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## A Histochemical Study of Substance P in the Rat Spinal Cord: Effect of Transcutaneous Electrical Nerve Stimulation

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### Abstract

The effects of pain stimulant and transcutaneous electrical nerve stimulation (TENS) on changes in substance P (SP) levels were investigated in the dorsal root ganglion (DRG) and dorsal horn of the spinal cord of Sprague-Dawley rats. The rats were divided into three groups: control, formalin stimulation and formalin + TENS. In all rats, the right sciatic nerve was attached to a hook electrode for recording, and the right lower leg was connected to a ring electrode. As a pain stimulant for the formalin stimulation group and formalin + TENS group, 50  $\mu$ l of a 5% formalin was injected into the right paw. At the same time, TENS (50 Hz, 50 V) for 5 minutes was applied via the ring electrode to the formalin + TENS group. After completing the stimulation, the components of the sciatic nerve, i.e. the DRG of the fourth to sixth lumbar spinal roots and a part of spinal cord, were removed. SP-like immunoreactivity (SP-LI) in the DRGs was quantified by the ELISA method. For the spinal cord, immunohistochemical staining for SP was carried out using an avidin-biotin immunoperoxidase method. Using an NIH image, the SP grains in the nerve ends, which were seen as dark brown stains on the Rexed laminae I and II in the dorsal horn of the spinal cord, were counted within a 450  $\mu$ m<sup>2</sup> area. The results showed that SP-LI levels of both the DRG and the dorsal horn of the spinal cord in the formalin + TENS group were significantly reduced as compared with formalin stimulation group. Based on the fact that SP is a nociceptive neurotransmitter, the present study suggests that TENS reduces production of SP in the DRG, and shows analgesic effects by suppressing nociception via C-fiber in the peripheral nerves.

(J Nippon Med Sch 2002; 69: 428–433)

**Key words:** transcutaneous electrical nerve stimulation, substance P, rat spinal cord

### Introduction

Transcutaneous electrical nerve stimulation (TENS) is a method for releasing pain by transcutaneously stimulating a nerve causing pain with the use of an electrode attached to the skin. This method is being used in the fields of rehabilitation and pain clinic as an analgesic therapy

for chronic low back pain, post-traumatic or postoperative pain, pain due to peripheral nerve disorders and so forth. The method has been developed based on the gate control theory<sup>1</sup>, which was originated by Melzack and Wall in 1965. However, the mechanism of releasing pain has not yet been clarified, although hypotheses such as endogenous pain inhibition<sup>2,3</sup>, blockage of pain transmission in the peripheral nerve<sup>4,5</sup> and effects on

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the sympathetic nerve<sup>6,7</sup> have been reported. In addition, the clinical efficacy of TENS has been reported to be 42% to 75%, which is not consistent<sup>8,9</sup>, and there are even reports on the placebo effect of TENS<sup>10,11</sup>.

It is well known that nerve peptides play important roles in either the transmission or modification of pain. A variety of peptides exist in the dorsal root ganglion (DRG) and dorsal horn of the spinal cord. Among them, substance P (SP) is found in large quantities in the DRG, and has been receiving much attention for its involvement in the transmission of pain.

The aim of the present study is to clarify the analgesic effect and mechanism of TENS by quantifying the activity of SP in the DRG and the dorsal spinal horn in a rat model of acute pain.

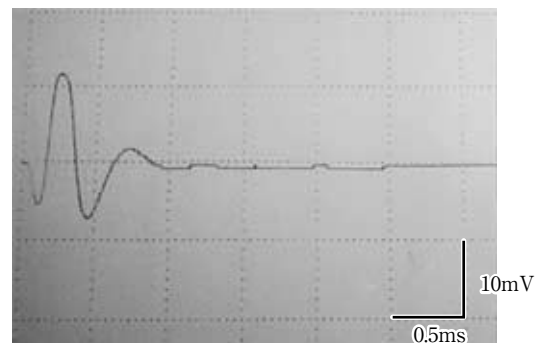
### Materials and Methods

Forty Sprague-Dawley adult male rats weighing 320 g to 350 g were used. The experiments were conducted according to the regulations established by the Animal Ethical Committee of Nippon Medical School with prior approval (number 12-45). Under intraperitoneal anesthesia with Nembutal (sodium pentobarbital: 20-30 mg/kg), the right sciatic nerve, was exposed. Laminectomy was performed through the posterior midline incision, and the components of the sciatic nerve, i. e. the DRG of the fourth to sixth lumbar spinal roots and spinal cord, were exposed. A hook electrode for recording was installed to a fixture and attached to the sciatic nerve. As an indifferent electrode, a silver-silver chloride electrode was installed dorsal-subcutaneously. For the purpose of transcutaneous stimulation, a ring electrode was installed on the right lower leg, leaving a 2 cm space between each terminal. Using a Neuro Pack 2 (Nihon Kohden Corporation Japan), TENS was performed by applying rectangular waves at 0.1 msec in pulse width, 50 Hz in stimulation frequency and 50 V in stimulation voltage. In the formalin test following the method of Dubuisson and Dennis<sup>12</sup>, 50  $\mu$ l of a 5% formalin was subcutaneously injected into the right paw as a pain stimulant (Fig. 1).

The rats were divided into three groups. The



Fig. 1 As a pain stimulant, 50  $\mu$ l of a 5% formalin was subcutaneously injected into the right paw, and at the same time, a ring electrode was installed on the right lower leg for applying TENS (50 Hz, 50 V).



TENS

Fig. 2 Electrogram of sciatic nerve evoked biphasic pattern induced by TENS (Pulse width: 0.1 msec, stimulation frequency: 50Hz, stimulation strength: 50 V)

control group (n=11) had installation of a ring electrode on the lower leg for just 5 minutes. The formalin stimulation group (n=15) had installation of a ring electrode on the lower leg, which was left for 5 minutes after completion of the subcutaneous injection of formalin. The formalin+TENS group (n=14) received both subcutaneous injection of formalin and subsequent TENS for 5 minutes

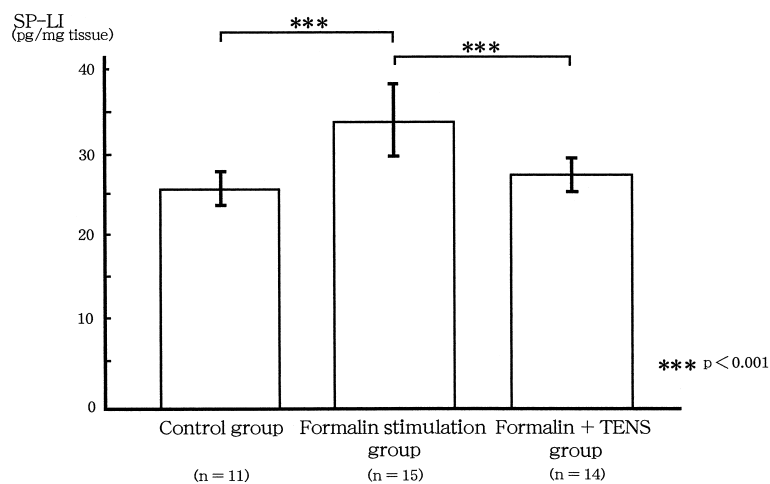


Fig. 3 Quantification of SP-LI in the DRG

SP-LI in the DRG was quantified by the ELISA method.

Compared with the control group and formalin + TENS group, the formalin stimulation group showed a significant increase in SP-LI level in the DRG ( $p < 0.001$ ).

through the ring electrode. For the formalin + TENS group, the derivation of sciatic nerve-evoked biphasic action potential was verified in every rat prior to the experiment (Fig. 2).

After completing the stimulation, the components of the sciatic nerve, i.e. the DRGs of the fourth to sixth lumbar spinal roots and a part of the spinal cord, were removed. The DRG was frozen in liquid nitrogen and stored at  $-20^{\circ}\text{C}$ . The spinal cord was fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4). During the experiment, the room temperature was adjusted to 23 to  $24^{\circ}\text{C}$  using an air conditioner.

### 1. Quantification of SP by the ELISA Method

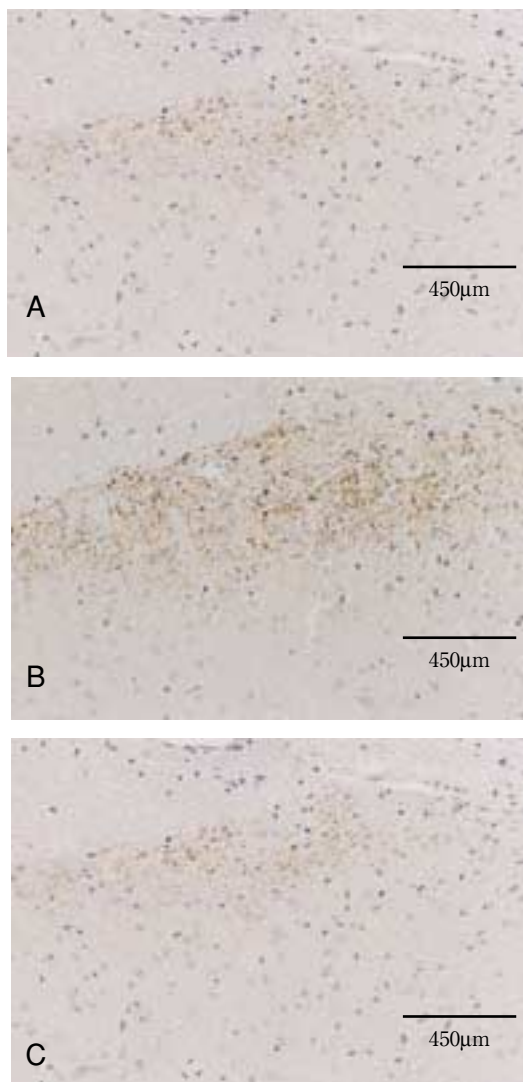
The DRGs were weighed and then added to 500  $\mu\text{l}$  of 2 M acetic acid, homogenized with Polytron homogenizer, and centrifuged at 15,000 rpm for 10 minutes. The supernatant was diluted for quantification. Using a SP EIA kit (Cayman Chemical Co, Ann Arbor, MI), SP-like immunoreactivity (SP-LI) was determined. A microplate adsorbed with anti-rabbit mouse monoclonal antibodies was treated with 50  $\mu\text{l}$  test fluid, and sequentially with 50  $\mu\text{l}$  enzyme-labeled antigens (SP acetylcholinesterase tracer), and 50  $\mu\text{l}$  SP antiserum. The microplate was incubated at  $4^{\circ}\text{C}$  for 18 hours. After rinsing thoroughly with phosphate buffer, 200  $\mu\text{l}$  Ellman's reagent was applied, and the plate was placed in the

dark at room temperature for 90 minutes in order to activate reaction. The absorbance at 412 nm was determined using a microplate reader. The value was divided by the weight of the test specimen, and the value per 1 mg of the test specimen was regarded as SP-LI.

The SP-LI levels are given as mean  $\pm$  SEM, and ANOVA was used for statistic analysis.

### 2. Immunohistochemical Staining

The spinal cords fixed for 24 hours in 4% paraformaldehyde in phosphate buffer (pH 7.4) were embedded in paraffin, and sectioned at 2.5  $\mu\text{m}$  in thickness. Immunohistological staining based on the peroxidase-labeled antibody method<sup>13</sup> was conducted using a Vecstastain ABC-PO kit (Vector Labs, Burlingame, CA). The sections were de-paraffinized. Endogenous peroxidase activity was inhibited by 0.3 %  $\text{H}_2\text{O}_2$  in methanol for 30 minutes, and non-specific bindings were inhibited by normal goat serum for 30 minutes. The sections were treated sequentially with 1:2,000 anti-SP polyclonal antibodies (Chemicon International Inc., Temecula, CA) for 1 h, 1:500 biotinylated secondary antibody for 1 h, and then avidin-biotinylated enzyme complex (ABC) for 1 h. The sections were then immersed in diaminobenzidine (DAB) for 20 min for color development. Nuclear staining was done with hematoxyline.



**Fig. 4** Localization of SP in the dorsal horn of the spinal cord in rats shown by immunohistochemical staining ( $\times 66$ )  
 All three groups showed nerve endings containing SP, which were observed as dark brown stains in the Rexed laminae I and II.  
 (A) Control group  
 (B) Formalin stimulation group  
 (C) Formalin + TENS group

## Results

### 1. Quantification of SP in the DRG

The mean levels of SP-LI in the DRGs of the control group, formalin stimulation group and formalin + TENS group were respectively  $25.5 \pm 2.1$ ,  $34.5 \pm 3.3$  and  $27.1 \pm 2.2$  pg/mg tissue. Compared with the control group, the formalin stimulation group

showed a statistically significant increase in SP-LI level ( $p < 0.001$ ). Compared with the formalin stimulation group, the formalin + TENS group showed a statistically significant decrease in SP-LI level ( $p < 0.001$ ). No significant difference was found in the SP-LI levels between the control group and the formalin + TENS group (**Fig. 3**).

### 2. Immunohistochemical staining and Quantification of SP in the Spinal Cord

The control group, formalin stimulation group and formalin + TENS group all showed nerve endings containing SP, observed as dark brown stains in the Rexed laminae I and II of the spinal cord (**Fig. 4**). The quantification of SP-LI in the spinal cords was conducted by examining three areas ( $450 \mu\text{m}^2/\text{area}$ ) in the dorsal horn. Using an NIH image, the number of grains stained dark brown in color within each area was counted, and the mean value was calculated. The mean levels of SP-LI in the spinal dorsal horns of the control group, formalin stimulation group and formalin + TENS group were respectively  $167.3 \pm 23.0$ ,  $300.0 \pm 24.3$  and  $211.8 \pm 9.3$  grains/area. Compared with the formalin stimulation group, the formalin + TENS group showed a statistically significant decrease in SP-LI level ( $p < 0.01$ ). Compared with the control group, the formalin stimulation group showed a statistically significant increase in SP-LI level ( $p < 0.001$ ). No significant difference was found in the SP-LI levels between the control group and the formalin + TENS group (**Fig. 5**).

These results indicate that in both the DRG and dorsal horn, TENS suppresses the increase of SP-LI level due to stimulation of pain with formalin.

## Discussion

Since the gate control theory developed by Melzack and Wall<sup>1</sup> in 1965, the effect of TENS has been annotated academically, and is widely used for treating patients with pain. In the gate control theory, the activity of thick sensory fibers ( $A\beta$ -fiber) inhibits synaptic transmission of the thin fibers ( $A\delta$ -fiber, C-fiber), which transmit nociception. When thick sensory nerves with low thresholds are

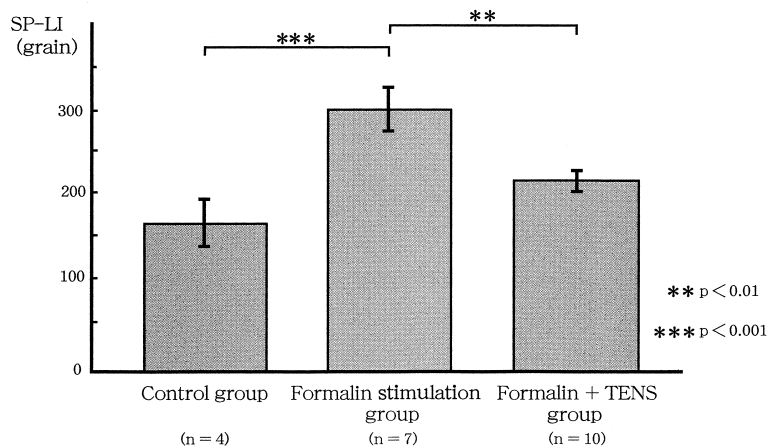


Fig. 5 Quantification of SP-LI in the dorsal horn of the spinal cord

For the spinal cord, immunohistochemical staining for SP was carried out using an avidin-biotin immunoperoxidase method.

Using an NIH image, the SP grains in the nerve ends, which were seen as dark brown stains on the Rexed laminae I and II in the dorsal horn, were counted within a  $450 \mu\text{m}^2$  area.

Compared with the control group and formalin + TENS group, the formalin stimulation group showed a significant increase in SP-LI level in the dorsal horn of the spinal cord ( $p < 0.001$ ).

selectively stimulated by weak electrical stimulation that provides no pain, the transmission of pain can be inhibited in the dorsal horn, leading to pain relief. Today, we have the gate control theory II, discussing the existence of a strong inhibitory system descending from the brain stem against the gate. However, there have been cases with prolonged analgesic effects for hours even after stopping the electrical stimulation. Since these phenomena cannot be explained with the gate control theory, other analgesic mechanisms have also been discussed.

Reports on animal experiments for clarifying the analgesic mechanism of TENS have been comparatively small in number. Ignelzi et al<sup>4</sup> applied low frequency TENS at 15 Hz in 60 V to 150 V to 10 matured cats, and investigated changes in the evoked potential. They reported that A $\delta$ -fiber evoked potential was selectively suppressed, but recovered shortly after completing the stimulation. Hamade et al<sup>5</sup> made a study using A $\delta$ -fibers of the sciatic nerve in the rat, in which TENS was applied at high frequency (100 Hz) and low frequency (20 Hz). They reported that although suppression of evoked potential was observed for both applications, the phenomenon was gone shortly after completing the TENS in both cases. In both of the reports, they suggest the reversible blocking effects of A $\delta$ -fiber in the peripheral nerve as the analgesic mechanism of

TENS. Based on the fact that TENS is applied subcutaneously, Hamade et al also suggested the involvement of adaptability of sensory receptors and sensory nerve terminals distributed beneath the skin.

In the present experimental study on the analgesic mechanism of TENS, SP of C-fiber specific neurotransmitter was quantified in order to observe the role of C-fiber, which is another pain fiber. SP is a polypeptide consisting of 11 amino acids, and is produced in nerve cells of the DRG. A study<sup>14</sup> using immunohistochemical stain has shown that 10 to 20% of primary afferent fibers ended at the spinal dorsal horn include SP. A neuron containing SP transmits unmyelinated C-fiber bidirectionally towards the center and periphery of the nervous system. The nerve endings at the center are found in the Rexed laminae I and peripheries of II<sup>15</sup>. The nerve endings at the periphery are found as free endings in the skin and peripheries of blood vessels. In the present study, the dark brown-stained nerve endings of SP were also found in the Rexed laminae I and II of the dorsal horn by immunohistochemistry.

The primary afferent fibers, which transmit nociception from periphery to the spinal cord, originated in the peripheral nerves, run into the spinal cord via the dorsal roots, and end up at the dorsal horn for the most part. Using radioimmunoassay,

Otsuka et al<sup>16</sup> has verified that when the spinal cord isolated from a newborn rat is stimulated at the dorsal roots under perfusion, the SP level in the perfusate increases. SP is released from nerve ends in response to stimulation, and provokes excitability effect of the spinal neurons. At present, it is believed that SP is a pain transmitter of the primary afferent fiber. Therefore, we consider that the elevation of the DRG SP-LI level observed in the formalin stimulation group in the present study was caused by an increase in SP production induced by the pain stimulation with formalin. The elevation of the SP-LI level in the dorsal horn of the spinal cord should have been caused by the increase of nociception via C-fiber to the dorsal horn that is caused by excitement of cutaneous neurons containing SP due to formalin. The decrease in SP-LI level in the DRG and the dorsal horn of the formalin + TENS group indicates that TENS suppresses the elevation of SP level caused by the subcutaneous injection of formalin. The present study suggests that TENS reduces production of SP in the DRG, and shows analgesic effects by suppressing the activity of C-fiber in the peripheral nerves.

### References

- Melzack R, Wall PD: A new theory. *Science* 1965; 150: 971-979.
- Abram SE, Reynolds AC, Cusick JF: Failure of naloxone to reverse analgesia from transcutaneous electrical stimulation in patients with chronic pain. *Anesth Analog* 1981; 60: 81-84.
- Sjolund BH, Eriksson MBE: The influence of naloxone on analgesia produced by peripheral conditioning stimulation. *Brain Reserch* 1979; 173: 295-301.
- Ignelzi RJ, Kyquist JK: Direct effect of electrical stimulation on peripheral nerve evoked activity: implications in pain relief. *J Neurosurg* 1976; 45: 159-165.
- Hamade S, Nara I, Tachino K, Tsujihara M: Effect of Transcutaneous Electrical Nerve Stimulation on First-Order Neuron Activity. *Rigaku Ryoho to Sagyo Ryoho* 1988; 22: 259-262.
- Leandri M, Brunetti O, Parodi CI: Telethermographic findings after transcutaneous electrical nerve stimulation. *Physical Therapy* 1986; 66: 210-213.
- Abram SE, Asiddao CB, Reynolds AC: Increased skin temperature during transcutaneous electrical stimulation. *Anesth Analg* 1980; 59: 22-25.
- Saeki S, Ogawa S: Pain relief induced by peripheral stimulation. *Pain Clinic* 1998; 19: 19-24.
- Magora F, Alajemoff L, Tannenbaum J, Magora A: Treatment of pain by transcutaneous electrical stimulation. *Acta anaesth scand* 1978; 112: 884-888.
- Evans FJ: The placebo response in pain reduction. *Advances in Neurology* 1974; 4: 289-296.
- Thorsteinsson G, Stonnington HH, Stillwell KS, Elveback LR: Transcutaneous electrical stimulation: A double-blind trial of its efficacy for pain. *Ach Phy Med Rehabil* 1977; 58: 8-13.
- Dubuisson D, Dennis SG: The formalin test: A quantitative study of the analgesic effects of morphine, and brain stem stimulation in rats and cats. *Pain* 1977; 4: 161-174.
- Streit WJ, Schulte BA, Balentine JD, Spicer SS: Evidence for glycoconjugate in nociceptive primary sensory neurons and its origin from the golgi complex. *Brain Res* 1986; 377: 1-17.
- Tuchscherer MM, Seybold VS: Immunohistochemical studies of substance P, cholecystokinin-octapeptide and somatostatin in dorsal root ganglia of the rat. *Neuroscience* 1985; 14: 593-605.
- Hokefelt T, Kellerth JO, Nilsson G, Pernow B: Experimental immunohistochemical studies on the localization and distribution of substance P in cat primary sensory neurons. *Brain Res* 1975; 100: 235-252.
- Otsuka M, Konishi S: Release of substance P-like immunoreactivity from isolated spinal cord of newborn rat. *Nature* 1976; 264: 83-84.

(Received, April 10, 2002)

(Accepted, May 10, 2002)