

## Suppression of neuropathic pain by peripheral electrical stimulation in rats: $\mu$ -opioid receptor and NMDA receptor implicated

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### Abstract

Peripheral electrical stimulation (PES) has been utilized to manage chronic pain associated with nerve injury. However, the data on clinical effectiveness are conflicting and the neurophysiological mechanism is not well known. This study was designed to assess whether PES relieved neuropathic pain and its possible mechanisms. The neuropathic pain model was made with lumbar 5th (L5) and 6th (L6) spinal nerve ligations in rats. Nociceptive responses of the rats were assessed by the cold plate test (the number and duration of paw lifts that occurred in 5 min on a  $5 \pm 1^\circ\text{C}$  cold plate). PES with a frequency of 2 Hz and at increasing strengths was given for 30 min via stainless-steel needles inserted into standard acupoints on the leg and back, respectively. Immunohistochemistry was used to examine the immunoreactivity of the NMDA receptor 1 (NR1) subunit in the spinal cord dorsal horn. The results are as follows: (1) PES relieved neuropathic pain and the effect was blocked by 1.0 mg/kg naloxone. (2) The effect of one session of PES lasted up to 12 h. (3) Repetitive PES showed a cumulative effect and no tolerance was observed. (4) There was a significant increase of NR1 immunoreactivity in the superficial laminae of the spinal cord of neuropathic pain rats as compared with naive rats. This increase could be reversed by repetitive 2 Hz PES. These results suggest that PES can relieve neuropathic pain, and that  $\mu$ -opioid receptors and NMDA receptors are involved in the effect of PES.

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### Introduction

Neuropathic pain is a devastating consequence of central or peripheral nerve injury. It is characterized by spontaneous pain, hyperalgesia (increased pain due to painful stimuli) and allodynia (pain due to normally non-painful stimuli). Because of its long-duration, slow recovery and difficulty in management, neuropathic pain has been regarded as one of the most obstinate pain syndromes in the clinic. Stimulation-produced analgesia has been subjected to intensive laboratory and clinical investigation since its introduction by Wall and Sweet in 1967 (Wall and Sweet, 1967). Now, it is available as a treatment option in most chronic pain clinics (Day, 2000; Ghoname et al., 1999; Stanton-Hicks and Salamon, 1997). However, much

of the published research shows conflicting data about its clinical efficacy (Carroll et al., 2001; Milne et al., 2001; Osiri et al., 2000; Proctor et al., 2002). In the clinic, patients may present at varying stages in the disease process. Furthermore, patient perception of pain as an indicator of treatment effectiveness is complicated by the motivational affective component of pain. All these make the interpretation of results very difficult. After an extensive review of the transcutaneous electrical nerve stimulation (TENS) literature, Sluka and Walsh (2003) concluded that randomized controlled trials (RCTs) are very important to evaluate the true efficacy of TENS. On the other hand, animal models of pain can overcome the shortcomings in the clinical research design, and allow us to study the neurobiological mechanism of electrical stimulation-produced analgesia. Previous studies showed that peripheral electrical stimulation (PES) inhibited radiant heat-induced pain and acute or chronic inflammatory pain in rats, as well as thermal hyperalgesia in a rat model of peripheral neuropathy (Han et al., 1984; Hsieh et al., 2000; Inoue et

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al., 2003; Liu et al., 1997; Luo et al., 1995; Sluka et al., 1998). In this study, we used a well-controlled animal neuropathic pain model to determine the effectiveness of PES treatment. We also explored the possible mechanism of PES actions. The use of PES in pain clinics is supported by the Gate Control theory and the release of endogenous opioids (Han, 1993; Han et al., 1984, 1991; Melzack and Wall, 1965; Sluka et al., 1999). The *N*-methyl-D-aspartate (NMDA) receptor plays an important role in pain transmission and central sensitization (Aanonsen and Wilox, 1987; Willis, 2002). Ample evidence has demonstrated that neuropathic pain may be associated with an increased function of NMDA receptors (Boyce et al., 1999; Leem et al., 1996; Mao et al., 1993; Qian et al., 1996; Tal and Bennett, 1994). NMDA receptors include NR1, NR2A-D and NR3 subunits in rats (Mori and Mishina, 1995; Masu et al., 1993). Among these NMDA receptor subunits, the NR1 subunit is an essential component for the formation of functional NMDA receptors (Mori and Mishina, 1995; Zou et al., 2002). In the present study, the NR1 subunit was examined in spinal cords of rats with neuropathic pain and rats with PES-treated neuropathic pain using immunohistochemistry to study the mechanism of the long-term analgesic effect of PES on neuropathic pain.

## Materials and methods

### *Animals*

Male Sprague–Dawley rats, provided by the Institute of Animal Research of the Chinese Academy of Sciences, weighing 230–250 g at the beginning of the experiment, were used throughout the experiment. The experimental protocols were approved by the Animal Use and Care Committee of Peking University Health Science Center.

### *Model of neuropathic pain (Chung model)*

The neuropathic pain model was made by ligation of the right L5 and L6 spinal nerves as described by Kim and Chung (1992). Briefly, under 10% chlorohydrate anaesthesia (3 ml kg<sup>-1</sup> body weight, i.p.), the right paraspinal muscles were separated from spinal processes by an incision at L4 to S2 levels. The right L5 and L6 spinal nerves were exposed and tightly ligated with 6–0 silk threads. The muscle and skin were then sutured and the wound treated with topical antibiotics.

### *Cold plate test*

The cold plate test was done as described by Choi et al. (1994) and Jasmin et al. (1998). Each rat was placed in a transparent plastic box (21 × 21 × 28 cm) on a brass plate kept at a cold temperature (5 ± 1°C). After 5 min of adaptation, the numbers of paw lifts and the time that the

rat held its paw off the plate in the next 5 min were recorded.

### *Inclined-plate test*

The rat was placed crosswise to the long axis of an inclined plane. The initial angle of the inclined plane was 50°. The angle was then adjusted in 5° increments. The maximum angle of the plane on which the rat maintained its body position for 5 s without falling was determined according to the method reported by Rivlin and Tator (1977, 1978).

### *PES*

A rat was kept in a specially designed plastic holder, with its hind legs and tail protruding (Han et al., 1984). Two pairs of stainless steel needles of 0.4-mm diameter were inserted into the acupoints ST 36 (5 mm lateral to the anterior tubercle of the tibia), and Jiaji (5 mm lateral to the L5 dorsal spine) on both sides. ST 36 and Jiaji on the same side were connected to form a circuit. Constant current square-wave electric stimulation produced by a HANS LH-800 programmed pulse generator (manufactured by Beijing University of Astronautics and Aeronautics Aviation) was given via the two needles, for a total of 30 min. The frequency of stimulation used was 2 Hz, and the pulse width was 0.6 ms. The intensity of the stimulation was set at 3, strengths in increasing order (0.5, 1 and 2 mA) 10 min for each intensity, lasting for a total of 30 min.

### *Immunohistochemistry*

The rats were deeply anesthetized with an over-dose of 10% chlorohydrate (4.5 ml·kg<sup>-1</sup> body weight, i.p.). They were then perfused transcardially with 150–200 ml normal saline followed by 200 ml fixative consisting of 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). The L5 spinal segment was removed, post-fixed in 4% paraformaldehyde in PB for 4–6 h, and then transferred to 20% sucrose for 24–48 h. Transverse sections of 12-μm thickness were cut with a cryostat. One of every 5–6 sections through the L5 spinal segment was collected and was mounted on gelatin-subbed slides for immunohistochemical labeling for the NMDA receptor R1 subunit (NR1). Six sections from every rat were collected, with 5–6 rats in each group.

The sections were stained immunohistochemically for NR1 using the ABC method. To reduce the possibility of the DAB reacting with endogenous peroxidases in red blood cells in the tissues, and additionally to increase the penetration of the antibody into the tissue, the tissue sections were rinsed in 0.3% H<sub>2</sub>O<sub>2</sub> methanol solution for 30 min before incubation with NR1 antisera. The tissue sections were then incubated sequentially in rabbit anti-NR1 (1:1,600, Upstate Biotechnology) overnight at 4°C,

biotinylated goat anti-rabbit IgG for 1 h at room temperature and avidin–biotin–peroxidase reagent (Histostain™-SP kit, SP-9001-3, Zymed) for 30 min at room temperature. All incubation steps were preceded by three rinses in PBS (5 min). The tissue was immunoreacted for NR1 in 0.05% diaminobenzidine tetrahydrochloride (DAB) and 0.03% H<sub>2</sub>O<sub>2</sub> in 0.01 M PBS for 2–3 min to yield a brown reaction product. The DAB step was preceded and followed by three rinses in 0.01 M PBS for 5 min. The sections were dehydrated in a series of dilutions of ethanol in water and xylene, and the slides were then mounted. To confirm the specificity of the immunolabeling, control slides were exposed to diluted normal goat serum instead of the primary antibody. Control slides that omitted the primary antibody were consistently negative. The specificity of these antisera has also been tested in previous studies (Zou et al., 2000, 2002).

### Data analysis

Data were processed with the Graphpad Prism 3.0 software. Results are presented as mean  $\pm$  SEM. Comparison between the means of groups were analyzed with *t* tests, and one-way or two-way ANOVA where appropriate, followed by Student's Newman–Keul's test. The level of statistical significance was taken as  $P < 0.05$ .

## Results

### Effect of PES on motor function

We used the inclined-plate test to study whether PES had some adverse effect on motor function. The control, needling and PES group rats were tested before and at 2, 6, 12, 24, and 48 h after PES. The results showed that there was no significant difference among the control,

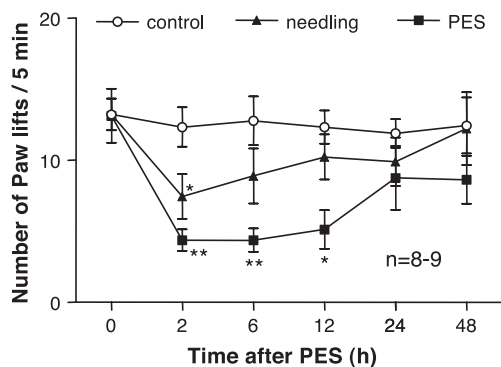


Fig. 1. Time-course of the inhibitory effect of PES (the first session) on neuropathic pain (time–effect curve). Vertical bars represent SEM. Differences between groups were analyzed with two-way ANOVA followed by Student's Newman–Keul's test. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the pre-stimulation in the same group.

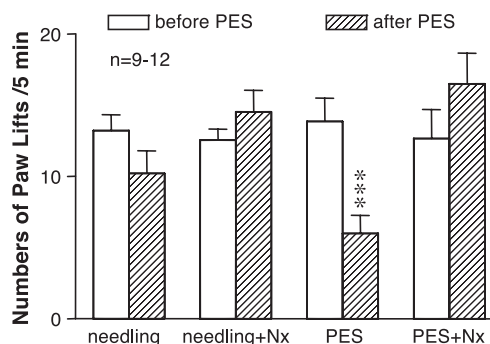


Fig. 2. Naloxone blocked the inhibitory effect of PES on neuropathic pain. \*\*\* $P < 0.001$ , compared with the pre-PES treatment in the same group (*t* test). Nx: 1 mg/kg naloxone.

needling and PES groups in the average maximum angle before and after PES (data not shown).

### Effect of PES on neuropathic pain

On the 8th day after L5/L6 spinal nerve ligation, the results of the cold plate test were assessed. The number of paw lifts for naive, sham surgery, and Chung surgery rats is  $1.2 \pm 0.4$  ( $n = 40$ ),  $2 \pm 0.41$  ( $n = 40$ ),  $14.4 \pm 1$  ( $n = 40$ ), respectively. Rats with a L5/L6 spinal nerve ligation that had more than eight paw lifts on a cold plate in 5 min were considered to have neuropathic pain. The neuropathic pain rats were randomly assigned into three groups, with 11–12 rats in each group. Group 1 was given PES; group 2 had needle insertion without electrical stimulation; and group 3 was restrained in the holder without needle insertion. The number of paw lifts on a cold plate in 5 min was observed at 2, 6, 12, 24 and 48 h after the termination of the PES. The results are shown in Fig. 1. In the control (simple restraint) group, the number of paw lifts (12–15 lifts/5 min) was quite stable over a period of 48 h. In the group with needling without electrical stimulation, paw lifts decreased slightly only at 2 h after PES ( $P < 0.05$  as compared to the pre-PES level). In contrast, in the PES group, the number of paw lifts decreased dramatically from the basal level of  $13.1 \pm 1.9$  lifts/5 min down to  $4.4 \pm 0.75$  lifts/5 min ( $P < 0.01$ ) 2 h after the PES and remained low at 6 h ( $P < 0.01$ ) and 12 h ( $P < 0.05$ ).

### Naloxone blockade

On the 8th day after L5/L6 spinal nerve ligation, the cold plate test was assessed as above. Then, the neuropathic pain rats were divided into four groups ( $n = 9–10$ ). Groups 1 and 2 were given PES; groups 3 and 4 were given only needling as control. Groups 1 and 3 were given subcutaneous injections of 1 mg/kg naloxone, and groups 2 and 4 were given normal saline 15 min before PES or needling. The results are shown in Fig. 2. The number of paw lifts on the cold plate was measured 15 min before and 12 h after

needing or PES. The numbers of paw lifts in the PES plus normal saline group were significantly reduced after PES. However, there was no significant change in the PES plus naloxone group. The numbers of paw lifts also showed no significant change in the two needling groups. These results indicate that the effect of PES on neuropathic pain could be blocked by pre-treatment with naloxone.

#### Effect of repeated PES on neuropathic pain

The above results showed that PES inhibited neuropathic pain. To observe whether PES has a cumulative analgesic effect with multiple treatments, the neuropathic pain rats were divided into two groups ( $n = 12$ ) on the 8th day after L5/L6 spinal nerve ligation. The control group was simply restrained in the holder, and the experimental group was restrained and given PES once every 4 days for 10 sessions. The number and duration of paw lifts was assessed before each session of PES. Results are shown in Fig. 3. While the numbers of paw lifts at the starting points of the two groups were almost identical, they separated from each other gradually (ANOVA,  $P < 0.05$ ) (Fig. 3A), suggesting that the degree of neuropathic pain decreased along with the repeated PES treatments. Fig. 3B shows the change of the cumulative duration of paw lifts in 5 min, another index representing the degree of neuropathic pain. This index

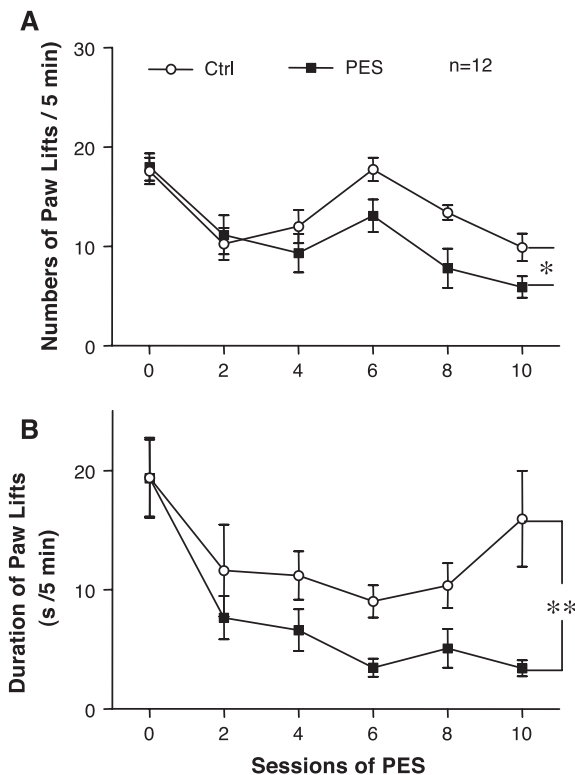


Fig. 3. The analgesic effects of repetitive PES on neuropathic pain. The numbers of paw lifts (A) and the duration of paw lifts (B) in 5 min on cold plate was detected at the beginning of each session of PES. \* $P < 0.05$ , compared with control group from the 6th to 10th session of PES (28th day to 44th day after operation) (two-way ANOVA).

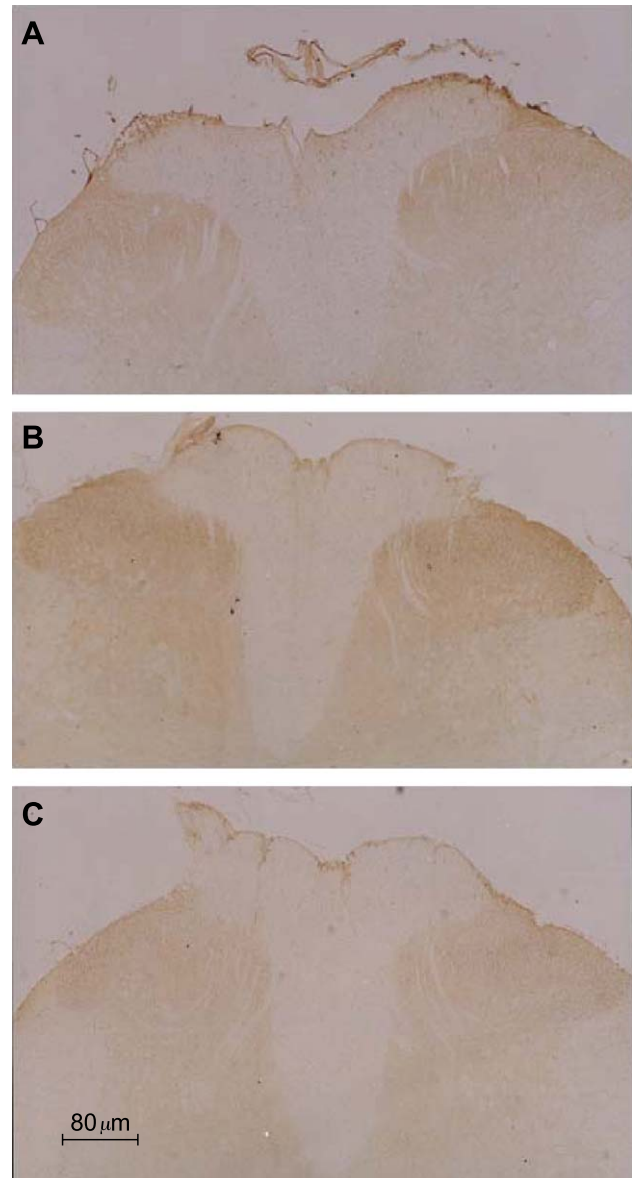


Fig. 4. Immunostaining of NMDA receptor subunit 1 (NR1) in sections of the dorsal horn in the L5 spinal cord segment of rats. (A) naïve rat, (B) rat with neuropathic pain, (C) rat with neuropathic pain plus PES treatment.

showed a gradual recovery from neuropathic pain even in the control group, but the degree of recovery was more substantial in the PES group (ANOVA,  $P < 0.01$ ).

#### NMDA receptor expression in the spinal cord dorsal horn and the effect of repeated PES in neuropathic pain rats

After 10 sessions of PES, expression of the NMDA receptor 1 subunit (NR1) was examined in the L5 spinal cord of a PES (10 sessions) group, a control group (restraint only) and a naïve group, respectively. NR1 immunoreactivity was enhanced significantly in the superficial laminae of spinal cord dorsal horn in neuropathic pain rats (control group) as compared with the naïve rats.



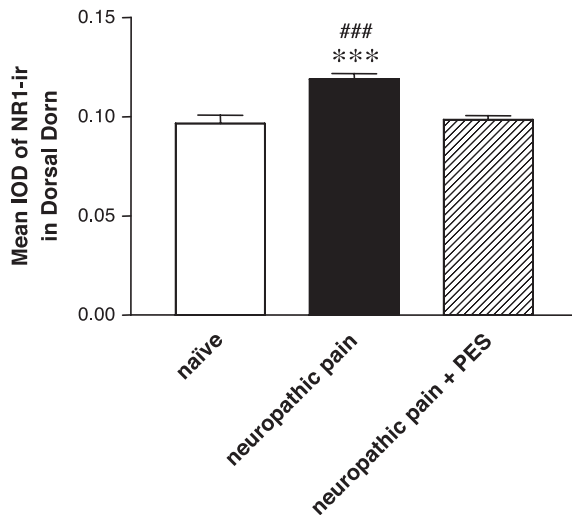


Fig. 5. Statistical analysis of the mean integrated optical density (IOD) of NR1 immunostaining shown in Fig. 4. Rats were divided into three groups: naïve, neuropathic pain and neuropathic pain plus PES. \* $P < 0.05$ , \*\*\* $P < 0.001$ , compared with naïve group. # $P < 0.05$ , ### $P < 0.001$ , compared with neuropathic pain plus PES group (one-way ANOVA).

This change was symmetrical on the two sides. No significant difference was found between the PES group and the naïve group (Fig. 4). Histological imaging was further analyzed densitometrically with an MCID-M4 Microimaging Collection and Analysis System (Canadian Imaging Research Inc.). The mean integrated optical density (IOD) of NR1-ir in the superficial laminae (I–II) was measured, and the data are shown in Fig. 5. The increased expression of NR1 immunoreactivity in the neuropathic pain rats was significantly suppressed by PES.

## Discussion

### *The analgesic effect of PES on neuropathic pain*

In this study, we demonstrated a long-lasting reduction of neuropathic pain following single session of PES. The analgesic effect could outlast the duration of conditioning stimulation by at least 12 h. However, previous studies demonstrated that the analgesic effect of PES in normal rats lasted only 30 min after PES termination (Wang et al., 1992). Ample electrophysiological experiments also showed that the effect of PES is short lasting in normal animals (Garrison and Foreman, 1997); Lee et al., 1985. These results suggest that the effects of PES are different when applied in the normal versus the neuropathic pain state. Sluka et al. (1998) demonstrated a long-lasting reduction of the secondary hyperalgesia induced by joint inflammation following application of transcutaneous nerve stimulation (TENS). Moreover, Ma and Sluka (2001) also showed that reduction in inflammation-induced sensitization of dorsal horn neurons by TENS is long lasting. In addition, Melzack (1975) and Thorsen and Lumsden (1997) also found a long-

term remission of neuropathic pain with brief, intense transcutaneous electric nerve stimulation. Our present study is consistent with the above results, supporting the possibility that PES may be more effective in pain states than in the normal state. Furthermore, after several sessions of PES, the analgesic effect of PES increased gradually and persisted during the whole treatment period. No PES tolerance was observed. The results suggest that repeated PES might be useful in treating chronic pain in the clinic.

### *The possible mechanism of PES effect*

As shown by the results of this study, PES can inhibit neuropathic pain. Furthermore, this effect could be completely prevented by pre-treatment with a moderate dose (1 mg/kg) of naloxone. This suggests that PES might exert its effect via activation of endogenous opioid receptors, possibly  $\mu$  receptors. Previous studies in our lab have amply shown that low-frequency (2 Hz) stimulation can mobilize endogenous opioid peptides, such as endomorphins and enkephalin, which act on the corresponding  $\mu$  and  $\delta$  opioid receptors to induce analgesia (Chen and Han, 1992; Han and Wang, 1992; Han et al., 1984, 1999) in acute pain models. Moreover, the analgesia produced by low-frequency TENS in arthritic rats also can be prevented by blocking spinal  $\mu$  opioid receptors (Sluka et al., 1999). The present study demonstrated that PES-released endogenous opioid peptides might be also important in analgesia in chronic neuropathic pain.

Not only the endogenous opioid system, but also many other transmitter and receptor systems (Han, 1993) are activated by PES. Studies of the hippocampus and other cerebral regions have demonstrated that long-term potentiation (LTP) in synaptic efficacy may be produced postsynaptically by alteration in patterns of presynaptic stimulation. Considerable evidence has established that intense, recurrent and/or sustained noxious stimulation of C fibers leads to an increase in synaptic efficacy and WDR neuron excitability in the DH (Sandkuhler, 2000; Sandkuhler and Liu, 1998; Svendsen et al., 1998; Willis, 1997, 2002). Processes leading to central sensitization are related to the processes underlying LTP and similarly involve the engagement of NMDA receptors (Svendsen et al., 1998; Willis, 1997, 2002). Previous studies, using electrophysiological and pharmacological approaches, demonstrated that NMDA receptors have an important role in neuropathic pain (Boyce et al., 1999; Leem et al., 1996; Mao et al., 1993; Qian et al., 1996; Tal and Bennett, 1994). In the present study, we found a significant increase in NR1 immunoreactivity in the superficial laminae of the dorsal horns of rats with neuropathic pain. Hara et al. (1995) reported a significant decrease of NR1 immunoreactivity in the dorsal horn of the neuropathic pain rat model produced by chronic constriction injury (CCI). This discrepancy may be accounted for by differences in the animal model used (Hama et al. used the CCI model), the time points of observation, and the methods

used for detection of pain behavior. Nociceptive behavior assessed by the cold plate test as an indicator of neuropathic pain was used in our study, whereas thermal hyperalgesia was used in the Hara et al. paper. On the other hand, Zou et al. (2002) demonstrated that only phosphorylated NR1 in spinal dorsal horn was enhanced after intradermal injection of capsaicin, but not NR1 itself. The sensitization of spinal dorsal horn neurons in the capsaicin model is expressed very rapidly and reversibly; it started at 15 min after capsaicin injection and lasted only 3 h (Dougherty et al., 1994). However, the sensitization of spinal dorsal horn neurons induced by peripheral nerve injury underlying chronic, pathological, painful states lasts a long time, and may be associated with alterations in gene expression of NR1, and ultimately, morphological changes in NR1 expression.

PES prevented the increased expression of NR1 subunits. This result suggests that PES can inhibit central sensitization induced by nerve injury by preventing the increased expression of NR1 subunits.

## Conclusions

PES produced a long lasting analgesic effect on neuropathic pain. Both opioid receptors and NMDA receptors (NR1) may be involved in effects mediated by PES on neuropathic pain.

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