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ORIGINAL PAPER

Downregulation of the spinal NMDA receptor NR2B subunit during electro-acupuncture relief of chronic visceral hyperalgesia

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Abstract The involvement of spinal NR2B, a *N*-methyl-D-aspartate (NMDA) receptor subunit, in the therapeutic effect of electro-acupuncture (EA) on chronic visceral hyperalgesia was investigated. Chronic visceral hyperalgesia was induced using an irritable bowel syndrome (IBS) model in rats. Graded colorectal distention (CRD) stimuli at strengths of 20, 40, 60 and 80 mmHg were applied, and behavioral tests were performed to measure the abdominal withdrawal reflex (AWR) in response to the CRD stimuli and assess the severity of the visceral hyperalgesia. Rats were randomly divided into four groups: normal intact (control) group, IBS model (model) group, EA-treated IBS rats (EA) group and sham EA-treated IBS rats (sham EA) group. For the EA treatment, electric stimuli were applied through needles inserted into two acupoints [Zu-san-li (ST-36) and Shang-ju-xu (ST-37)] in both hind limbs, while the sham EA treatment consisted of only the insertion of needles into these same acupoints without an application of electric stimuli. Our results showed that AWR scores of the model group responding to CRD stimuli of 20, 40, 60 and 80 mmHg were significantly increased. These increased scores subsequently decreased following EA treatment ($P < 0.05$) compared with those for the other groups. The expression of NR2B in the superficial laminae (SDH, laminae I and II), nucleus proprius (NP, laminae III and IV), neck of the dorsal horn (NECK, laminae V and VI) and central canal region (lamina X) at thoracolumbar (T13-L2) and lumbosacral (L6-S2) segmental level significantly increased in the model group versus the control group ($P < 0.05$) and significantly decreased after EA treatment ($P < 0.05$). There were no significant changes in neither AWR scores nor expression of the NR2B subunit in these spinal regions after the sham EA treatment. These results confirm that EA can relieve chronic visceral hyperalgesia in IBS model rats and suggest that such an effect is possibly mediated through the downregulation of the NR2B subunits of NMDA at the spinal level.

Keywords Chronic visceral hyperalgesia · Irritable bowel syndrome · Electro-acupuncture · NMDA receptor · NR2B · Spinal cord

Introduction

Visceral hyperalgesia is a pathological state in which the sensory threshold of pain decreases or the sense of pain increases due to factors such as tissue injury, inflammation and persistent exposure of tissues and organs to noxious stimuli. In this state, nerves or neurons can discharge in the absence of obvious stimuli or discharge extensively with only innoxious stimuli (e.g., mechanical distention). As a result, the silent afferents or neurons in the nervous system are overexcitated [1–4].

Peripheral and central sensitization are considered the two neurobiological mechanisms underlying chronic visceral hyperalgesia. However, some visceral disorders or diseases, such as irritable bowel syndrome (IBS) with chronic visceral hyperalgesia, lack obvious involvement of peripheral sensitization [5]. Studies focusing on the central sensitization mechanism have demonstrated that this kind of sensitization is a key causal factor for a pathophysiological situation of chronic visceral hyperalgesia [1, 6, 7].

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In addition, many neurotransmitters, such as excitatory amino acids, have been reported to be involved in chronic visceral hyperalgesia [8–10].

N-methyl-D-aspartate (NMDA) receptors are excitatory amino acid neurotransmitter receptors in the central nervous system. It has been shown that peripheral nociceptive visceral signaling can activate NMDA receptors, thereby excite neurons in the spinal dorsal horn and facilitate nociceptive transmission in the spinal cord [11–14]. Intrathecal injection of MK-801, an antagonist of NMDA receptor, blocks this activation of NMDA receptors [15]. NMDA receptors are composed of a glycine-binding NR1 subunit, with a glutamate-binding NR2 (A–D) and/or glycine-binding NR3 (A, B) subunit. A functional NMDA receptor requires NR1 subunits and at least one of the NR2 subunits [16–18]. At the spinal level, these subunits of the NMDA receptor are involved in the formation and development of chronic visceral hyperalgesia. In a chronic visceral hyperalgesia rat model, Zhou et al. reported upregulation of the NR1 subunit of the NMDA receptor in the spine [19], while Lin et al. observed significantly higher expression of the NR2B subunit in the spinal dorsal horn [20, 21].

IBS is a typical example of chronic visceral hyperalgesia and is a highly prevalent and challenging clinical problem that affects 5–15 % of the general population. The condition may profoundly impair the sufferer's quality of life and is costly to both patients and society [5]. Persistent and recurrent abdominal pain or discomfort is a main symptom and a key diagnostic marker for IBS diagnosis. Experimental modeling of IBS in adult rats can be achieved through colorectal distention (CRD), a method shown by Al-Chaer et al. to produce a condition similar to chronic visceral hyperalgesia [22].

Using this CRD-induced animal model of IBS, we observed that electro-acupuncture (EA) can relieve the symptoms of chronic visceral hyperalgesia [23]. The pain relief mechanism can be broadly understood as an acupuncture-activating endogenous pain modulation system [24]. However, in-depth understanding requires further clarification of the neurobiological mechanisms. In the study reported here, we focused on the relationship between the spinal NR2B subunit of the NMDA receptor and the effect of EA on chronic visceral hyperalgesia with the aim to obtain evidence supporting this effect of EA.

Materials and methods

Animals

Male Sprague–Dawley neonatal rats (age <8 days) were purchased from the Department of Laboratory Animal Science, Shanghai Medical College, Fudan University, China. A total of 32 rats were used for the study. Ten male neonatal rats were housed with one nursing female adult rat in a plastic cage until 25 days of age when they were separated from the adult female and housed four to a cage with access to food and water ad libitum. All rats were kept under a light/dark cycle of 12 h light and 12 h dark. All experimental protocols pertaining to the rats conformed to the National Institutes of Health Guidelines for the ethical use of animals in order to minimize the number of animals used and the effect of the experimental treatments.

Production of chronic visceral hyperalgesia model

The chronic visceral hyperalgesia model was induced according to the method of Al-Chaer et al. [22]. Mechanical CRD was applied to the rats from 8 to 21 days after their birth. The procedure has been described in detail by Cui et al. [23]. In brief, a silica gel balloon was inserted into the descending colon of the awake rat from the anus to a depth of 2 cm. Once in place, the balloon was distended with air (volume 0.01–0.35 ml; volume was increased as the rats grew) for 1 min and then deflated and withdrawn. The distention was repeated twice daily for 2 weeks, with a 30-min interval between insertion/distension. Rats in the control group were handled similarly to model rats but without insertion of a balloon and distention; rather, they were touched gently on the perineal area daily with a sterile cotton swab. After the 2-week stimulation period, both model and control rats were housed in cages until they reached adulthood (at least 6 weeks of age), and then they were observed by in behavioral test for the assessment of visceral hyperalgesia.

Behavioral test

The assessment of visceral hyperalgesia was conducted by observing and scoring the abdominal withdrawn reflex (AWR) of rats in response to CRD stimulation. Prior to observation, a distensible latex balloon (length 2 cm) was fixed to one end of a polyethylene tube; the other end of the tube was connected to a 10-ml syringe and a sphygmomanometer through a T-connector (Fig. 1; [25]). The balloon was then inserted into the colorectum of the conscious rat (Fig. 1); the rat was kept in a transparent cubicle (20 × 8 × 8 cm) on a platform and allowed to adapt to the situation for 20 min. A series of graded CRD was applied at strengths of 20, 40, 60 and 80 mmHg to observe the AWR. Each distention lasted for 20 s, with a 4-min interval between each distension. Each graded CRD was repeated four times, and the mean value of the four responsive AWR scores was used for analysis. The AWR was scored semi-quantitatively according the scale adopted by Al-Chaer et al., with five possible values (0–4; Fig. 2; [22]). Those

rats which successfully developed the IBS model (n = 24) were randomly divided into three groups: the IBS model (model) group, IBS rats treated by EA (EA) group; IBS rats treated by sham EA (sham EA) group. Eight normal intact rats formed the control group.

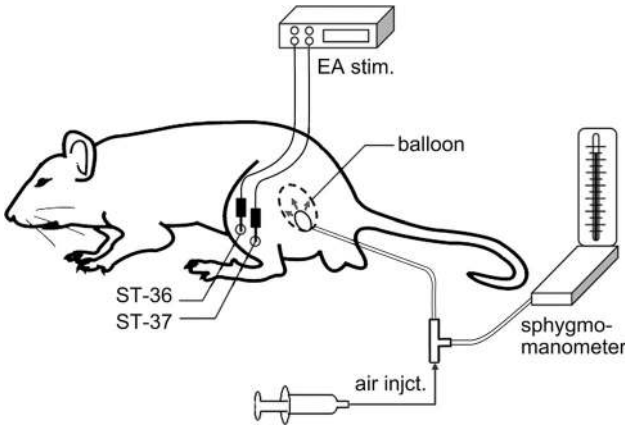

Fig. 1 Schematic diagram of the rat model for colorectal distention (CRD) stimulation and electro-acupuncture (*EA*) treatment (revised according to Wang and Li [25]). Stimulation of colorectal distention was achieved by inserting a distensible balloon into the colorectum and injecting a volume of air into the balloon to maintain a pressure of 20, 40, 60 and 80 mmHg, respectively. For EA treatment, two pair of needles were inserted into two acupoints [*ST-36* (Zu-san-li acupoint) and *ST-37* (Shang-ju-xu acupoint)] on both hind limbs. Electric stimuli were applied to the needles with disperse-dense waves at strengths and times ranging from 5 Hz for 5 s to 25 Hz for 10 s alternately in frequency and 1 mA in intensity, for a total treatment time of 30 min. *EA stim.* EA stimulator, *air injct.* air injection

EA treatment

The EA treatment was carried out by inserting two pairs of needles (diameter 0.25 mm) to a depth of 5 mm into two acupoints, namely, Zu-san-li (ST-36; located 5 mm lateral of the anterior tubercle of the tibia and 10 mm below the knee joint) and Shang-ju-xu (ST-37; located 5 mm lateral of the anterior tubercle of the tibia and 15 mm below the knee joint) on both hind limbs (Fig. 1). Each pair of needles was connected to the output terminals of an EA instrument (model SDZ-IV; Suzhou Medical Appliance Factory, Jiangsu Yuwell Medical Equipment Inc., Suzhou New District, P.R. China) (Fig. 1). Electric stimuli were given in the form of disperse-dense waves, in alternating frequency and duration between 5 Hz for 5 s and 25 Hz for 10 s. The stimuli were 1 mA in intensity, and each EA treatment session lasted for 30 min. Following the EA treatment session, the rat was transferred to an observation cubicle without any stimulation for 30 min; behavior tests were then performed between 30 and 90 min after the EA treatment. The course of EA treatment spanned 7 days, and EA was applied to rats on every other day during the study period. The sham EA treatment was carried out by inserting needles to the acupoints in a similar procedure as that in the EA treatment but without giving electrical stimulation. Rats in the Control and model groups were not treated by EA or sham EA, but their AWR was assessed following the same protocol as that used for the EA and sham EA groups.

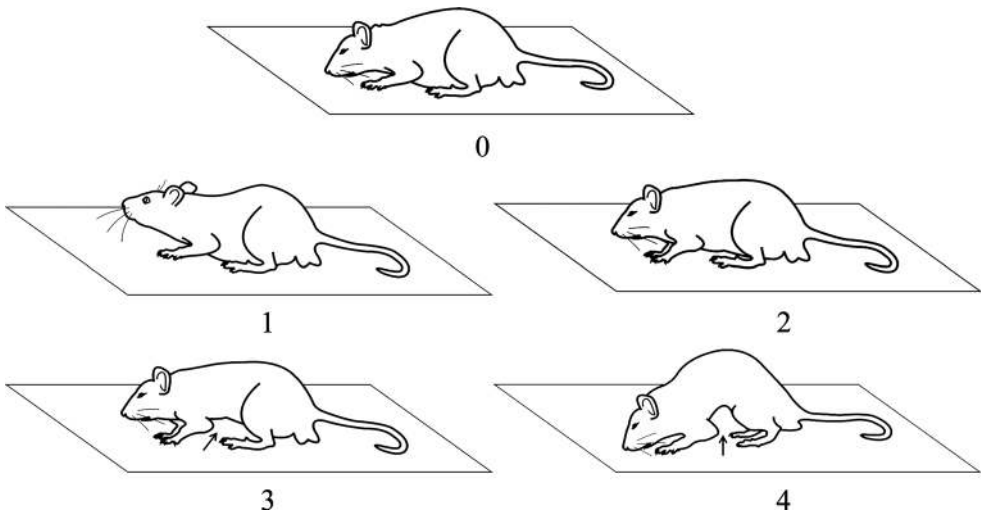

Fig. 2 Schematic diagram of changes in rat behavior in response to graded CRD based on abdominal withdrawn reflex (AWR) scores (revised according to Al-Chaer et al. [22]). *0* No behavior response to CRD; *1* brief head or body movement at the onset of stimulus; *2* contraction of the abdominal muscles, but no lifting off the platform; *3* a strong contraction of the abdominal muscles and lifting off the platform, but no lifting the pelvic structure off the platform; *4* a severe contraction of the abdominal muscles based on body arching and lifting of the pelvic and scrotum. *Arrows in panels* indicate lifting of the abdomen

Immunohistochemical staining of NR2B in the spinal dorsal horn

Preparation of spinal slices

After the final AWR assessment (7th day in the EA treatment course), rats were deeply anesthetized with an intraperitoneal injection of 10 % chloral hydrate (0.3 ml/100 g) and perfused with 4 % paraformaldehyde through a needle inserted into the ascending aorta from the left ventricle for tissue fixation. The spinal cord was dissected and removed at the thoracolumbar (T13-L2) and lumbosacral (L6-S2) segmental levels (Fig. 3) [26]. The spinal tissue was stored overnight at 4 °C for postfixation and then submerged in a phosphate buffered solution (PBS) with 30 % sucrose with for an additional 12 h. The spinal tissue was cut into 30-μm-thick slices using a freezing microtome (model CM 3050S; Leica Biosystems, Wetzlar, Germany).

Immunohistochemical staining of NR2B

The spinal slices from different spinal segments and groups were washed three times, 5 min each wash, in PBS at approximately 25 °C to remove residual traces of paraformaldehyde. The slices were then immersed in an 0.3 % H_2O_2 methanol solution for 10 min to block endogenous peroxide activity and then washed once again three times, 5 min each wash, in PBS. The spinal slices were subsequently incubated in a solution of normal goat serum (Wuhan Boster Bio-Engineering Ltd.t Co., Wuhan P.R. China) at room temperature for 30 min, followed by incubation in the primary antibody (rabbit anti-NMDA NR2B antibody, at 1:1000 dilution; Sigma-Aldrich Corp., St. Louis, MO) solution for 48 h at 4 °C. After a further three washes of 5-min duration each in PBS, the spinal slices were transferred to goat anti-rabbit immunoglobulin G conjugated with fluorescein isothiocyanate (1:400 dilution; Sigma-Aldrich) solution for 2 h at room temperature. The spinal slides were then subjected to a final series of three 5-min washes in PBS and mounted onto microscope slides, covered with Vectastain Hard Set Mounting Medium and viewed by fluorescence microscopy (model 90I; Nikon Corp., Tokyo, Japan). Nonspecific staining was determined by omitting the primary antibodies. To quantify NR2B immunoreactivity, we randomly selected five spinal slices from different spinal cord segments per animal. Images of the spinal dorsal horn and central canal region were captured with a CDD spot camera mounted on a Nikon optical microscope at ×100 magnification. Neurons staining positive for NR2B were counted in each subregion of the spinal dorsal horn and central canal region, and the average number of neurons for each rat which stained positive for NR2B at the T13-L2 and L6-S2 segmental levels was used in the data analysis.

Statistical analysis

All AWR scores recorded in response to graded CRD stimulation with all rats in each group were directly used for statistical analysis. The data were presented as mean ± standard error and analyzed by using a statistical software program (SPSS, ver. 20.0; IBM Co., Armonk, NY). Differences in AWR scores and number of the positive NR2B-staining neurons among different groups were analyzed by one-way analysis of variance followed by the

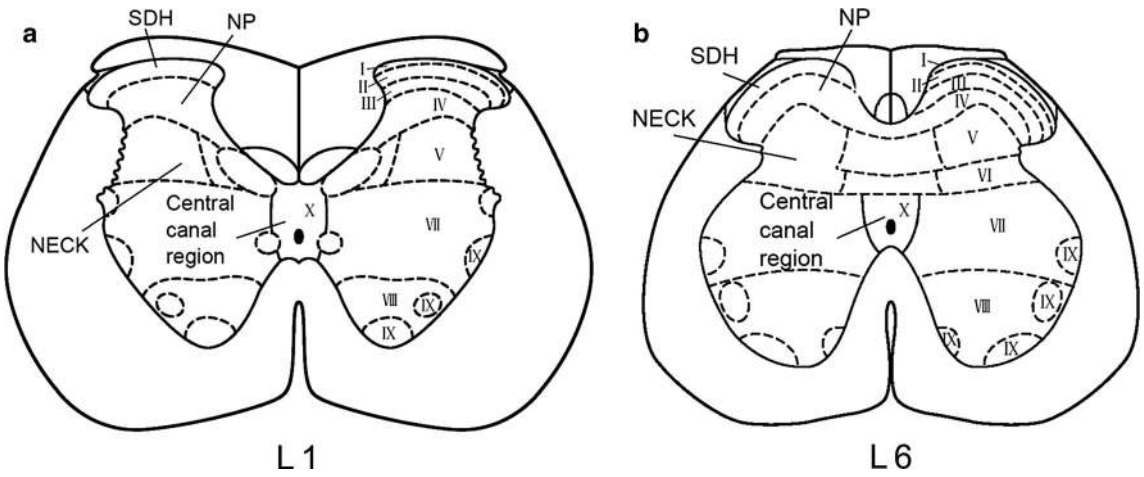

Fig. 3 Atlas of the spinal dorsal horn sliced at the thoracolumbar (T13-L2) and lumbosacral (L6-S2) segmental levels (modified according to Paxinos and Watson [26]). **a** and **b**: schematic diagrams of transversal photograph at the L1 level (**a**) and L6 level (**b**). *SDH* Superficial laminae (laminae I and II), *NP* nucleus proprius (laminae III and IV), *NECK* neck of the dorsal horn (laminae V and VI), central canal region: around the central canal (lamina X)

Bonferroni's test. A P value of <0.05 implies statistical significance.

Results

Repetitive CRD induced chronic visceral hyperalgesia

After applying repetitive CRD during the neonatal period to produce the chronic visceral hyperalgesia rat model, we tested all rats, including the control rats, with graded CRD at strengths of 20, 40, 60 and 80 mmHg, starting from an age of 6 weeks, for five times in total (days 1, 3, 5, 7 and 9). The AWR scores for the model rats showed a significant increase in response to all intensities of CRD compared with those for the control ones. In response to CRD at 20, 40, 60 and 80 mmHg, the mean AWR scores were 1.09 ± 0.06, 1.79 ± 0.06, 2.78 ± 0.06 and 3.50 ± 0.05, respectively, in the model rats versus 0.55 ± 0.05, 1.20 ± 0.05, 1.96 ± 0.06 and 3.08 ± 0.07, respectively, in the control ones (Fig. 4). The AWR responses to graded CRD stimuli remained stable during and even after the observation days, lasting for as long as 12 weeks post-stimulation (data not shown).

EA treatment attenuated chronic visceral hyperalgesia

A course of EA treatments was applied to the model rats. Upon completion of the treatment course (the 4th session on day 7), the AWR scores for the EA group had decreased significantly compared to those for the model group (IBS-modeled rat without EA treatment) (Fig. 5). In response to CRD at 20, 40, 60 and 80 mmHg, the AWR scores were 0.67 ± 0.10, 1.21 ± 0.10, 2.08 ± 0.10 and 3.08 ± 0.11, respectively, in the EA group versus 1.08 ± 0.13, 1.67 ± 0.12, 2.88 ± 0.13 and 3.50 ± 0.10, respectively, in the model group. The AWR scores in the sham EA group did not change significantly compared to those for the model group (Fig. 5).

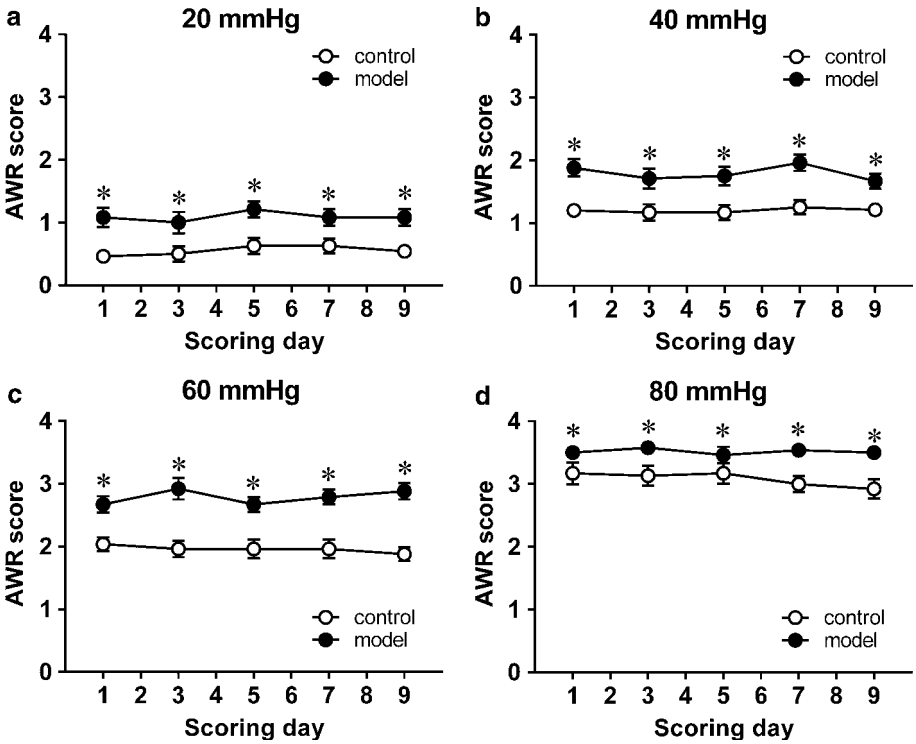

Fig. 4 AWR scores in response to graded CRD in control vs. model groups, measured on days 1, 3, 5, 7 and 9 ($n = 8$ rats in each group). a–d Sets of AWR scores in control and model groups in response to CRD stimuli at strengths of 20, 40, 60 and 80 mmHg, respectively. *Abscissa* Time course of AWR scoring every other day from day 1 to day 9, *ordinate* AWR scores. Data are presented as mean ± standard error (SE). *Significant difference at $P < 0.05$ vs. control. *Model* Rats which developed inflammatory bowel syndrome (IBS) experimentally induced by CRD as described in section "Production of chronic visceral hyperalgesia model", *Control* rats Intact normal rats which were not subjected to insertion of a balloon and distention, but received gentle touching on the perineal area daily with a sterile cotton swab

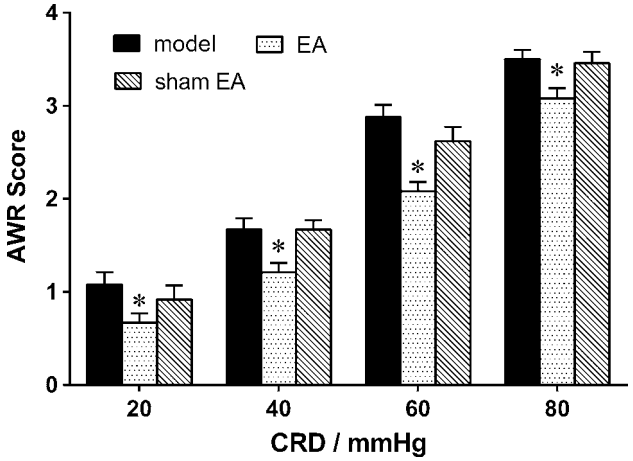

Fig. 5 AWR scores for model, EA and sham-EA groups ($n = 8$ rats in each group). *Abscissa* Four intensities of CRD stimuli (20, 40, 60 and 80 mmHg), *ordinate* AWR scores. Data are presented as the mean ± SE. *Significant difference at $P < 0.05$ vs. model. Model rats ($n = 24$) were randomly divided into three groups: *Model* IBS model (rats were not subjected to EA treatment), *EA* IBS rats subjected to EA treatment, *sham-EA* IBS rats subjected to sham EA treatment

Expression of spinal NR2B subunit in the spinal dorsal horn

Expression of spinal NR2B subunit at the thoracolumbar (T13-L2) segmental level

The immunohistochemical examination was focused on determining NR2B subunit expression in areas of the superficial laminae (SDH; in laminae I and II), nucleus proprius (NP; in laminae III and IV), neck of the dorsal horn NECK; in laminae V and VI) and central canal region (around the central canal, in lamina X) in the spinal dorsal horn at theT13-L2 segmental level.

The number of NR2B-positive neurons in the spinal dorsal horn areas (laminae I–VI) and central canal region (laminae X) at the T13-L2 segmental level are shown in Fig. 6, and the statistical analysis of the number of NR2B-positive neurons in all areas at the T13-L2 segmental level is shown for all rat groups in Fig. 7. The results show a significant increase in NR2B expression in the observed areas of the model rats compared with those in control rats.

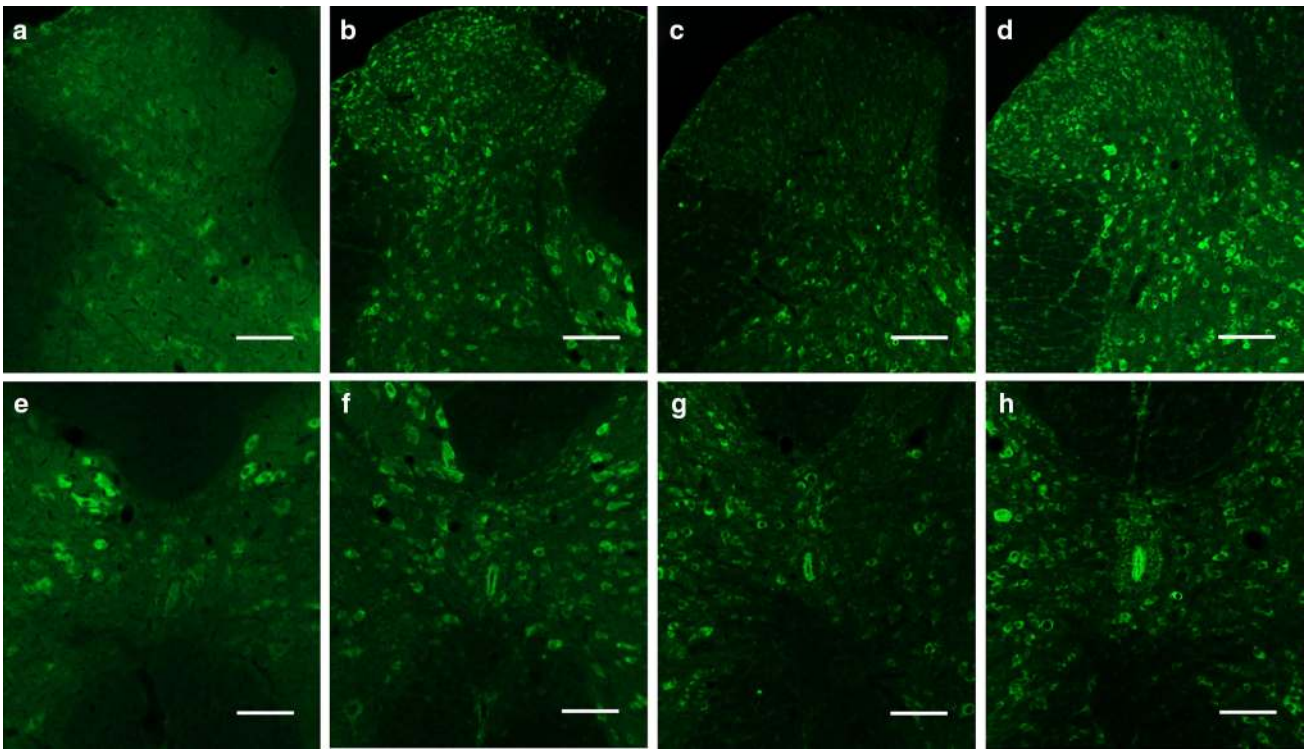

Fig. 6 Neurons showing positive immunoreactivity for the glutamate-binding NR2 subunit (NR2B) of the *N*-methyl-D-aspartate (NMDA) receptor in transverse sections of the spinal dorsal horn and central canal region at the thoracolumbar (T13-L2) segmental level (magnification ×100). **a** Low expression of NR2B in the spinal dorsal horn of the control rat; **b** high expression of NR2B in the spinal dorsal horn of the model rat (without any treatment); **c** low expression of NR2B in the spinal dorsal horn of the EA-treated model rat; **d** high expression of NR2B in the spinal dorsal horn of the sham EA-treated model rat; **e** low expression of NR2B in the central canal region of the control rat; **f** high expression of NR2B in the central canal region of the model rat (without any treatment); **g** low expression of NR2B in the central canal region of the EA-treated model rat; **h** high expression of NR2B in the central canal region of the sham EA-treated model rat. *Scale bar* 100 μm

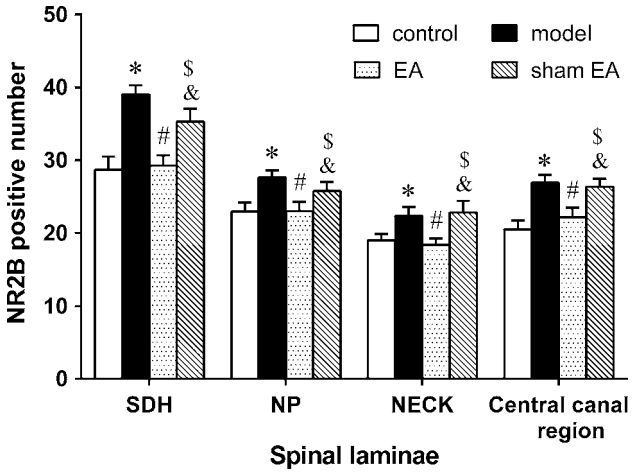

Fig. 7 Number of NR2B-positive immunoreactive neurons at the thoracolumbar (T13-L2) segmental level in the four rat groups (*model*, *EA*, *sham EA*, *control*; $n = 8$ rats in each group). *Abscissa* Number of NR2B-staining neurons, *ordinate* observed areas (see caption to Fig. 3 for definition of abbreviations) in the spinal dorsal horn observed at low magnification (×100) under a microscope. Data are presented as the mean ± SE. *Significant difference at $P < 0.05$ vs. control, $^{\#}$significant difference at $P < 0.05$ vs. model, $^{\$}$significant difference at $P < 0.05$ vs. control, $^{\&}$significant difference at $P < 0.05$ vs. EA

The mean number of NR2B-positive neurons in the SDH, NP, NECK and central canal regions was 39.02 ± 1.27, 27.68 ± 0.93, 22.35 ± 1.26 and 26.9 ± 1.1, respectively, in model rats versus 28.68 ± 1.83, 22.93 ± 1.27, 19 ± 0.83 and 20.48 ± 1.24, respectively, in control rats. EA treatment significantly decreased this observed increased NR2B expression in model rats to 29.25 ± 1.42, 23 ± 1.28, 18.38 ± 0.91 and 22.15 ± 1.32 in the SDH, NP, NECK and central canal regions, respectively. There were no significant changes in NR2B expression in model rats subjected to the sham EA treatment. Additionally, there were significant changes in NR2B expression in the sham EA group versus the EA and normal groups, respectively (Fig. 7).

Expression of spinal NR2B subunit at lumbosacral (L6-S2) segment level

The number of NR2B-positive neurons in the spinal cord dorsal horn areas (laminae I–VI) and central canal region (laminae X) at the L6-S2 segmental level is shown in Fig. 8, and the statistical analysis of the number of NR2B-positive neurons in all areas at the T13-L2 segmental level is shown for all rat groups in Fig. 9. The results show a significant increase in NR2B expression in the observed areas of model rats compared with those in control rats. The mean number of NR2B-positive neurons in the SDH, NP, NECK and central canal regions was 35.73 ± 1.69, 27.3 ± 1.98, 26.9 ± 1.83 and 25.83 ± 1.89, respectively, in model rats versus 27.63 ± 1.47, 21.73 ± 0.98, 20.05 ± 1.71 and 20.95 ± 1.29, respectively, in control rats. The effect of the EA treatment was to significantly decrease the increased NR2B expression observed in model rats to 27.53 ± 1.07, 21.33 ± 1.31, 20.85 ± 1.41 and 20.43 ± 1.19 in the SDH, NP, NECK and central canal regions, respectively. There were no significant changes in NR2B expression in model rats treated by sham EA. Additionally, significant changes in NR2B expression in the sham EA group versus the EA and normal groups, respectively (Fig. 9).

Discussion

As mentioned in the "Introduction", certain chronic visceral hyperalgesia can occur in absence of identifiable pathological changes in the peripheral organs (i.e. peripheral sensitization). Defined as functional abdominal pain, IBS is just such a case, being primarily caused by central sensitization (i.e. the sensitization of the central nervous system) that has been shown in animal models to be induced by repetitive peripheral visceral noxious inputs during postnatal development of the central nervous system [1, 8, 23]. Such sensitization may be due to the vulnerability and susceptibility of the neonatal nervous system to plastic changes. In this study, we established a rat model of IBS using the procedure reported by Al-Chaer et al. [22] whereby neonatal rats received repetitive CRD stimuli and then developed chronic visceral hypersensitivity in the central nervous system in adulthood. Using the rats which developed IBS as a chronic visceral hyperalgesia model (model group), we found that the model rats were more sensitive to CRD stimulation than the normal control rats and that the sensitization was stable and long-lasting. This result indicates that CRD stimuli during the neonatal period of the rat can cause chronic visceral hyperalgesia in the adult rat, which is in line with the findings of other studies [12, 13].

A key step in the development of central sensitization is the activation of the NMDA receptor, which belongs to the family of glutamate receptors, in dorsal horn neurons of the spinal cord. NMDA modulates neuronal reception and transmission of peripheral nociceptive inputs [15]. It has been shown that the application of sustainable CRD stimuli to neonatal rats activates NMDA receptors in the second-order sensory neurons of pain pathways within the spinal cord. This activation can increase the number of various neurotransmitters, such as glutamate, substance P, aspartate, among others, facilitate the responses of the neurons receiving noxious visceral inputs and result in visceral hyperalgesia and central hyperexcitability [13, 14].

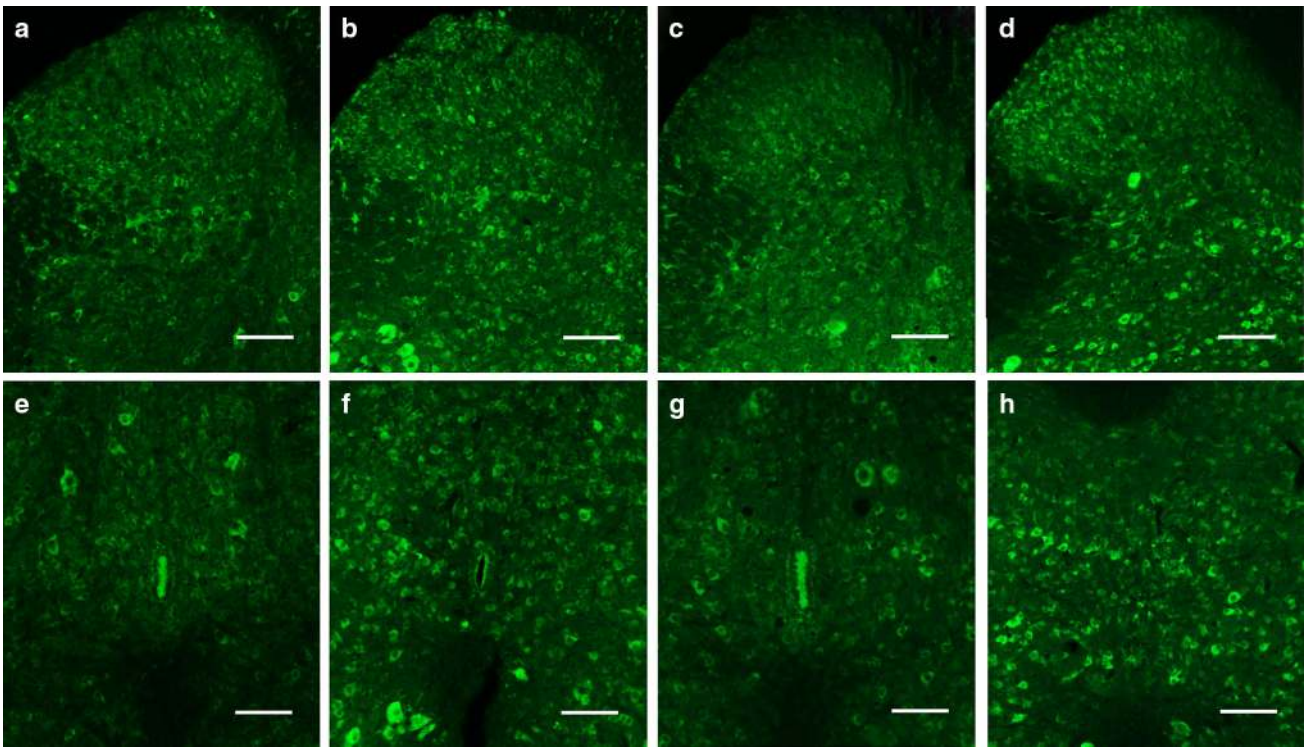

Fig. 8 NR2B-positive immunoreactive neurons in transverse sections of the spinal dorsal horn and central canal region at the lumbosacral (L6-S2) segmental level (magnification ×100). **a** Low expression of NR2B in the spinal dorsal horn of the control rat; **b** high expression of NR2B in the spinal dorsal horn of the model rat (without any treatment); **c** low expression of NR2B in the spinal dorsal horn of the EA-treated model rat; **d** high expression of NR2B in the spinal dorsal horn of the sham EA-treated model rat; **e** low expression of NR2B in the central canal region of the control rat; **f** high expression of NR2B in the central canal region of the model rat (without any treatment); **g** low expression of NR2B in the central canal region of the EA-treated model rat, **h** high expression of NR2B in the central canal region of the sham EA-treated model rat. *Scale bar* 100 μm

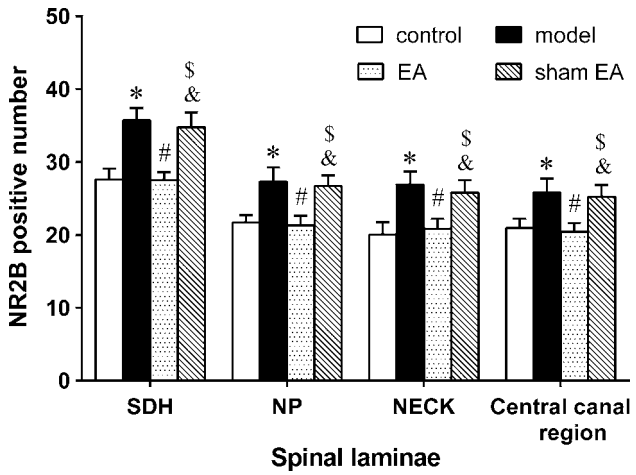

Fig. 9 Number of NR2B-positive immunoreactive neurons at the lumbosacral (L6-S2) segmental level in the four rat groups (*model*, *EA*, *sham EA*, *control*; $n = 8$ rats in each group). *Abscissa* Number of NR2B-staining neurons, *ordinate* observed areas (see caption to Fig. 3 for definition of abbreviations) in the spinal dorsal horn observed at low magnification (×100) under a microscope. Data are presented as the mean ± SE. *Significant difference at $P < 0.05$ vs. control, $^{\#}$significant difference at $P < 0.05$ vs. model, $^{\$}$significant difference at $P < 0.05$ vs. control, $^{\&}$significant difference at $P < 0.05$ vs. EA

It would appear that a functional NMDA receptor requires NR1 subunits and at least one of the NR2 subunits [16–18]. Studies have also shown that selective NR2B antagonists of the NR2B subunit, such as CP-101 and -606 and Ro 25-6981, can produce antinociception [4, 20]. Taken together, these findings suggest that NR2B may be specifically required for the anxiolytic effect. The NR2B subunit has a denser and more defined distribution in the spinal dorsal horn and around central canal (lamina X) [20]. In our study, we demonstrated that the expression of NR2B in neurons in the model rats was enhanced in areas such as the SDH (superficial laminae), NP (nucleus proprius) and NECK (neck of the dorsal horn) in the spinal dorsal horn and central canal region, at both the thoracolumbar and lumbosacral segmental levels. This enhanced expression appeared to be associated with postnatal development of visceral hypersensitivity in rats.

Traditional acupuncture and its more modern alternative electro-acupuncture are key components of traditional Chinese medicine and known effective therapeutic strategies for treating a variety of diseases, including pain [24, 27, 28]. The technique is easy to apply, is of low cost and

causes fewer side effects than other medications. EA with alternating frequencies of 5/25 Hz is extensively used in clinical practice in China. The results of animal studies [29–31] show that analgesia can be induced by 2/15 Hz EA and that this effect is mediated by the simultaneous promotion of the release of enkephalin, endomorphin and dynorphin and activation of their receptors in the spine. Findings from clinical [32] and animal [33] studies also demonstrate that the optimal duration of EA treatment is 30–45 min. Based on these previously reported findings, we chose these parameters for EA treatment in our study. We found that EA treatment decreased the sensitivity of the model rats with chronic visceral hyperalgesia to CRD stimulation, which is consistent with the results of a previous study by our group [23]. This result shows that EA can be an effective method for relieving chronic visceral pain. Many studies have attempted clarify the neurobiological mechanism of acupuncture or EA treatment. Qi et al. showed that EA plays an inhibitory role in rats with chronic visceral hypersensitivity through modulating c-fos protein in the spinal dorsal horn [34], while other studies have revealed that EA attenuates IBS-related visceral hypersensitivity through decreasing the NR1 level in the spinal cord and rostral ventromedia medulla [19, 35]. In our study, we showed that this increased expression of NR2B in the model rats could be reduced by EA but not by the sham EA treatment and that this effect of EA on NR2B expression was paralleled by a change in AWR scores. These results may suggest that the effect of EA on the model rats could be related to the downregulation of NR2B expression in the spinal dorsal horns at the thoracolumbar and lumbosacral segmental levels.

In a previous study we showed that expression of the NR1 subunit of the NMDA receptor of IBS model rats was higher than that in normal control rats and that it decreased after EA treatment, as shown by reverse transcription-PCR (RT-PCR) studies [19]. Based on the results of their western blot analysis, Luo et al. reported that the expression of the spinal NR2B subunit significantly increased by 55 % in IBS model rats versus control rats [21]. RT-PCR and western blot, both well-known quantitative procedures for detecting the expression of mRNAs and proteins, will be used in a future study to be carried out by our group to further confirm the role of the NR2B subunit of NMDA receptor in the pathogenesis of chronic visceral hyperalgesia and the therapeutic effect of EA based on our present results.

In conclusion, the results of our study provide evidence that: (1) NR2B subunit receptors in the spinal cord dorsal horn and central canal region are involved in the development of chronic visceral hyperalgesia in rats induced by repetitive neonatal mechanical stimuli to the visceral organ; (2) repeated EA can attenuate such chronic visceral hyperalgesia; (3) the therapeutic effect of EA might be mediated through downregulation of the NR2B subunit receptors in neurons in the spinal cord dorsal horn.

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