by EA and guanethidine on colitis rats ($^{***}P < .001$ vs. control; $^{\#\#\#}P < .001$ vs. colitis. n = 6 for each group). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4D) DRGs. Thus, the projected neurons in L6 DRG were overlapped from both the colon and ST 36.

L6 DRG was chosen to observe the role of sympathetic sprouting to the sensory neurons induced by colitis. By employing TH to label sympathetic fibers and CGRP to label sensory neurons in DRG, the distribution of TH and CGRP immune-positive neurons and nerve fibers were observed. In the control and vehicle groups, there were few TH and CGRP positive neurons scattered in L6 DRG, and no abnormal growth of sympathetic fibers was found in any sections. However, colitis rats exhibited enhanced TH (yellow arrows, Fig. 5Ac-d) and CGRP (blue arrows, Fig. 5Ac-d) positive neurons and fibers in L6 DRG, but no double labeled neurons or fibers were observed. Sprouting of sympathetic fibers into the DRG cell layer toward sensory neurons was clearly observed in L6 DRG of the colitis rats (white arrows, Fig. 5A, i-j). The length of fibers in L6 DRG of colitis rats further increased on day 7 (colitis vs. vehicle: 16.8 ± 3.9 mm vs. 2.9 ± 0.6 mm, $P < .001$) and persisted on day 14 (33.7 ± 4.3 mm, $P < .001$) in colitis rats, as well as the number of TH-IR (colitis vs. vehicle: 7d: 35.8 ± 5.5 vs. 11.0 ± 2.9; 14d: 44.5 ± 4.8, $P < .001$) and CGRP-IR neurons (colitis vs. vehicle: 7d: 246.2 ± 6.2 vs. 84.2 ± 8.1; 14d: 369.3 ± 5.8, $P < .001$) (Fig. 5B). EA significantly inhibited the expression of TH in fibers (4.2 ± 0.7, $P < .001$) and neurons (13.5 ± 3.0, $P < .001$), as well as number of CGRP-IR neurons (91.7 ± 7.7, $P < .001$) in L6 DRG on day 7, with a similar value on day 14 (Fig. 5B). The same trends occurred after guanethidine application, indicating similar effects of EA and guanethidine. Also, there were some TH-IR fibers and CGRP-IR neurons in L5 DRG (Fig. 6), but no obvious changes were observed after TNBS irritation (Fig. 6Ac-d, Fig. 6B).

4. Discussion

The present study demonstrated that both colonic hypersensitivity and secondary hindpaw hyperalgesia were elicited in TNBS-induced colitis rats. Hyperalgesia was caused by overexpressed TH-IR neurons

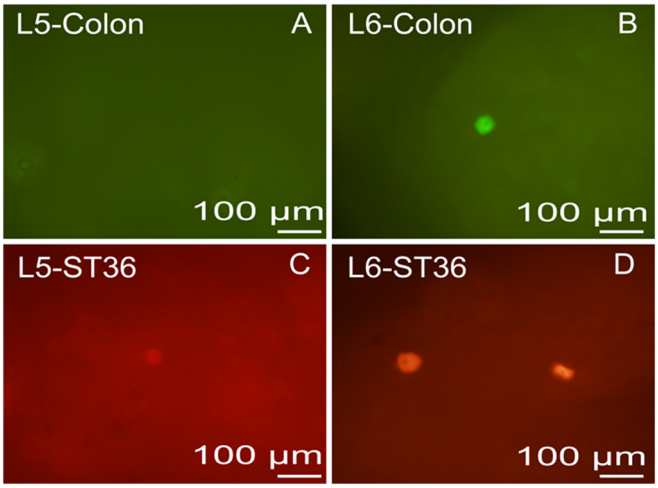

Fig. 4. Retrograde labeling of the colon and ST 36 in L5 and L6 DRGs. CTB-488 labeled colon afferent neurons scattered in L6 DRG (B), but not in L5 (A). CTB-555 labeled sensory neurons from ST 36 were distributed in both L5 (C) and L6 DRGs (D).

and excessive sprouting of TH-IR fibers toward CGRP-positive sensory neurons in L6 DRG. Electroacupuncture (EA) at acupoints ST 36 and ST 37 alleviated the hyperalgesia and attenuated inflammatory damage in the distal colon by inhibiting TH expression in L6 DRG.

Chronic pain originating in internal organs can present in other areas of the body. Patients with visceral pain often exhibit a wide variety of somatic symptoms, including back pain, migraine headaches, and muscle pain (Mayer and Raybould, 1990; Verne et al., 2001). In some animal models such as chronic colitis (Zhou et al., 2008) and uterine inflammation (Wesselmann and Lai, 1997), somatic hyperalgesia also appeared similar to the referred pain in humans. Possible explanations of the neural pathways mediating referred pain has are the axon reflex and antidromic activation of afferent fibers, and an interneuron communication in the spinal cord via dorsal root reflexes (DRRs), resulting in neurogenic plasma extravasation and induction of neurogenic inflammation in the related dermatomes (Lobanov and Peng, 2011). Viscerosomatic facilitation has also been found to be related to central sensitization (Verne et al., 2012). Noradrenergic sprouting within the DRG was first observed in peripheral nerve injury (Mclachlan et al., 1993). This observation was followed by the discovery of sensory-sympathetic coupling in rat and human DRG following peripheral nerve injury (Shinder et al., 1999). In neuropathic pain, basket-like skeins formed by exuberant sprouting of sympathetic postganglionic axons around the somata of primary sensory neurons in DRGs were also observed in chronic constriction injury rats (Guo et al., 2017). Furthermore, sprouting TH-IR nerve fibers were also found to form basket-like structures around colon-innervating DRG neurons in colonic inflammation (Xia Jr et al., 2011), within the occurrence of referred somatic hyperalgesia (Lü et al., 2019). The present study demonstrated that TH-IR sprouting toward sensory neurons in DRGs is one of the mechanisms of referred somatic hyperalgesia due to visceral inflammation.

Under physiologic conditions, sympathetic fibers labeled by TH only participate in modulating the concomitant blood vessels surrounding the sensory neurons but do not correlate with sensory neurons within the DRG (Xie et al., 2010). In nociception/pain, TH-IR fibers were found increased as indicative of sympathetic sprouting (Brumovsky, 2016). Thus, in the present study TH was applied to label the sympathetic nerve fibers. Increasing attention is being paid to the importance of TH-IR DRG neurons in sensation and pain mechanisms (Brumovsky, 2016). In this study, we also observed the excessive numbers of sympathetic fibers that sprouted toward sensory neurons as well as the increasing number of TH-IR neurons in L6 DRG, which played an important role in colonic hypersensitivity and secondary hind paw hyperalgesia after TNBS irritation.

As a rate-limiting enzyme, TH is broadly expressed in noradrenergic and dopaminergic neurons in the central nervous system, as well as in the peripheral sympathetic system. The dopaminergic nature of TH-expression neurons has also been confirmed, and dopamine receptors 1 to 5 (D1-5Rs) have also been reported in rat DRG neurons. It has been shown that dopamine inhibited transient receptor potential vanilloid type 1 (TRPV1) receptor in dorsal root ganglia nociceptive neurons via D1/D5 dopamine receptors but not D2 dopamine receptors (Chakraborty et al., 2016). In fact, this dopamine induced TRPV1 inhibition was through presynaptic inhibition. Up to now, as discussed by Wood (Wood, 2008), the role of central dopamine in pain and analgesia depends on the level at which the effects are evaluated (spinal vs. supraspinal), the pain model employed, and the related receptors. The roles of noradrenaline in pain modulation also vary from an antinociceptive to a pronociceptive. The expression of novel noradrenergic receptors induced by injuries may have contributed to the peripheral pronociceptive effect of noradrenaline, while the peripheral antinociceptive effect of noradrenaline is associated not only with sprouting of sympathetic nerve fibers (as we observed), but also with the pronociceptive alterations in the ion channel properties in the primary afferent nociceptors (Pertovaara, 2013). The central noradrenergic system contributes to feedback inhibition of pain following injuries, predominantly through action on α2-adrenocepters. It has been observed noradrenaline strongly down-regulates the activity of presynaptic TRPV1 channels in DRG neurons through α2-adrenocepters on the central terminals of nociceptors (Chakraborty et al., 2017). Taken together, previous studies showed that the roles of dopamine, noradrenaline in pain and analgesia are mainly related to the central descending inhibition of pain. In addition, a sensory neuron source of peripheral noradrenaline release was also suggested (Brumovsky et al., 2012). Therefore, the mechanism of catecholaminergic neurons of the DRG in pain modulation still needs to be further investigated. So far studies have shown that the TH expression is down-regulated by peripheral nerve injury, however, changes in TH expression after insults of tissue inflammation remain unclear (Brumovsky, 2016). Of interest is that differences have been observed when comparing non-visceral and visceral TH-expression DRG neurons. Firstly, non-visceral TH-expression DRG neurons are non-peptidergic and do not bind IB4. In contrast, a large percentage of TH- expression neurons targeting the colorectum or the bladder of the mouse are peptidergic (Brumovsky et al., 2012). Among the TH-expressed DRG neurons, visceral sources were small- and medium-sized, while non-visceral sources were small-sized. Therefore, non-visceral and visceral TH-expressed DRG neurons may play different roles in pain modulation.

EA at ST 36 and ST 37 has long been used to treat chronic colitis in animal and human studies (Li et al., 2015; Yang et al., 2014). EA has been demonstrated to ameliorate chronic pain by the descending pain inhibitory pathways involving spinal opioids, adrenergic, dopaminergic, serotonergic, and cholinergic receptors, as well as the reduction in nerve growth factor (NGF)-stimulated hyperalgesia (Fang et al., 2017; Zhao, 2008; Aloe and Manni, 2009). Recently, EA was found to alleviate colonic hypersensitivity and secondary hind paw hyperalgesia after colonic inflammation by restoring the enhanced threshold of C-fiber-evoked field potentials and facilitating spinal long-term potentiation in colitis rats, and suppression of exaggerated nociceptive signaling transmission in the spinal dorsal horn (Lü et al., 2019). However, whether EA is involved in sensory-sympathetic coupling in DRGs remains unclear.

In the present study, it was observed that EA and guanethidine significantly reduced TH expression in L6 DRG of colitis rats, and alleviated local inflammatory response as well as visceral and referred somatic hypersensitivity. Guanethidine is a sympatholytic antagonist that blocks signal transmission of noradrenergic neurons by displacing norepinephrine, which is selectively concentrated by noradrenergic

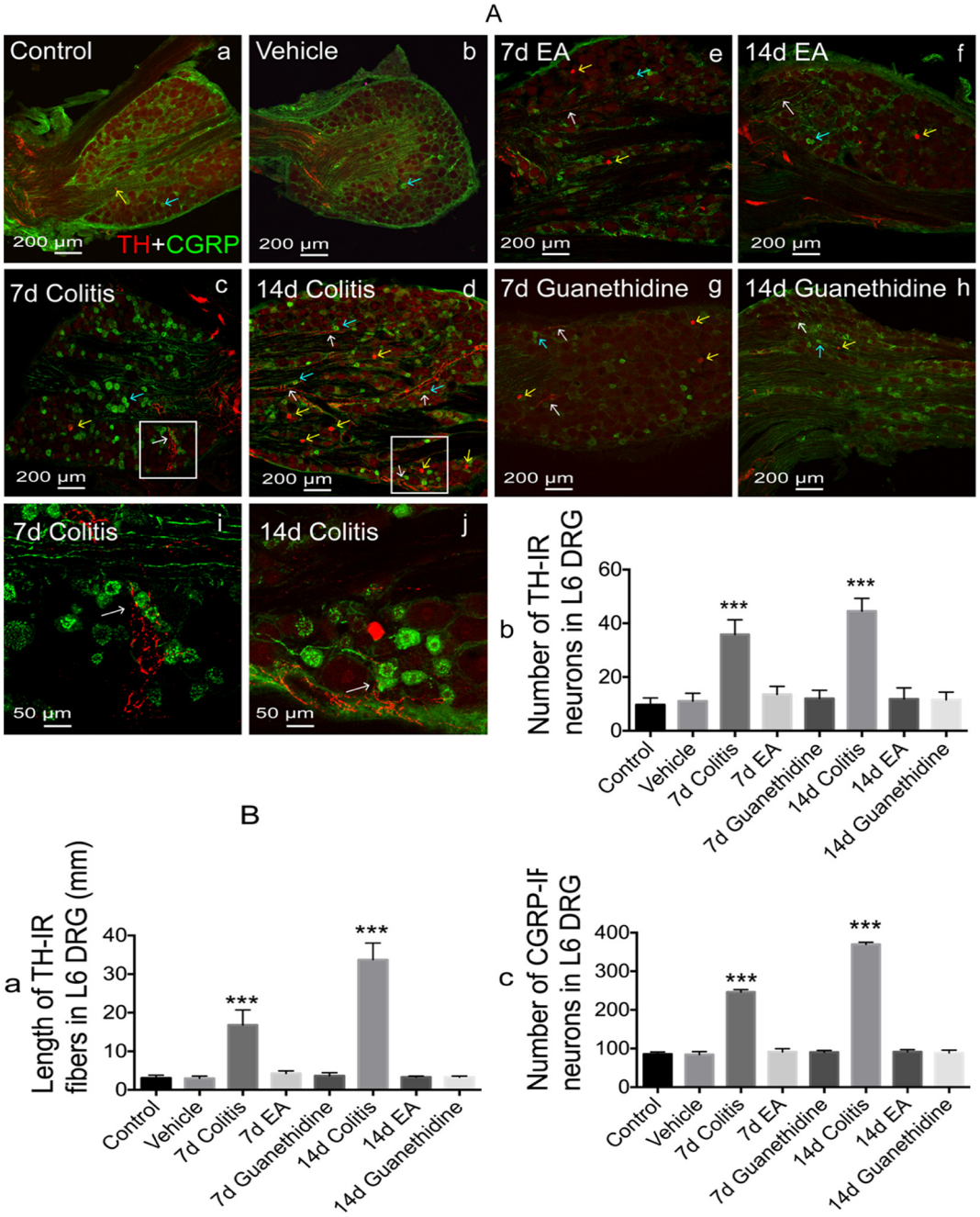

Fig. 5. TH and CGRP immunoreactivity were expressed in L6 DRG. More immunoreactive neurons of TH (yellow arrows) and CGRP (blue arrows) as well as TH immunoreactive fibers (white arrows) sprouting toward the sensory neurons were demonstrated on day 7 (Ac, i) and day 14 (Ad, j) of colitis rats compared with the control (Aa) and vehicle (Ab) animals. Ai and Aj were magnified images of Ac and Ad respectively. EA (Ae-f) and guanethidine (Ag-h) inhibited the expressions of TH and CGRP as well as sympathetic sprouting in L6 DRG. Bar graphs represent the statistical results. Lengths of TH-IR fibers (Ba) as well as TH -IR (Bb) and CGRP -IR (Bc) neurons were significantly increased in L6 DRG of colitis rats and were inhibited by both EA and guanethidine (***$P < .001$ vs. control, n = 6 for each group). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nerve endings, and has a strong affinity for its target tissues. When guanethidine enters the nerve endings, norepinephrine is displaced from intraneuronal storage and is gradually depleted and prevented from being reabsorbed from the synaptic space, which leads to sympathetic blockade (Pertin et al., 2007). Based on these results, it is speculated that similar to guanethidine, EA alleviated visceral hypersensitivity and referred pain by inhibiting TH expression in DRG, which may be one of the mechanisms by which EA attenuates symptoms of colitis.

The effects of neurochemical changes on damaged DRG neurons in the appearance of TH-IR neurons/fibers have also been suggested. Morphological studies showed co-expression of TH with neurochemical components in DRG neurons including NPY (Xue et al., 1987; Marchand et al., 1999) and SP (Leblanc, 1990). NGF sources within the DRG have also been suggested to be responsible for initiating sympathetic sprouting following peripheral axotomy (Jones, 1999). The relationship between neurochemical changes in the damaged neurons and the appearance of TH-IR neurons/fibers should be investigated in the future.

Thus, our study is the first to observe that the application of electroacupuncture leads to the suppression of TH-IR expression in the dorsal root ganglia. Further research is needed to explore how electroacupuncture suppresses TH-IR expression in visceral inflammation.

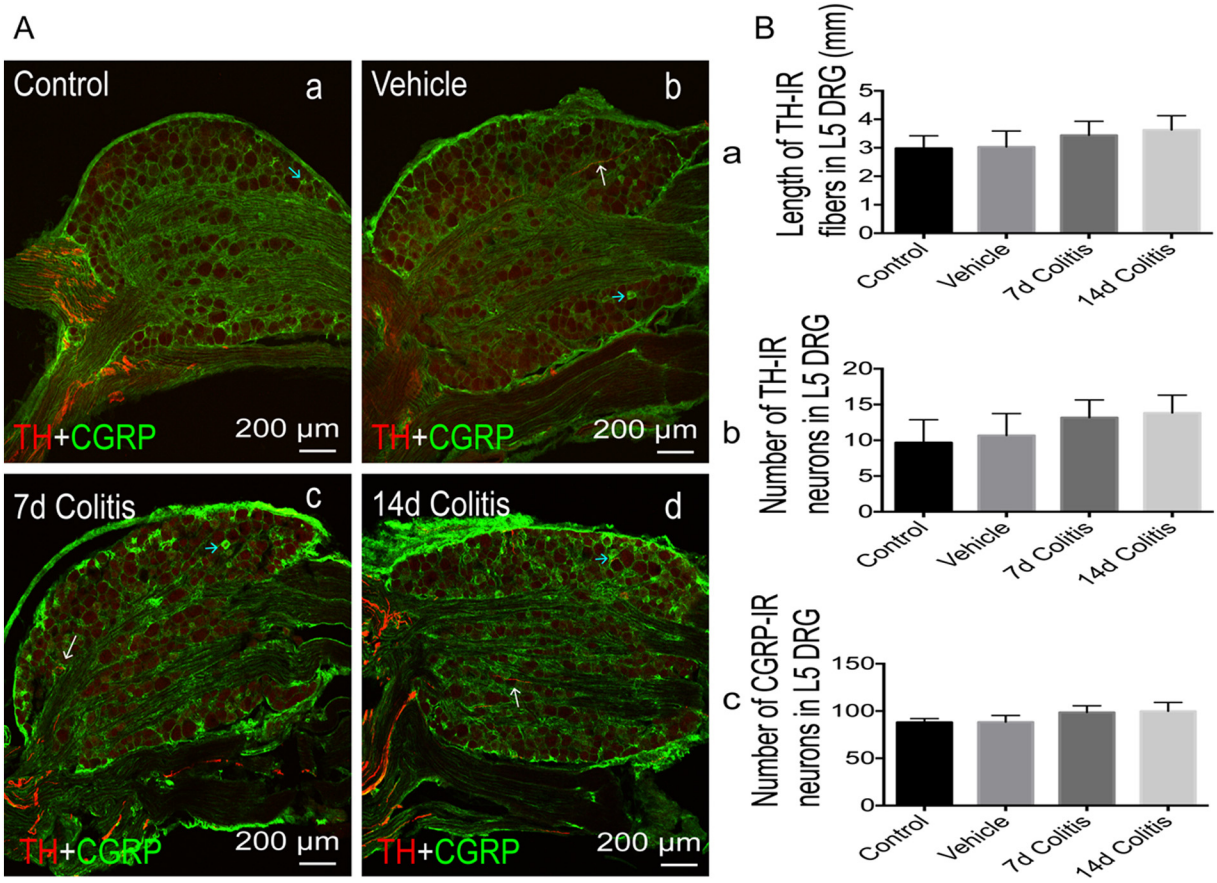

Fig. 6. TH and CGRP immunoreactivity were expressed in L5 DRG. Some TH-IR fibers (white arrow) and CGRP-positive sensory neurons (blue arrows) were observed in L5 DRG among the 4 groups (Aa-d). There was no difference among the 4 groups on the length of TH-IR fibers (Ba), the number of TH-IR (Bb) and CGRP-IR (Bc) neurons. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

5. Conclusion

Increased TH-IR neurons and excessive TH-IR sprouting toward sensory neurons in L6 DRG of colitis rats occurred in the development of colonic inflammation accompanying visceral and referred hindpaw hypersensitivity after TNBS irritation. Electroacupuncture at ST 36 and ST 37 acupoints alleviated colonic inflammation and attenuated hypersensitivity, which is likely mediated by inhibiting TH-IR sprouting in L6 DRG.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

X. H. Jing conceived and designed the experiments; Y.L. Wang, Y.S. Su, W. He performed the experiments; X. H. Jing, Y.L. Wang, Y.S. Su, W. He wrote the paper. All the authors approved the final version of the manuscript.

Declaration of Competing Interests

The authors declare no financial interests or other conflicts of interest.

Submission declaration and verification

The work done has not been considered or published elsewhere in any journal.

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