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# Repeated electro-acupuncture attenuates chronic visceral hypersensitivity and spinal cord NMDA receptor phosphorylation in a rat irritable bowel syndrome model

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ABSTRACT

Acupuncture has been used in clinical trials for the treatment of abdominal pain in patients with irritable bowel syndrome (IBS). However, scientific evidence is still lacking and the underlying mechanism remains largely unexplored. The aim of this study was to examine the effects of repeated administration of electro-acupuncture (EA) on chronic visceral hypersensitivity and on the phosphorylation of spinal cord *N*-methyl-D-aspartic acid (NMDA) receptors in a rat model of IBS. The results showed that repeated administration of EA at bilateral points of Zu-san-li (ST-36) and Shang-ju-xu (ST-37) significantly attenuated chronic visceral hypersensitivity induced in young adult rats by neonatal colon irritation. Such an effect was not seen in either of the two controls: sham-EA at ST-36 and ST-37 without electrical stimulation and EA at control points (BL-62 and tail). Furthermore, rats with chronic visceral hypersensitivity exhibited high-level expression of phosphorylated NMDA receptor subunit 1 (pNR1) in the spinal cord (L4–L5 segments), which was markedly attenuated by EA treatment. In addition, EA at ST-36 and ST-37 neither altered the pain threshold of normal rats nor affected the expression of pNR1 in the lumbosacral spinal cord. Altogether, these data indicate that the EA-mediated attenuation of chronic visceral hypersensitivity is correlated with the down-regulation of NMDA receptors phosphorylation at the spinal level.



## Introduction

Irritable bowel syndrome (IBS) is a common disease characterized by abdominal pain and altered bowel function (diarrhea, constipation or both) that can interfere with the patient's life. The persistent and recurrent abdominal pain is one of the main reasons why people suffering from IBS seek medical help. As no optimal treatment has been found so far, some complementary and alternative therapies (such as acupuncture and massage) have come into use. Clinically, acupuncture has been recognized as a method to relieve IBS-induced abdominal pain (Chan et al., 1997; Xing et al., 2004). In addition, some experimental studies (Cui et al., 2005; Tian et al., 2006; Wu et al., 2008) have shown the inhibitory effect of electro-acupuncture (EA) on chronic visceral hypersensitivity in IBS. These findings suggest that acupuncture may become a promising supplementary treatment for chronic visceral pain in IBS. Nevertheless, some researchers have questioned whether the efficacy of acupuncture is a placebo response (Forbes et al., 2005; Schneider et al., 2006). It is necessary to provide more convincing data to validate the efficacy of acupuncture and to understand the underlying mechanism.

There is clear agreement that the generation of chronic visceral hypersensitivity is closely related to the phenomenon of central sensitization at the spinal level, which is manifested as an increase in dorsal horn neuronal excitability (Bueno et al., 1997; Gebhart, 1999; Al-Chaer and Traub, 2002). Pivotal in the development of spinal cord central sensitization is the activation of the *N*-methyl-D-aspartate (NMDA) receptor, a glutamate-gated ionotropic channel, in neurons that receive primary afferent input (Haley et al., 1990; Woolf and Thompson, 1991). Importantly, recent studies indicate that phosphorylation of NMDA receptor subunit 1 (NR1), an essential subunit of the NMDA receptor, may play a key role in pain hypersensitivity. In these studies, spinal cord NR1 phosphorylation has been demonstrated to be enhanced following inflammation or tissue injury during the development of acute (Zou et al., 2000; Brenner et al., 2004; Caudle et al., 2005; Zhang et al., 2005; Kim et al., 2006; Zhang et al., 2008) or persistent (Gao et al., 2005; Ultenius et al., 2006) somatic pain. This leads to the conjecture that that NR1 phosphorylation is involved in the generation of visceral hypersensitivity.

In the present study, we hypothesized that the development of IBS-related visceral hypersensitivity is correlated with spinal cord NR1 phosphorylation and acupuncture treatment can alter the phosphorylation level of spinal cord NR1. To test this hypothesis, we examined the

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effects of EA on visceral perception and spinal cord NR1 phosphorylation in a rat IBS model. Since the treatment of patients by acupuncture over a period of several days is common practice, we here focused on the effects of repeated administration of EA and set strict controls (EA at control points, sham acupuncture without electric stimulation, and EA in normal animals).

## Materials and methods

### *Animals*

Male Sprague–Dawley rats (younger than 8 days) were obtained from the Experiment Animal Center, Chinese Academy of Sciences Shanghai Branch. Rats were housed in plastic cages containing corn chip bedding and were maintained on a 12 h light:12 h dark cycle (07:00–19:00 h, light cycle; 19:00–07:00 h, dark cycle). Every 12 male neonates were housed with a nursing adult female rat until they were 25 days old. The adult female rat had access to food and water ad libitum. After being separated from the adult female rat, weaned rats were housed with access to food and water ad libitum. Each cage contained 4 rats. All rats in the study were used strictly in accordance with the National Institutions of Health Guide for the Care and Use of Laboratory Animals.

### *Study design*

As in the previous report (Al-Chaer et al., 2000), model rats were subjected to 2 weeks of mechanical colon irritation followed by a resting period of another 2 weeks. To examine the relationship between the expression of phosphorylated NR1 (pNR1) and visceral hypersensitivity, subsequent experiments were conducted in the week (the 6th week) right after the resting period. Behavioral tests and electrophysiological recordings were performed on rats for the assessment of the model. Eight model rats were chosen for abdominal withdrawal reflex (AWR) test, 8 for pain threshold pressure (PTP) measurement, and 8 for electromyogram (EMG) recordings. These model rats were randomly divided into 4 groups ($n$=6 in each group): no treatment; repeated administration of EA at bilateral Zu-san-li (ST-36) and Shang-ju-xu (ST-37); sham acupuncture at bilateral ST-36 and ST-37 without electrical stimulation; and repeated administration of EA at control points (BL-62 and tail). The experimental design was shown in Fig. 1.

### *Neonatal colon irritation*

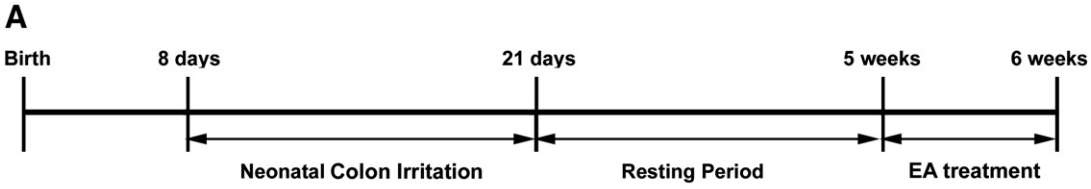
Neonatal rats of 8 days old received colorectal distension (CRD) on a daily basis between the ages of 8 and 21 days. The procedure was modified from previous report (Al-Chaer et al., 2000). Briefly, a silica gel balloon (length: 20.0 mm; diameter: 2.0 mm) was inserted rectally into the descending colon of the rat. The balloon was distended with 0.2 ml of air for 1 min, and then deflated and withdrawn. The distention was repeated twice a day at a 30-min interval.

### *Behavioral tests*

Rats were deprived of food but had free access to water 24 h before behavioral tests. A distension balloon (4 cm in length and made of the finger of a latex glove) was carefully inserted through the anus into the rectum and descending colon of the rat. The tube of the balloon was connected via a Y connector to a 10-ml syringe and a sphygmomanometer. The rat was then placed in a small cubicle (18 cm × 7 cm × 7 cm) on a platform and adapted for 20 min. Graded CRD stimulation (20, 40, 60, and 80 mm Hg) was applied in an ascending manner at 4-min intervals. Each pressure lasted for 20 s and was repeated 3 times to attain an accurate estimate. AWR was scored by two blinded observers according to the scale of Al-Chaer et al. (2000): 0, no behavior response to CRD; 1, head or body movement at the onset of the stimulus; 2, a mild contraction of the abdominal muscles, but no lifting the abdomen off the platform; 3, a strong contraction of the abdominal muscles and lifting the abdomen off the platform, no lifting the pelvic structure off the platform; 4, body arching and lifting the pelvic structure and scrotum. PTP is defined as the CRD pressure that evokes the visually identifiable abdominal wall contraction (Al-Chaer et al., 2000, Yang et al., 2006, Tian et al., 2006).

### *Electrophysiological measurement*

Rectus abdominis EMG recordings were performed on rats under mild and stable anesthesia. Rats were initially anesthetized with pentobarbital (40 mg/kg, intraperitoneally) and fixed in a supine position. Body temperature was monitored and kept around 37 °C by a heat blanket. CRD stimulation was applied in a manner similar to that in the AWR test. Electrodes were placed in bilateral rectus abdominis of the rat. Then EMG responses to graded CRD stimulation (20, 40, 60, and 80 mm Hg) were recorded and analyzed with Powerlab system




B

| | |
|---|---|
| IBS (n=6) | No EA treatment |
| EA (n=6) | EA at ST-36 and ST-37 |
| Sham-EA (n=6) | Sham-EA without stimulation |
| Control-point-EA (n=6) | EA at control points |

**Fig. 1.** A. Description of the experimental procedure. Colon irritation began at an age of 8 days and continued for 2 weeks. Neonatal colon irritation was followed by a resting period of 2 weeks. Repeated administration of EA was applied in the 6th week. B. Model rats were divided into 4 groups.

(Powerlab 8 and Chart 5.0, AD Instrument, Australia). Anesthesia was maintained with continuous infusion of pentobarbital (8 mg/kg/h, intraperitoneally) through a peristaltic pump.

*EA treatment*

EA treatment was applied once per day for 3 successive days. Stainless steel needles (0.25 mm in diameter, Suzhou Medical Appliance, China) were inserted bilaterally at a depth of approximate 5 mm into the points of Zu-san-li (ST-36, 5 mm lateral to the anterior tubercle of the tibia and 10 mm below the knee joint) and Shang-ju-xu (ST-37, 5 mm lateral to the anterior tubercle of the tibia and 20 mm below the knee joint). Each pair of needles was connected with the output terminals of an acupoint stimulator (Model LH202H Hans, Beijing Huawei Medical Instrument, P.R. China). Alternating trains of dense–sparse frequencies (100 Hz for 1.05 s and 4 Hz for 2.85 s alternately) were used for 30 min. The intensity was 1 mA.

*Western blot assay*

Right after behavioral tests rats were sacrificed and L4–L5 spinal cord segments were quickly removed and homogenized at 4 °C in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM $Na_3VO_4$, 5 mM EDTA, 2 mM benzamidine, 1 mM henylmethylsulfonyl fluoride. Total protein was extracted for Western blot analysis. The protein concentration of the supernatant was measured using BCA Kit I (Beyotime Biotechnology, P.R. China). The supernatant, equal amounts of 30 μg protein, was fractionated by 10% (w/v) SDS-PAGE gel and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with immunoaffinity-purified antibody to phospho-NR1 (Ser896 cat# 06-640 or Ser897 cat# 06-641, 1: 250, Upstate Biotechnology), or monoclonal anti-GAPDH antibody (1:10,000, Sigma, St. Louis, Missouri) at room temperature for 2 h. Then the blots were incubated with horseradish peroxidase conjugated IgG (1:4000) for 2 h at room temperature, and visualized with enhanced chemiluminescence (ECL kit; Beyotime Biotechnology, P.R. China). The blots were exposed to autoradiographic film which was scanned into a computer. The intensity of the immunoreactive bands of interest was quantified using densitometric scanning analysis. The amount of protein was expressed in terms of the ratio of the pNR1 to GAPDH density.

*Data analysis*

Data were expressed as mean±SEM. Statistical evaluation was performed using SPSS v.14 (SPSS, Inc.). Graphs were generated using Sigma Plot v.9 (Systat, Inc.). AWR and EMG data were analyzed using the general linear model (GLM) in SPSS to perform two-way repeated-measures ANOVA. PTP and Western blot data were analyzed by unpaired *t* test or one-way ANOVA. An LSD and S–N–K post hoc test was used after one or two-way ANOVA analysis where appropriate. Differences with $P<0.05$ were considered to be significant.

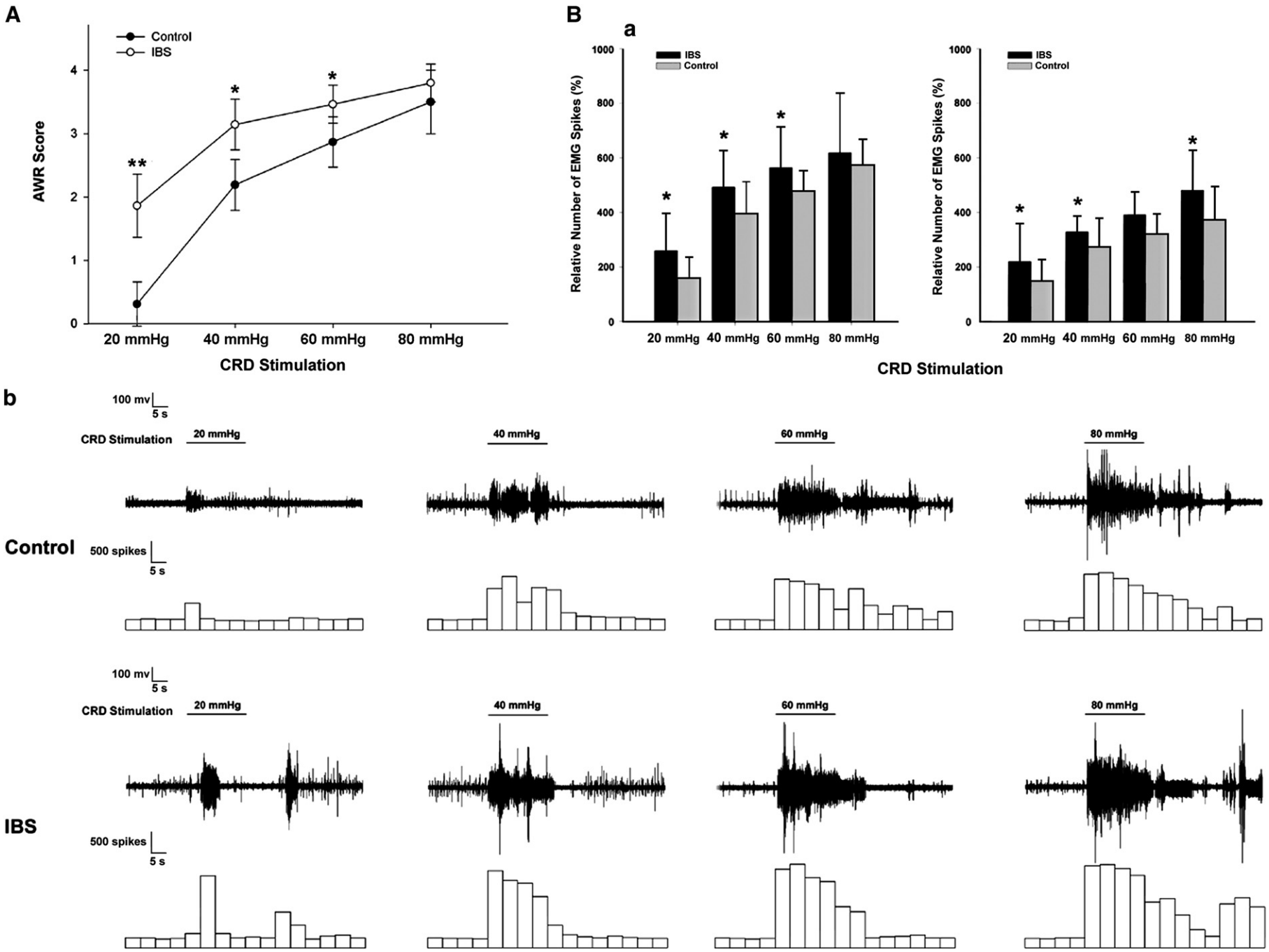
*Results*

The first step was to evaluate the model in the 6th week. Rats were tested for sensitivity to CRD stimulation. PTP measurement showed that an average CRD pressure of 20.1±5.3 mm Hg ($n=8$) evoked identifiable contraction of the abdominal wall in rats exposed to neonatal colon irritation (we will call them IBS rats in the following). This was much lower than the pressure (36.4±6.2 mm Hg, $n=12$) in normal control rats ($P<0.01$, unpaired *t* test). As shown in Fig. 2A, a significant increase in AWR grade in response to increasing CRD pressure was observed in both the control ($n=6$) and the IBS ($n=8$) groups. The AWR test revealed that rats treated with neonatal colon irritation exhibited increased visceral sensitivity to CRD. Data were analyzed by two-way repeated-measures ANOVA with neonatal treatment as the between group factor and distension pressure as the repeated factor. As shown in Fig. 2A there was a significant effect of neonatal treatment ($F=27.734$, $P<0.01$) and of distention pressure ($F=491.472$, $P<0.001$) on AWR responses. Significant differences between the IBS and the control groups were found at distention pressures of 20 ($P=0.005$), 40 ($P=0.024$), and 60 ($P=0.033$) mm Hg. Yet significance was not reached at 80 mm Hg ($P=0.488$). In a separate experiment, EMG activity was measured in response to graded CRD. As shown in Fig. 2B(a), EMG responses were significantly higher in the IBS group ($n=8$) compared to the control group ($n=6$) (two-way repeated-measures ANOVA, neonatal treatment: $F=35.350$, $P<0.01$ in the left panel and $F=22.840$, $P<0.01$ in the right panel). In the left panel, differences in the responses were statistically significant at the pressures of 20 ($P=0.012$), 40 ($P=0.032$), and 60 ($P=0.026$) mm Hg. In the right panel, *P* values were 0.023, 0.018, 0.135 and 0.037 at the pressures of 20, 40, 60 and 80 mm Hg, respectively. Fig. 2B(b) shows the representative EMG recordings of the IBS and control rats. Notice that although the responses of control and model rats were not statistically different at a high distention pressure (80 mm Hg); the responses at relatively low pressures showed substantial differences. Taking into account that a CRD pressure of around 20 mm Hg, which is the subthreshold for normal rats, led to identifiable abdominal wall contraction in IBS rats, we thus generally demonstrate that neonatal colon irritation induces visceral hypersensitivity during postnatal development.

In the second step, all model rats were randomly divided into 4 groups as shown in Fig. 1 and EA treatment was applied. To maximally reduce the effects of anesthesia on subsequent experiments, only PTP and AWR assessments were performed on conscious rats to test the effects of EA. As shown in Fig. 3A, after 3 applications of EA at bilateral ST-36 and ST-37 in the rats with chronic visceral hypersensitivity, PTP in response to CRD was significantly increased. The average PTP of the EA group was 35.6±7.3 mm Hg, much higher than that of the IBS group. In contrast, there was no significant difference in PTP between the sham-EA (19.7±6.6 mm Hg) and the IBS (20.2±5.4 mm Hg) groups. Fig. 3B showed the significant differences in AWR scores among different groups (two-way repeated-measures ANOVA with treatment as the between group factor: $F=19.417$, $P<0.01$). Post hoc analysis showed that AWR scores of the EA-treated rats were significantly less than the scores of IBS rats at distention pressures of 20 ($P=0.004$), 40 ($P=0.007$), and 60 ($P=0.023$) mm Hg. Similar to what was found in PTP measurement sham-EA produced no inhibitory effects on responsive AWR induced by graded CRD stimulation. These data indicate that repeated administration of EA at ST-36 and ST-37 attenuates chronic visceral hypersensitivity in IBS rats. Notice that comparison between control-point-EA and IBS groups revealed that neither PTP values nor AWR scores were significantly different. This suggests that the two control points are not effective in relieving the experimental visceral pain.

After behavioral tests L4–L5 spinal cord segments of all rats were removed for Western blot assay. As clearly shown in Fig. 4A,

**Fig. 2.** A. AWR scores measured in control and model rats subjected to graded colorectal distension (CRD) stimulation (neonatal treatment, $F=27.734$, $P<0.01$; distension pressure, $F=491.472$, $P<0.001$, two-way repeated-measures ANOVA). *$P<0.05$ vs. control, **$P<0.01$ vs. control. B. (a). Bar graphs showing the average EMG spikes recorded in control and model rats subjected to graded CRD stimulation (neonatal treatment, $F=35.350$, $P<0.01$ in the right panel; $F=22.840$, $P<0.01$ in the left panel, two-way repeated-measures ANOVA). *$P<0.05$ vs. control. The right panel differed from the left one in that an additional 40 s delay after withdrawing the stimulation was included in the time period. The spike counting number before CRD (counted for 20 s) was taken as 100% and the spikes in response to CRD was expressed as percentage with respect to spikes before CRD stimulation. (b). Representative EMG discharges (the upper panel) and spikes (the lower panel) of the same rats as (a).


A
Control
IBS
AWR Score
CRD Stimulation
20 mmHg
40 mmHg
60 mmHg
80 mmHg
B
a
IBS
Control
Relative Number of EMG Spikes (%)
CRD Stimulation
b
100 mv
5 s
CRD Stimulation
Control
500 spikes
IBS

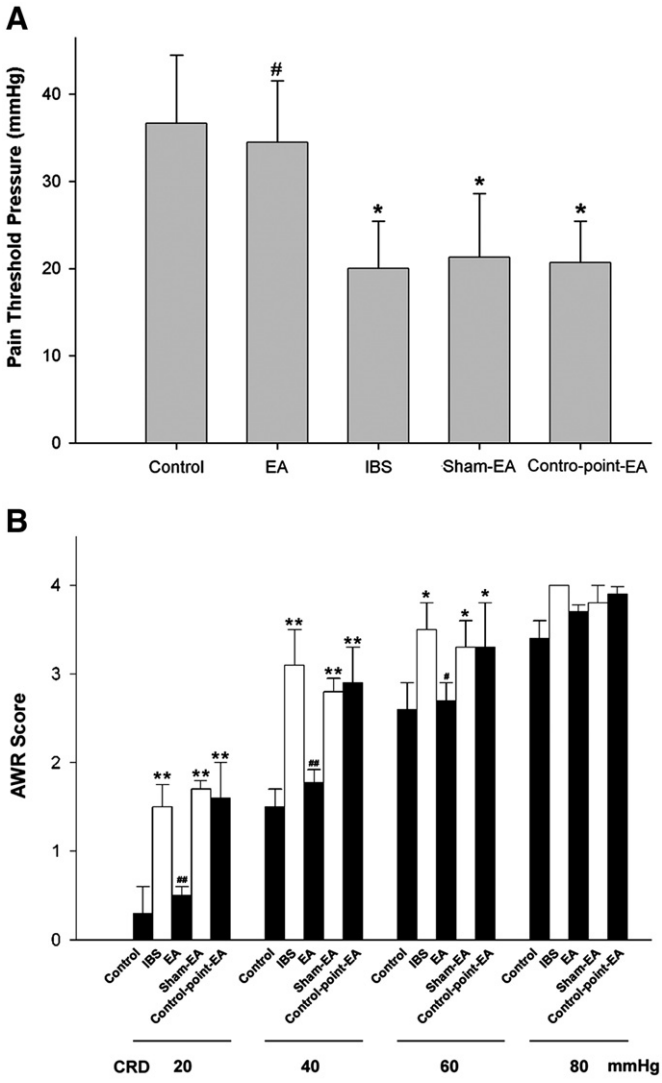



**Fig. 3.** A. Pain threshold pressure (PTP) measured in response to continuous CRD in control, IBS, EA, sham-EA, control-point-EA groups. *$P$<0.01 vs. control, #$P$<0.01 vs. IBS, one-way ANOVA. B. AWR scores measured in response to graded CRD in the same groups as A (two-way repeated-measures ANOVA with treatment as the between group factor: $F$=19.417, $P$<0.01). *$P$<0.05 vs. control, **$P$<0.01 vs. control, #$P$<0.05 vs. IBS, # #$P$<0.0s1 vs. IBS.

pNR1 immunoreactivity was obviously enhanced in the IBS group. In Fig. 4B, semi-quantitative analysis showed a significant increase in pNR1 expression in IBS rats compared with normal control rats. This result suggests that neonatal colon irritation induces hyperphosphorylation of NR1 at the sites of Ser896 and Ser897. In the EA group, repeated administration of EA at ST-36 and ST-37 significantly attenuated phosphorylation of NR1 at both sites compared with the IBS group. However, such an effect was observed neither in the sham-EA nor in the control-point-EA group. We tested the level of the reference protein GAPDH in all samples and no significant difference was observed. In addition, behavioral tests had no effect on the subsequent Western blot assay because EA-treated rats with/without the tests exhibited nearly the same level of pNR1 expression (Fig. 4C).

For further demonstration we explored the effects of repeated administration of EA at ST-36 and ST-37 on normal rats in a separate experiment. No significant difference in PTP between normal (34.8±6.3 mm Hg) and EA-treated normal (35.8±5.5 mm Hg) rats was observed ($n$=6 in each group, $P$>0.05, unpaired $t$ test). As shown in Fig. 5A, the AWR scores for all CRD pressures (20, 40, 60, and 80 mm Hg) were statistically indistinguishable in both groups. In addition, for normal rats EA did not alter the expression level of phosphorylated NR1 either at Ser 896 or at Ser 897 (Fig. 5B).

## Discussion

Over the past decade the role of visceral hypersensitivity in the pathophysiology of functional bowel disorders, especially IBS, has attracted much interest. Visceral hypersensitivity is thought to be a pain state caused by central sensitization, which leads to abnormal perception of otherwise painless (allodynia) or only slightly painful (hyperalgesia) stimuli (Mayer and Gebhart, 1994). The etiopathogenesis of this visceral hypersensitivity is probably multifactorial. It is believed that exposure to adverse events at an early stage in postnatal life is one of the main causes (Chitkara et al., 2008). This may be due to the fact that the neonatal nervous system appears to be particularly vulnerable and susceptible to plastic changes. Based on this view, Al-Chaer et al. established a model in which rats were subjected to neonatal colon irritation and developed chronic visceral hypersensitivity with motility disturbances in adulthood (Al-Chaer et al., 2000; Ma et al., 2002). In this study, we reproduced this model and found that model rats were more sensitive to innocuous CRD stimulation, and exhibited greater responses to noxious CRD stimulation compared with normal rats. These results indicate that the visceral perception of neonatally irritated rats has characteristics of both allodynia and hyperalgesia. Therefore, our study supports the conclusion that colon irritation on neonates subsequently leads to visceral hypersensitivity in adulthood.

Acupuncture has been claimed as an effective treatment for certain pain. The analgesic action of acupuncture in IBS-induced abdominal pain has been reported in clinical trials (Chan et al., 1997; Xing et al., 2004); however, the mechanism remains largely unknown. The animal model at hand successfully produced the specific characteristics of this affliction. Thus it provides a valuable tool to investigate the effect and related mechanism of acupuncture treatment on IBS-induced visceral pain. A recent study on rats exposed to neonatal colon irritation (Tian et al., 2006) shows that the inhibitory effect of EA on chronic visceral hypersensitivity is mediated through serotonin (5-HT) signaling pathway in the colon. As argued in another study (Wu et al., 2008), the mechanism of EA-mediated attenuation of IBS-related visceral hypersensitivity involves the prevention of mucous mast cell activation and the decrease of secretion of both substance P and vasoactive intestinal peptide. However, mechanism at the spinal level has not been investigated. Moreover, available research data have not come to consensus about the EA effect on spinal cord NMDA receptors that are important for the development of pain hypersensitivity. In this work we put the focus on this issue.

Abundant data confirms that the phenomenon of NMDA receptor-mediated central sensitization is a key mechanism involved in pain hypersensitivity. In somatic tissues, the stimulation of nociceptive afferents following an injury or inflammation results in an increased and sustained release of glutamate from the central terminals of the primary afferent fibers. This leads to the activation of postsynaptic NMDA receptors in the spinal cord neurons and consequently to the amplification of pain (Woolf and Thompson, 1991; Woolf and Costigan, 1999; Woolf and Salter, 2000). In a similar manner, visceral stimuli can also produce central sensitization via NMDA receptors. There is a growing body of scientific evidence showing that spinal administration of NMDA aggravates behavioral and neural responses to acute noxious/innocuous colorectal stimuli and that the administration of NMDA receptor antagonists attenuates these responses (Kolhekar and Gebhart, 1996; Zhai and Traub, 1999; Ji and Traub, 2001; Traub et al., 2002; Gaudreau and Plourde, 2004). However, to our knowledge, only a few studies have dealt with the contribution of spinal cord NMDA receptors to central sensitization in chronic visceral pain. Rice and McMahon investigated the role of spinal cord NMDA receptors in central sensitization in an animal model of persistent

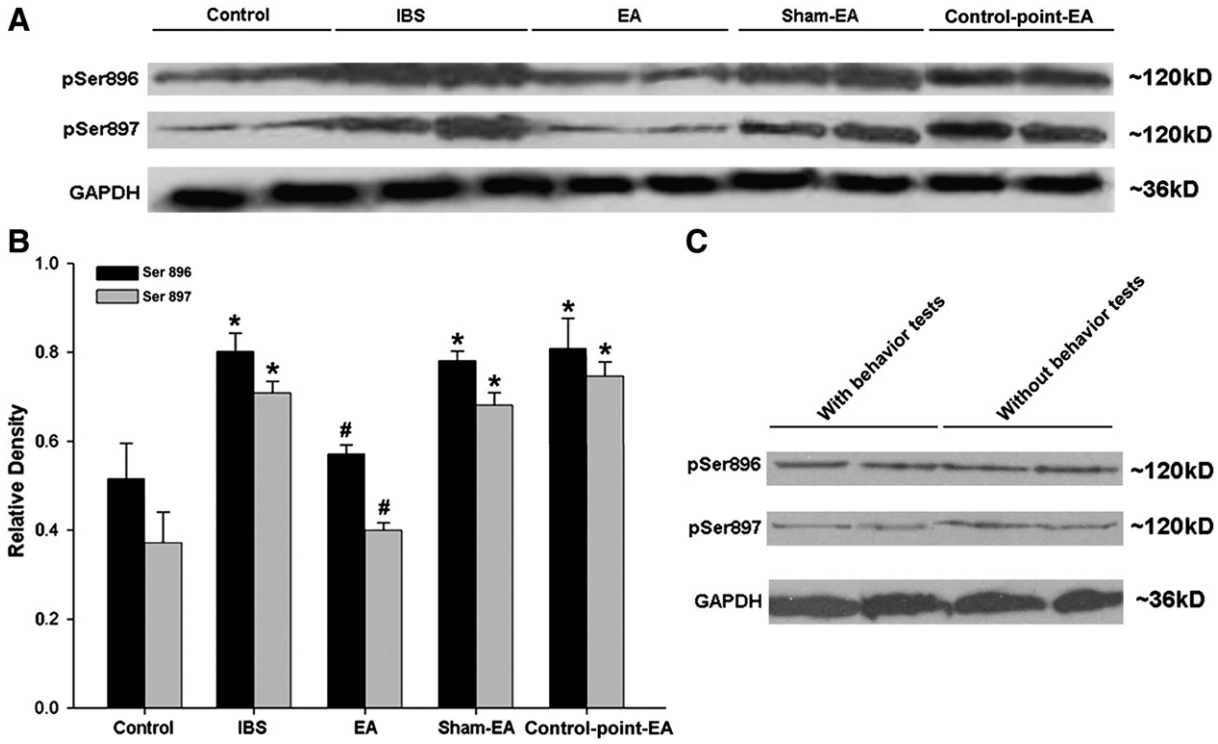



**Fig. 4.** A. Representative immunoblots showing the effect of repeated administration of EA on NR1 phosphorylation in the lumbosacral spinal cord. Equal amount of protein was loaded for Western blot assay. Numbers labeled on the right of the blots indicate molecular weight in kilo Daltons. B. Bar graph summarizing the relative density of the immunoblot bands corresponding to pNR1. *$P<0.01$ vs. control, #$P<0.01$ vs. IBS, one-way ANOVA. C. Representative immunoblots of spinal cord pNR1 in EA-treated rats with/without behavioral tests.

visceral pain (Rice and McMahon, 1994). Lin and Al-Chaer observed the differential effects of glutamate receptor antagonists on dorsal horn neurons responding to CRD in a neonatal colon irritation rat model (Lin and Al-Chaer, 2005). In this study, we demonstrated the enhanced phosphorylation of spinal cord NR1 in rats exposed to neonatal mechanical colon irritation, which coincided with the visceral hypersensitivity in postnatal development. After EA treatment, expression of phosphorylated NR1 decreased while the pain threshold of the rats increased. It is known that activities of NMDA receptors can be regulated by protein phosphorylation (Raymond et al., 1994; Llansola et al., 2005). Our data thus present further evidence that spinal cord NMDA receptors participate in the generation

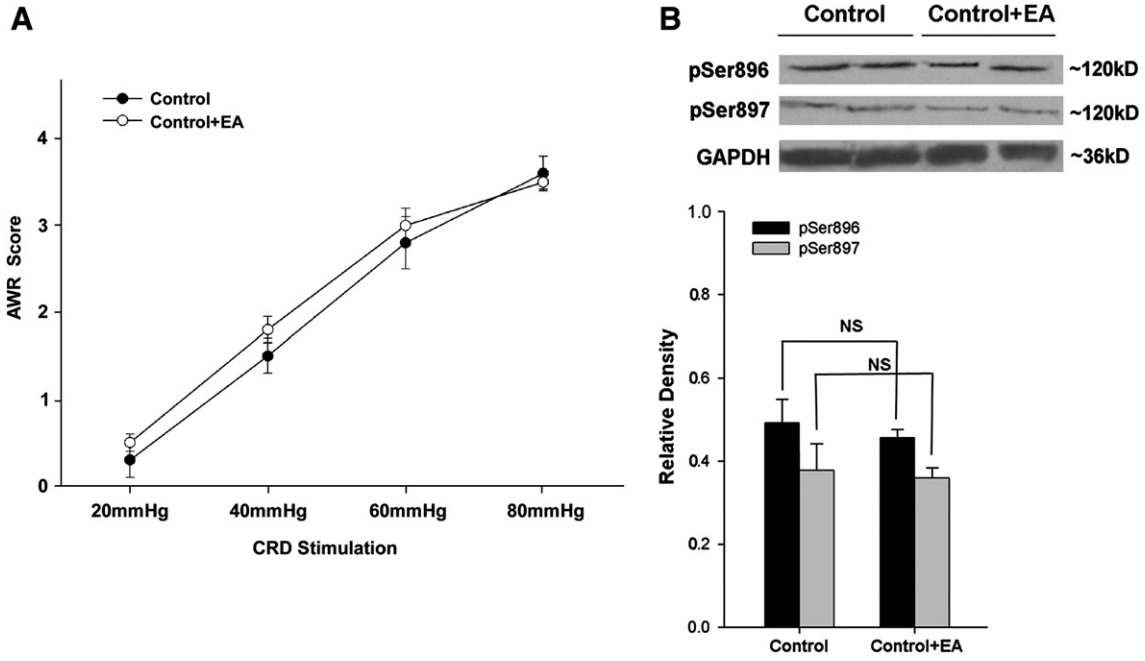



**Fig. 5.** A. AWR scores measured in normal and EA-treated normal rats. The scores of both groups in response to graded CRD (20, 40, 60, and 80 mm Hg) were not different in the statistical sense (two-way repeated-measures ANOVA with treatment as the between group factor: $F=0.324$, $P<0.05$). B. Representative immunoblots and quantitative analysis of the relative density of pNR1 in the same groups as in A. NS: no significant difference, $P>0.05$, unpaired $t$ test.

of chronic visceral hypersensitivity. The data also indicate that the effect of EA on visceral perception of IBS rats may be related to the down-regulation of NR1 phosphorylation in the spinal cord.

The role of NR1 phosphorylation at the sites of Ser 896/897 in the central sensitization of acute or inflammatory pain has been recently documented. NR1 phosphorylation at Ser 896 was demonstrated in spinal dorsal horn neurons following peripheral capsaicin injection (Zou et al., 2004) or noxious heat stimulation (Brenner et al., 2004). This phosphorylation is catalyzed by protein kinase C (PKC). Similarly, an accumulation of phosphorylated NR1 at a protein kinase A (PKA)-dependent site, Ser 897, was found in spinothalamic tract neurons following capsaicin injection (Zou et al., 2000, 2002). However, the involvement of spinal cord NR1 phosphorylation in chronic visceral hypersensitivity has not been studied. In this study, we found that neonatal repetitive colon irritation induced hyperphosphorylation of NR1 at Ser 896 and Ser 897 in the spinal cord, and EA attenuated such hyperphosphorylation at both sites. In addition, EA did not alter the phosphorylation level of spinal cord NR1 in normal rats either at Ser 896 or at Ser 897. It is known that NR1 phosphorylation at Ser 896 suppresses endoplasmic reticulum retention of NMDA receptors and promotes movement of the receptors from intracellular stores to cell membrane (Scott et al., 2001; Xia et al., 2001); while phosphorylation at Ser 897 facilitates accumulation of NMDA receptors in synapses (Crump et al., 2001). Moreover, PKC phosphorylation of Ser 896 acts coherently with PKA phosphorylation of Ser 897 to induce membrane insertion of NMDA receptors (Scott et al., 2003). Therefore, the findings in this study suggest that peripheral visceral stimuli might induce the migration of intracellular NMDA receptors in spinal cord. Presumably, the newly inserted NMDA receptors in cell membrane participate in enhancing synaptic activity and inducing central sensitization in chronic visceral pain. We propose that EA may modulate some kinases or phosphatases that mediate its effects on spinal cord NR1 phosphorylation and thus may affect the migration of NMDA receptors in neurons. To clarify this, needless to say, deserves more efforts.

Because of their contribution to the development of central sensitization, NMDA receptors have been targeted to develop anti-hyperalgesic drugs for IBS treatment (Bueno et al., 1997; Chovet, 2000). Blockade of these receptors remains of interest and much work is underway testing drugs that may modulate the NMDA receptor-somatostatin pathways (Talley, 2003). For example, the commercially available drug octreotide, which is a somatostatin analogue, has been reported to exert anti-hyperalgesic effects at the spinal level (Schwetz et al., 2004). Further evidence indicates that the drug can suppress glutamate responses through the activation of somatostatin 2 receptors (Peineaul et al., 2003). However, the drugs that help alleviate chronic visceral pain are either expensive or associated with various side effects that cannot be ignored. In this study we showed that EA at ST-36 and ST-37 significantly inhibited hyperphosphorylation of spinal cord NMDA receptors in the rat model of chronic visceral hypersensitivity. This result suggests that the activities of spinal cord NMDA receptors can be affected by EA treatment and that acupuncture can be a promising physical therapy for chronic visceral hypersensitivity. Yet we believe that the best control for an acupoint is to stimulate another acupoint known to have a different function (Mayer 2000; Zhou et al., 2005). Therefore, in addition to the point on tail, Shen-mai (BL-62) which is seldom used to treat patients with IBS was selected as the control point. It was found that, unlike at ST-36 and ST-37, EA at the control points altered neither the visceral perception nor the spinal cord NR1 phosphorylation in model rats, suggesting that stimulation at appropriate acupoints is essential to relieving IBS-induced chronic visceral hypersensitivity. We stress that sham acupuncture has been found to cause analgesia in human subjects before (Forbes et al., 2005; Schneider et al., 2006). Yet we believe this effect may be due to conscious perception that evokes placebo responses which cannot serve as the physiological basis of real acupuncture treatment.

In summary, our present findings provide evidence for the theory that attenuation of chronic visceral hypersensitivity can be mediated by repeated administration of EA. EA may be able to regulate the phosphorylation of NMDA receptors in the spinal cord. These results reinforce the potential of acupuncture as a therapy against chronic visceral pain for patients with IBS.

## Acknowledgements

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