

Electroacupuncture Attenuates Visceral Pain and Reverses Upregulation of TRPV1 Expression in Adult Rats with Neonatal Maternal Deprivation

Hongyan Zhu^{1*}, Shufen Hu^{2*}, Xiuhua Miao¹, Ying Xiao², Guangyin Xu^{1,2#}

¹Center for Translation Medicine, the Affiliated Zhangjiagang Hospital of Soochow University, Zhangjiagang, China

²Jiangsu Key Laboratory of Translational Research and Therapy for Neuro-Psycho-Diseases, Institute of Neuroscience, Soochow University, Suzhou, China

Email: #guangyinxu@suda.edu.cn

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Abstract

Irritable bowel syndrome (IBS) is characterized by chronic visceral hypersensitivity that accompanied by altered bowel movement. However, the treatment options are very limited. The aim of this study was to investigate effects of electroacupuncture (EA) on visceral hypersensitivity in a rat model of IBS and to explore the underlying mechanisms of EA effects. Visceral hypersensitivity was established by neonatal maternal deprivation (NMD) in male rats on postnatal days 2 - 15. Behavioral experiments were conducted at the age of 7 weeks. Treatment with EA at *Zusanli* (stomach-36, ST-36) significantly reduced abdominal withdrawal reflex (AWR) scores in NMD rats but not in age-matched healthy control rats. In addition, EA treatment hyperpolarized resting membrane potentials, increased the rheobase and reduced the numbers of action potentials evoked by 2 and 3 times rheobase current stimulation of dorsal root ganglion (DRG) neurons innervating the colon. NMD markedly enhanced expression of TRPV1 in colon related DRGs while EA treatment drastically suppressed the expression of TRPV1 in DRGs of NMD rats. These data suggest that EA treatment produced an analgesic effect, which might be mediated at least in a part by suppression of TRPV1 expression and by inhibition of neuronal excitability of primary sensory neurons in rats with chronic visceral pain.

*These authors contribute to this work equally.

#Corresponding author.

Keywords

Electroacupuncture, Irritable Bowel Syndrome, Chronic Visceral Pain, Dorsal Root Ganglion, TRPV1

1. Introduction

Irritable bowel syndrome (IBS) is one of the most common functional gastrointestinal disorders, featured by chronic visceral hypersensitivity (CVH) and altered bowel movement. The treatment of CVH in IBS patients remains challenging for the clinicians. Both acupuncture and electroacupuncture (EA) have been applied in treating many diseases including chronic pain for the past several decades [1]-[3]. It has been demonstrated that EA treatment produces a significant improvement both in general well-being and in symptoms of chronic visceral hypersensitivity [4]. Although these data suggest that EA could be a promising method to treat chronic pain in patients, scientific evidence is very little and frequently contradictory. Thus, further observations of the efficacy and the underlying mechanisms of EA are definitely needed.

We have previously reported that EA treatment significantly inhibited the neuronal excitability of dorsal root ganglion (DRG) neurons and attenuated the CVH in a rat model of IBS induced by neonatal colonic inflammation [5]. The sensitization of DRGs is considered to be a major cause of aberrant pain induced by neonatal maternal deprivation (NMD) [6] [7]. However, whether EA produced an inhibitory effect on the hyperexcitability of DRG neurons of adult rats with NMD is not clear. Emerging evidence has suggested that TRPV1 played an important role in inflammatory pain [8] and neuropathic pain [9]. A growing body of evidence suggests a pro-nociceptive role for TRPV1 in IBS [10] [11]. In a painful IBS model induced by neonatal colonic inflammation, TRPV1 expression is significantly increased in DRGs of rats [5]. However, roles of TRPV1 signaling pathway in NMD-induced visceral pain remain largely unknown. It would be of considerable interest to explore whether EA exerts its therapeutic effect via the inhibition of TRPV1 in DRGs of adult rats with NMD.

We therefore hypothesized that TRPV1 activation and sensitization of DRG neurons participated in NMD-induced visceral hypersensitivity and that EA treatment produced analgesic effects via suppression of TRPV1 expression in DRGs. To test this hypothesis, we investigated roles of TRPV1 and excitability of DRG neurons in adult NMD rats with EA treatment. Our results indicate that NMD-induced visceral hypersensitivity is likely mediated by upregulation of TRPV1 expression and an increase of cell excitability in DRG neurons, and that EA treatment relieves visceral hypersensitivity through the inhibition of TRPV1 upregulation and the reduction of excitability of DRG neurons innervating the colon in this setting.

2. Materials and Methods

2.1. Induction of Chronic Visceral Pain

Experiments were conducted on adult male Sprague-Dawley (SD) rats. Handling and care of rats were approved by the Institutional Animal Care and Use Committee of Soochow University and were in accordance with the guidelines of the International Association for the Study of Pain. As described previously, CVH was established by neonatal maternal deprivation (NMD) [12] [13]. Briefly, separating pups from the maternity cage and placed in isolated cages were performed 3 hours daily from postnatal day (PND) 2 to 15. The inside temperature of the isolated cages was kept around 32°C by using an electric blanket. After the separation, pups were returned to their dams. Pups in the control (CON) group were not exposed to handling and were maintained in their maternity cage with the dam. Only male pups were used in the present study. Experiments were performed at 7 weeks of age in a total of 38 rats (CON: n = 6 for behavior test, n = 7 for patch clamp recordings, n = 6 for western blot analysis; NMD: n = 6 for behavior test, n = 7 for patch clamp recordings, n = 6 for western blot analysis).

2.2. Behavioral Studies to Measure Nocifensive Responses to Graded CRD

CVH was determined by assessing the behavioral responses to colorectal distention (CRD) at the age of 7 weeks as described previously [14] [15]. Under mild sedation by 1% Brevital (25 mg/kg, i.p.), a flexible latex balloon (6 cm) attached to a tygon tubing was inserted 8 cm into the descending colon and rectum via the anus. The

plastic tube was kept in place by taping the tubing to the rat tail. The rat was positioned in small Lucite cubicles for 30 min to adapt. CRD was determined by quickly inflating the balloon to a constant pressure using a sphygmomanometer. The balloon was inflated to 20, 40, 60 and 80 mmHg, for 20 s followed by 2 min rest. Behavioral responses to CRD were determined by visual observation of the abdominal withdrawal reflex (AWR) by an observer in a blinded manner. The AWR score was assigned as follows: 0 with normal behavior, 1 with slight head movement without abdominal response, 2 with contraction of abdominal muscles, 3 with lifting of abdominal wall or 4 with body arching and lifting of pelvic structures.

2.3. Electroacupuncture Treatment

Electroacupuncture (EA) treatment was given at ST-36 (stomach-36, Zusanli) of control and NMD rats [1] [4]. A pair of stainless steel suturehook-shaped needles were inserted a depth of 5 mm into the skin and underlying muscles of bilateral ST-36. Stimulation was delivered through the needles inserted into the acupoints by an EA apparatus (Model G-6805-2; Shanghai Medical Electronic Apparatus Company, Shanghai, China) with a constant rectangular current of alternating trains of dense-sparse frequencies (100 Hz for 1.05 seconds and 2 Hz for 2.85 seconds alternately, pulse width, 0.1 ms). These parameters have been reported as the most optimum condition to induce maximal release of met-enkephalin and dynorphin A. Electrical stimulus intensity was determined as the threshold for a detectable muscle twitch (approximately 1 mA). The stimulation was applied for 30 minutes once to observe the acute effect or 30 minutes once per day for consecutive 5 days to observe the accumulative effect in these rats.

2.4. Cell Labeling

DRG neurons innervating the colon were labeled by injection of 1, 1'-dioleil-3, 3, 3', 3-tetramethylindocarbocyanine methanesulfonate (DiI, Invitrogen) into the colon wall before patch clamp recordings. Briefly, adult rats (~6 weeks old) were anaesthetized by a mixture of ketamine (80 mg/kg, i. p.) and xylazine (5 - 10 mg/kg, i. p.). Surgery was performed to expose the colon. DiI was injected in ~1 μ l volume at 10 sites on the exposed colon extending from the level of the bladder to ~6 cm in an oral direction [16]. The needle was left in place for 1 min to prevent DiI leakage. After surgery, these rats were returned to their house and were free access to drinking water and standard food pellets.

2.5. Dissociation of DRG Neurons and Patch Clamp Recordings

NMD (~7 weeks) or age-matched control rats were euthanized by cervical dislocation 10 days after DiI injection. Bilateral DRGs (T13-L2) were dissected out for patch clamp recording as described previously [13]. In brief, these DRGs were moved to an ice-cold, oxygenated fresh solution, containing (in mM): 130 NaCl, 5 KCl, 2 KH_2PO_4 , 1.5 CaCl_2 , 6 MgSO_4 , 10 glucose and 10 HEPES, pH 7.2 (Osmolarity: 305 mOsm). The dissecting solution also included collagenase D (1.8 - 2.0 mg/ml; Roche, Indianapolis, Indiana, USA) and trypsin (1.2 - 1.5 mg/ml; Sigma, St Louis, Missouri, USA). After incubation in this solution for 1.5 h at 34.5°C, DRGs were then taken out from the enzyme solution, washed and moved to 0.5 ml of the dissecting solution containing DNase (0.5 mg/ml; Sigma, St Louis, Missouri, USA). A single cell suspension was then formed by repeated trituration through flame-polished glass pipettes. The normal external solution included (in mM): 130 NaCl, 5 KCl, 2 KH_2PO_4 , 2.5 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose, with pH adjusted to 7.2 with NaOH, Osmolarity: 295 - 300 mOsm. DiI-labeled neurons were selected by their fluorescence under the fluorescent microscope (Olympus IX71) for the patch-clamp recordings. Recording pipettes were pulled from borosilicate glass tubing using a horizontal puller (P-97, Sutter Instruments). Patch pipettes had a resistance of 4 - 7 M Ω when they were filled with the pipette solution containing (in mM): 140 potassium gluconate, 10 NaCl, 10 HEPES, 10 glucose, 5 EGTA and 1 CaCl_2 , pH = 7.25 adjusted with KOH; Osmolarity = 292 mOsm. Resting potential (RP) and action potentials (APs) evoked by current stimulation were recorded by an HEKA EPC10 patch clamp amplifier (HEKA; Germany). Electrophysiological recordings were performed at room temperature of ~22°C. Data were saved in a personal computer for a later analysis using FitMaster (HEKA; Germany).

2.6. Western Blot Experiments

The protein expression of TRPV1 from colon DRGs (T13-L2) of control, NMD and NMD + EA rats was deter-

mined by western blotting, as described previously [13]. The primary antibodies were rabbit anti-TRPV1 (1:1000) and mouse anti- β -actin (1:1000). β -actin was used as a loading control. All protein samples were normalized to β -actin.

2.7. Data Analysis

Data are shown as mean \pm SD. Statistical analysis were determined using software including OriginPro 8 (OriginLab, US) and Matlab (Mathworks, US). Normality of all data was first examined before further analyses. Significance of all data was assessed using Wilcoxon rank sum test. The level of significance was set at $p < 0.05$.

3. Results

3.1. EA Treatment Attenuates AWRs in NMD Rats

To determine whether EA suppressed visceral hypersensitivity of NMD rats, CRD was applied to examine the behavior responses of NMD rats before (PRE) and after EA treatment. Compared with PRE-EA, EA treatment for 30 minutes markedly suppressed the AWRs at 0 and 30 min (**Figure 1(a)**, * $p < 0.05$, $n = 6$, Wilcoxon rank sum test). We next determined whether the same EA treatment also plays an effect on age-matched healthy control rats. EA treatment did not have any effect on AWRs in control rats when compared with PRE EA treatment (**Figure 1(b)**, $n = 6$). These results suggest that EA treatment plays a therapeutic effect on visceral hypersensitivity of NMD rats.

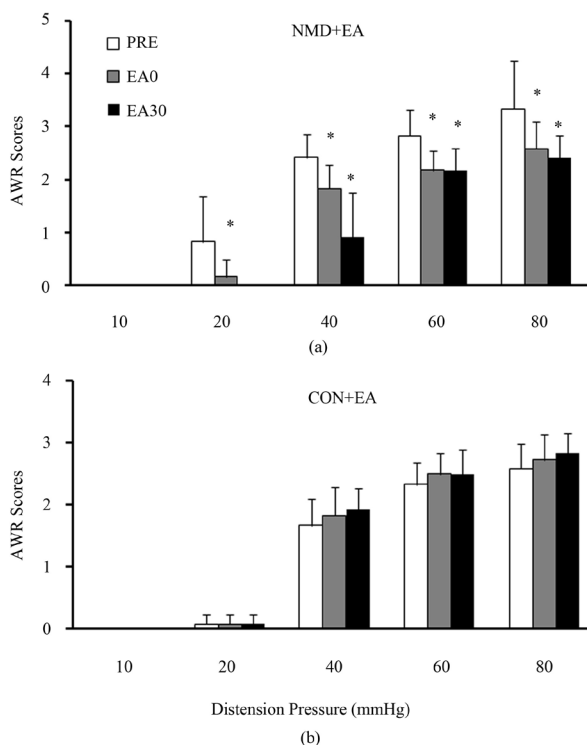


Figure 1. EA treatment attenuated abdominal withdrawal reflex (AWR) scores. (a) Time course of effect of one time EA treatment (30 min) on NMD rats. EA at ST-36 produced the analgesic effect in NMD rats when compared with PRE. This effect lasted for about 30 minutes within our observation time period. $n = 6$ rats, * $p < 0.05$. (b) Time course of effect of EA treatment on control rats. The EA treatment had no effect on AWRs in age-matched healthy control rats. $n = 6$ rats, $p > 0.05$ when compared with PRE.

3.2. EA Treatment Inhibits Neuronal Hyperexcitability of DRG Neurons Innervating the Colon

Since EA treatment attenuated the AWRs in NMD rats, we next investigated whether this therapeutic effect is mediated by suppression of excitability of colon neurons. To determine the effect of EA on neuronal excitability, patch clamp recordings were performed on colon specific DRG neurons that were labeled by DiI (**Figure 2(a)** and **Figure 2(b)**, arrow). EA treatment significantly hyperpolarized resting membrane potentials (RP, **Figure 2(c)**). The RPs were -49.71 ± 4.3 (n = 28 cells), -45.24 ± 3.47 (n = 41 cells) and -47.15 ± 3.46 (n = 13 cells) for CON, NMD and EA, respectively. In addition, EA treatment dramatically enhanced rheobase when compared with the NMD group (**Figure 2(d)**, CON: 0.24 ± 0.11 nA, n = 28; NMD: 0.17 ± 0.09 nA, n = 41; EA: 0.3 ± 0.1 nA, n = 13, **p < 0.01, compared with CON; #p < 0.05, compared with NMD. Wilcoxon rank sum test). EA treatment markedly decreased the number of action potentials (APs) evoked by 2X and 3X rheobase current stimulation (**Figures 3(a)-(c)**). The average numbers of APs evoked by 2X rheobase stimulation were 1.05 ± 0.49 for CON (n = 28 cells), 2.64 ± 3.01 for NMD (n = 41 cells) and 1.23 ± 0.44 for EA (n = 13 cells), respectively (**Figure 3(b)**, *p < 0.05, compared with CON; #p < 0.05, compared with NMD. Wilcoxon rank sum test). The average numbers of APs evoked by 3X rheobase stimulation were 1.96 ± 1.6 for CON (n = 28 cells); 3.5 ± 3.01 for NMD (n = 41 cells) and 1.00 ± 0.00 for EA (n = 13 cells), respectively (**Figure 3(c)**, *p < 0.05, compared with CON; #p < 0.05, compared with NMD. Wilcoxon rank sum test).

Additional membrane properties of DRG neurons innervating the colon were also examined and the results were shown in **Table 1**. Neither NMD nor EA treatment altered the cell size, input resistance, AP threshold, amplitude, overshoot, duration and latency of DRG neurons innervating the colon (**Table 1**).

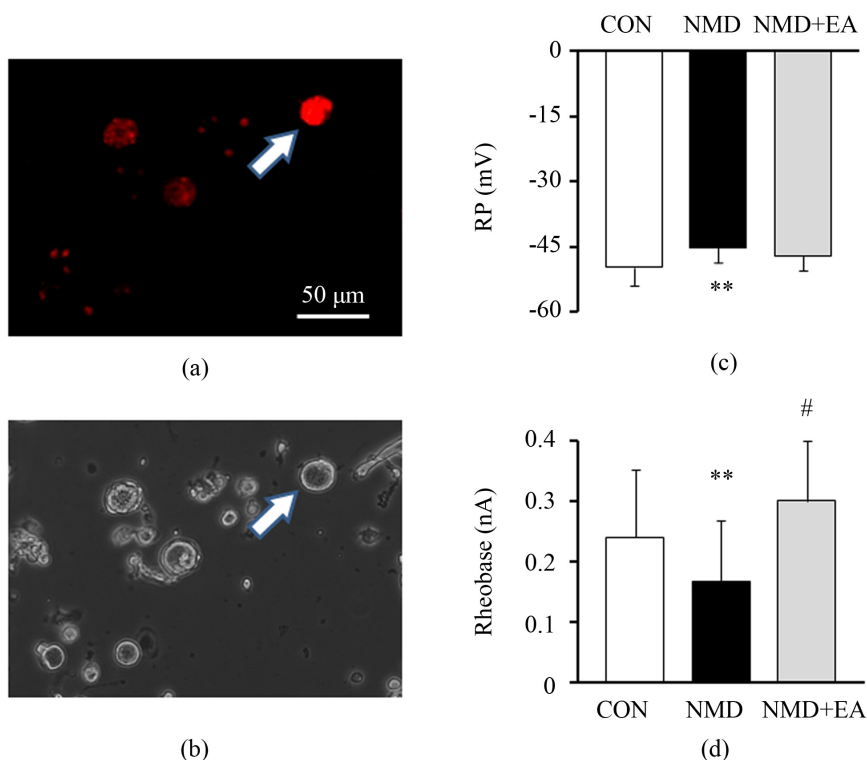


Figure 2. EA treatment hyperpolarized resting membrane potentials and enhanced rheobase. (a) DiI-fluorescence (upper) and (b) bright-field (lower) images of DRG neurons. A colon innervating DRG neuron is shown in red (arrow). Bar = 50 μ m. (c) Bar graph showed NMD significantly depolarized rest membrane potential (RP) while EA treatment reversed the depolarization of RP. n = 7 rats for each group, **p < 0.01, compared with CON. (d) Bar graph showed NMD markedly decreased rheobase of current stimulation of colon specific DRG neuron while EA treatment increased rheobase of current stimulation of colon specific DRG neuron. n = 7 rats for each group, **p < 0.01, compared with CON; #p < 0.05, compared with NMD.

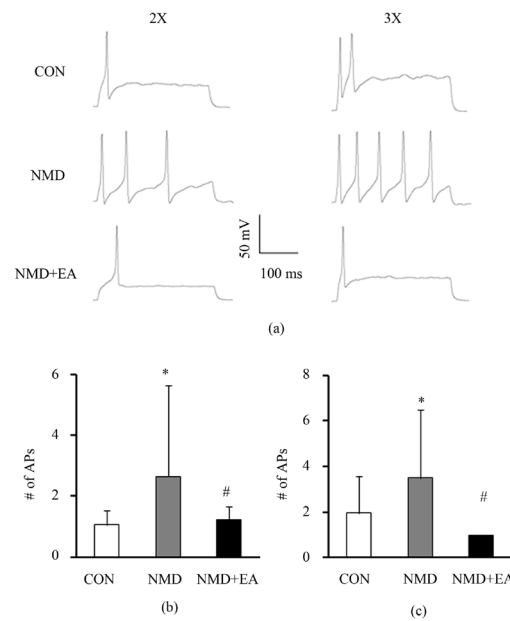


Figure 3. EA treatment reduced numbers of action potentials (APs) evoked by current stimulation. (a) Examples of APs by 2× and 3× rheobase current injection for control group (CON, top), NMD group (NMD, middle) and electroacupuncture group (NMD + EA, bottom). (b) Bar graph showed NMD significantly increased number of APs evoked by 2× rheobase current stimulation while EA treatment reversed NMD-induced increase in number of APs elicited by 2× rheobase current stimulation. $n = 7$ rats for each group, * $p < 0.05$, compared with CON; # $p < 0.05$ compared with NMD. (c) Bar graph showed NMD greatly increased number of APs evoked by 3× rheobase current stimulation while EA treatment reversed NMD-induced increase in number of APs elicited by 3× rheobase current stimulation. $n = 7$ rats for each group, * $p < 0.05$, compared with CON; # $p < 0.05$ compared with NMD.

Table 1. Membrane characteristics of colon-specific DRG neurons in control (CON), NMD and NMD+EA-treated rats.

	CON	NMD	NMD + EA
Cell Size (μm)	27.8 ± 2.8 ($n = 28$)	26.6 ± 2.35 ($n = 28$)	29.4 ± 2.08 ($n = 13$)
Rin ($\text{M}\Omega$)	674.0 ± 251.3 ($n = 28$)	703.5 ± 199.3 ($n = 28$)	560.4 ± 217.89 ($n = 13$)
RP (mV)	-49.7 ± 4.3 ($n = 28$)	$-45.2 \pm 3.47^{***}$ ($n = 28$)	-47.2 ± 3.46 ($n = 13$)
Rheobase (nA)	0.24 ± 0.11 ($n = 28$)	$0.17 \pm 0.09^{**}$ ($n = 28$)	$0.30 \pm 0.1^{\#}$ ($n = 13$)
# of AP (2xrheobase)	1.0 ± 0.4 ($n = 25$)	$2.6 \pm 3^*$ ($n = 27$)	$1.2 \pm 0.44^{\#}$ ($n = 13$)
# of AP (3xrheobase)	1.0 ± 1.6 ($n = 21$)	$3.5 \pm 3^*$ ($n = 25$)	$1.0 \pm 0.0^{\#}$ ($n = 8$)
AP Threshold (mV)	-16.8 ± 10.69 ($n = 28$)	-18.4 ± 7.22 ($n = 28$)	-16.3 ± 4.25 ($n = 13$)
AP Amplitude (mV)	75.7 ± 14.68 ($n = 28$)	71.0 ± 13.66 ($n = 28$)	79.3 ± 9.93 ($n = 13$)
AP Overshoot (mV)	26.0 ± 13.41 ($n = 28$)	25.8 ± 12.08 ($n = 28$)	32.2 ± 8.86 ($n = 13$)
AP Duration (ms)	3.3 ± 1.0 ($n = 28$)	3.3 ± 1.06 ($n = 28$)	2.9 ± 0.49 ($n = 13$)
Latency (ms)	72.8 ± 101.92 ($n = 28$)	55.1 ± 90.4 ($n = 28$)	50.9 ± 91.57 ($n = 13$)

Values are mean \pm SD, with sample size in parenthesis; Rin, Input resistance; RP, Resting membrane potential; AP, Action potential. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with CON; # $p < 0.05$, compared with NMD.

3.3. EA Treatment Suppresses TRPV1 Expression in DRGs of NMD Rats

To determine the expression of TRPV1 in DRGs of NMD rats, proteins isolated from both sides of T13-L2 DRGs of NMD and age-matched control rats were probed with anti-TRPV1 antibody. Anti-TRPV1 antibody labeled a 90 Kd molecular weight protein. Seven weeks after NMD, the relative densitometry of TRPV1 was 0.29 ± 0.087 ($n = 3$) and 0.56 ± 0.21 ($n = 3$) for control (CON) and NMD, respectively. The levels of expression of TRPV1 were significantly increased after NMD (Figure 4(a), * $p < 0.05$, Wilcoxon rank sum test). EA treatment was administrated at age of 6 weeks once daily for consecutive 5 days. The relative densitometry was 0.91 ± 0.05 ($n = 3$) and 0.59 ± 0.33 ($n = 3$) for NMD and EA, respectively. EA treatment significantly reduced expression of TRPV1 (Figure 4(b), * $p < 0.05$, Wilcoxon rank sum test). Thus, EA treatment reverses the upregulation of TRPV1 expression in colon DRGs isolated from NMD rats.

4. Discussion

In the present study we provided evidence to support our hypothesis that electroacupuncture at acupoints of ST-36 (stomach-36, Zusanli) relieved visceral hypersensitivity in adult rats with neonatal maternal deprivation, as indicated by the inhibitory effects of EA on AWR scores. In addition, our present study demonstrated that EA treatment suppressed TRPV1 expression and reduced cell excitability of DRG neurons innervating the colon, which might be the mechanism by which EA treatment produced analgesic effects on the visceral hypersensitivity. These findings further support the previous reports and provide additional evidence for EA treatment for visceral pain in a rat model of IBS [4] [17].

NMD-induced rat model of visceral pain is a frequently used animal model for study mechanisms of visceral pain. After EA treatment, NMD rats showed a significant reduction in visceromotor responses to colorectal distention while the same EA treatment did alter the AWRs in age-matched healthy control rats. This suggested that EA-produced analgesic effect is not a non-specific effect and most likely is disease-related, further confirming the specificity of EA effects. It seems that the analgesic effect induced by EA in various conditions may be mediated by different mechanisms depending on the specific conditions [12] [18]. We have previously reported that EA treatment inhibited cell excitability of DRG neurons in rats with neonatal colonic inflammation, another rat model of IBS [4]. In the present study, we further confirmed that EA reversed the hyperexcitability of DRG neurons innervating the colon. Moreover, the present findings show that TRPV1 expression was greatly upregulated in colon DRGs and that EA treatment reversed the up regulation of TRPV1 expression in NMD rats. To the best of our knowledge, this is the first report showing an involvement of electroacupuncture in the primary sensory neurons in a painful NMD rat model and inhibition of this pathway underlies the EA-induced analgesic effect in visceral hypersensitivity in this study.

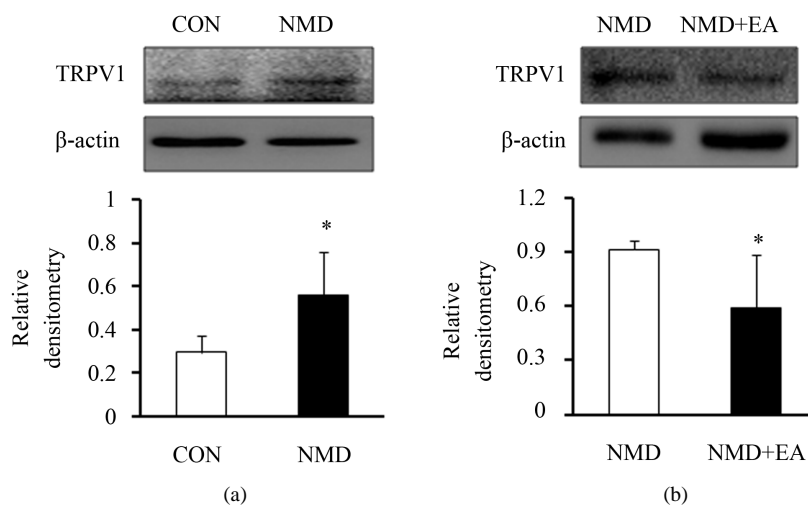


Figure 4. EA treatment suppressed TRPV1 expression. (a) NMD significantly increased TRPV1 expression of colon DRGs when compared with age-matched control group. $n = 3$ rats for each group, * $p < 0.05$. (b) EA treatment markedly decreased TRPV1 expression when compared with NMD group. $n = 3$ rats for each group, * $p < 0.05$.

One of the limitations of the present study is the use of male rats. The purpose of only using male rats is to exclude the possible effects of estrogen on female ones [19] [20]. The female hormones may induce visceral pain periodically compared with male ones although the prevalence of visceral pain is higher in female patients than in male ones. Another limitation is that only ST-36 acupoints were used in the present study. The reason why ST-36 was used is because we have previously showed that ST-36 is an effective acupoint in a rat model of IBS induced by neonatal colonic inflammation [4] and by chronic heterotypical stress as well [1]. Future studies are warranted to investigate the roles of female hormones and other acupoints in this model of visceral pain.

5. Conclusion

In conclusion, the present study demonstrated that NMD led to upregulation of TRPV1 expression and hyperexcitability of DRG neurons innervating the colon. EA treatment at ST-36 significantly attenuates the visceral hypersensitivity in association with reduced expression of TRPV1 and inhibition of colon specific DRG neurons in NMD rats.

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Disclosure

The authors declare no conflict of interest.

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