Early transcutaneous electrical nerve stimulation reduces hyperalgesia and decreases activation of spinal glial cells in mice with neuropathic pain

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A B S T R A C T

Although transcutaneous electrical nerve stimulation (TENS) is widely used for the treatment of neuropathic pain, its effectiveness and mechanism of action in reducing neuropathic pain remain uncertain. We investigated the effects of early TENS (starting from the day after surgery) in mice with neuropathic pain, on hyperalgesia, glial cell activation, pain transmission neuron sensitization, expression of proinflammatory cytokines, and opioid receptors in the spinal dorsal horn. Following nerve injury, TENS and behavioral tests were performed every day. Immunohistochemical, immunoblot, and flow cytometric analysis of the lumbar spinal cord were performed after 8 days. Early TENS reduced mechanical and thermal hyperalgesia and decreased the activation of microglia and astrocytes (P < 0.05). In contrast, the application of TENS at 1 week (TENS-1w) or 2 weeks (TENS-2w) after injury was ineffective in reducing hyperalgesia (mechanical and thermal) or activation of microglia and astrocytes. Early TENS decreased p-p38 within microglia (P < 0.05), the expression levels of protein kinase C (PKC-ε), and phosphorylated anti-phospho-cyclic AMP response element-binding protein (p-CREB) in the superficial spinal dorsal horn neurons (P < 0.05), mitogen-activated protein (MAP) kinases, and proinflammatory cytokines, and increased the expression levels of opioid receptors (P < 0.05). The results suggested that the application of early TENS relieved hyperalgesia in our mouse model of neuropathic pain by inhibiting glial activation, MAP kinase activation, PKC-ε, and p-CREB expression, as well as maintenance of spinal opioid receptors. The findings indicate that TENS treatment is more effective when applied as early after nerve injury as possible.

1. Introduction

Transcutaneous electrical nerve stimulation (TENS) has been successfully applied in clinical treatment of various types of pain, including: neuropathic pain, osteoarthritis-related pain, postoperative pain, and musculoskeletal pain [5,10,55]. Among these, peripheral neuropathic pain is considered one of the most suited for TENS treatment [16,26]. Several experimental studies also reported that TENS can reduce hyperalgesia in neuropathic pain. Although the methodologies (frequency, intensity, placement of electrode, and beginning period) differ among the studies, TENS results in consistent improvement of mechanical and thermal hyperalgesia [21,44], with reduction in the firing of spinal dorsal horn neurons induced by brush and pinch stimuli [33] and increased inhibitory input to the pain pathways at the spinal cord (SC) level [15]. Moreover, early TENS treatment applied soon after nerve injury seems to prevent the development of hyperalgesia caused by central sensitization in neuropathic pain [56]. While there is general agreement that TENS reduces pain via the activation of endogenous opioids [53,70], the precise mechanism of the
action of TENS has not been well documented in models of neuropathic pain.

Neuropathic pain is caused by damage to the somatosensory system, including peripheral or central nervous system [2,64]. It is characterized by spontaneous pain, hyperalgesia (enhanced pain evoked by noxious stimuli), and allodynia (pain evoked by normally innocuous stimuli) [50]. Research on neuropathic pain has focused on changes in neurons and neuronal function in peripheral and central nervous systems. However, recent research identified another mechanism of neuropathic pain that involves both inflammatory responses and glial activation in the spinal cord dorsal horn (SC-DH) [9,50,66,69]. Microglia is the first cell activated following peripheral nerve injury and remains active for several weeks [39,41,50,61]. Histological analysis often shows proliferation and hypertrophy of activated microglia [61,66]. The activated microglia release multiple proinflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor alpha (TNF-α), and IL-6 [9,41,68]. Furthermore, spinal astrocytes also release many proinflammatory cytokines. It is already known that these proinflammatory cytokines play an important role in pain hypersensitization in neurons [9,41]. One of the signaling molecules involved in proinflammatory cytokine processing is phosphorylated p38 (p-p38), including mitogen-activated protein (MAP) kinase family, which is selectively expressed in activated microglia of SC-DH [24]. Phosphorylation of p38 is typically triggered by cellular stress and proinflammatory cytokines. Furthermore, p-p38 is known to regulate the synthesis of numerous proinflammatory cytokines via transcriptional regulation [23,31]. This cascade plays a role in the initiation of neuropathic pain as well as in long-term maintenance of central sensitization in the SC. Thus, suppression of microglial activation and p-p38 activation in microglia should prevent the development of hyperalgesia caused by peripheral nerve injury.

Based on the above background, we hypothesized that TENS may attenuate neuropathic pain through the regulation of glial cell activation level. We used mice with neuropathic pain to investigate the effects of early TENS on hyperalgesia, activation of glial cells, sensitization of pain transmission neurons, and release of proinflammatory cytokines and opioid receptors in the spinal dorsal horn.

2. Materials and methods

2.1. Animals

All experimental procedures were approved by the Ethics Committee for Animal Experimentation of Fukui University. All animals were provided with care according to the guidelines of the International Association for the study of Pain [74]. Experiments were conducted in 124 adult male jcl:ICR mice (Clea, Tokyo, Japan), aged 9 weeks, with a mean body weight of 39.6 ± 3.0 g (±SD). All mice were housed (one per cage) under a 12-hour light-dark cycle, and all had access to water and food ad libitum.

2.2. Spared nerve injury

Spared nerve injury (SNI) was performed according to procedures described in detail previously [8], as adapted to mice [28,32]. Surgical procedures were performed under anesthesia using isoflurane (Forane; Abbot Japan, Tokyo, Japan). After shaving, the left hind limb was incised at mid-thigh level and the overlying muscles were retracted to expose the peripheral branches of the sciatic nerve (common peroneal, tibial, and sural nerves). The common peroneal and tibial nerves were tightly ligated with a 0-0 silk suture. Next, the ligated branches were transected distal to the ligature, and a 2-mm portion of each nerve was removed. Great care was taken to avoid any contact with or stretching of the intact sural nerve. The wound was closed with muscle and skin sutures. In the sham-operated group, the sciatic nerve and its branches were exposed without any lesion.

2.3. TENS application

The mice were lightly anesthetized for application of TENS. TENS was applied for 30 minutes through self-adhesive surface electrodes using a TENS stimulator device (Pulsecure-Pro KR-7; OG GIKEN, Okayama, Japan). The frequency of the stimulation was set at 100 Hz and the intensity was defined by the sensory threshold; the one immediately below the motor level (visible muscle contraction). The hair on the left lumbar area was removed and the area cleaned with water. The surface electrode was resized to 45 mm (length) by 5 mm (width) and placed as described previously [56]. The electrode was placed on the skin overlying the left paraspinous muscle, which is innervated by the dorsal rami of the left L1 to L6. This placement spans the dermatomes of SC segments that also innervate the painful (left) paw. Sham mice were lightly anesthetized and electrodes were placed on the left lumbar skin like the TENS group, but no TENS was applied.

2.4. Measurement of mechanical hyperalgesia

To test for mechanical hyperalgesia, the paw withdrawal threshold (g) to mechanical pressure was assessed using an Analgesia-meter (Ugo Basile, Comerio, VA, Italy) before surgery and every day after surgery. For the test, mice were placed in plastic boxes on a metal mesh floor and habituated to the test environment for 20 minutes. Next, the Analgesia-meter was pushed onto the lateral plantar surface of the left hind paw, which is innervated by the sural nerve. Paw withdrawal thresholds were determined with continuous increase in pressure applied on the planter surface of the hind paw. The pressure value was recorded at leg withdrawal. The reported results of the test represent the mean values of 5 consecutive tests applied at intervals of 5 minutes.

2.5. Measurement of thermal hyperalgesia

Thermal hyperalgesia was assessed using paw withdraw latency to a radiant heat stimulus as described by Hargreaves et al. [17] before surgery and every day after surgery. Before the test, mice were placed in a clear plastic cage and allowed approximately 20 minutes to acclimatize. Using a plantar test apparatus (Ugo Basile), radiant heat was applied to the lateral plantar surface of the left hind paw, which is innervated by the sural nerve. Thermal pain threshold was assessed 5 times, with 5 minutes separating each assessment. The cut-off latency was predetermined at 20 seconds to prevent paw injury. The average of 5 measurements was recorded as withdrawal latency.

2.6. Immunohistochemistry

Eight days after SNI or sham surgery, mice were anesthetized with isoflurane and perfused intracardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). After the perfusion, the L4-5 segment of the SC was removed, postfixed in the same fixative at 4°C for 24 hours, and then immersed in 20% sucrose in 0.1 M PBS at 4°C for 24 hours. Segments of the SC were embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and cut on a cryostat in serial 20-μm-thick sagittal frozen sections. The sections were serially mounted on glass slides, and fixed with 2% paraformaldehyde in 0.1 M PBS for 5 minutes, rinsed in 0.1 M PBS, and stored at −80°C.
For immunofluorescence staining, the sections were permeabilized with 0.3% Triton X-100 in 0.1 M PBS for 2 hours. To avoid nonspecific labeling, slides were incubated at room temperature for 1 hour in PBS containing 2% normal goat serum. The following primary antibodies were diluted in commercial diluents (Antibody Diluent with Background Reducing Components; DakoCytomation, Carpentry, CA, USA) and incubated overnight at 4°C with antigenic combination binding adaptor molecule 1 (Iba1) antibody (1:200, rabbit immunoglobulin G [IgG]; Wako Pure Chemicals, Osaka, Japan) for microglia, anti-glia fibrillary acidic protein (GFAP) antibody (1:200, mouse IgG; Abcam plc, Cambridge, UK) for astrocytes, monoclonal CD11b antibody (1:200, rat IgG; Abcam plc) for microglia, anti-phospho-p38 (p-p38) antibody (1:200, rabbit IgG; Cell Signaling Technology, Danvers, MA, USA), anti-γ isoform of protein kinase C (PKC-γ) antibody (1:200, rabbit IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-phospho-cyclic AMP response element-binding protein (CREB) antibody (1:200, rabbit IgG; Millipore, Billerica, MA, USA). The sections were then incubated for 2 hours at room temperature with Alexa fluor-conjugated 488 or 568 secondary antibodies (1:200, Molecular Probes, Eugene, OR, USA). For cell proliferation assays, the 5-bromo-2'-deoxy-uridine (BrdU) kit (Roche Applied Science, Indianapolis, IN, USA) was used for assessment of cell proliferation. BrdU was injected intraperitoneally at a dose of 1 mL/100 g body weight 1 hour before sacrifice and was labeled in sections according to the instructions provided by the manufacturer. Some sections were also counterstained with the nuclear marker DAPI (Abbott Molecular, Des Plaines, IL, USA). The sections were then washed, wet-mounted, and examined by epifluorescence. All images were obtained using a fluorescence microscope (Olympus AX80; Olympus Optical, Tokyo, Japan) or a confocal laser scanning microscope (model TCS SP2; Leica Instruments, Nussloch, Germany), using 405, 488, and 543 nm wavelengths of the argon/helium-neon laser for fluorescence excitation.

2.6.1. Semi-quantitative analysis of tissue staining

To quantify positive immunoreactivity in glial cells in the ipsilateral side of the superficial dorsal horn of the SC, 3–5 sections per animal were selected at random and analyzed. Highly magnified images (63× objective) were captured with a confocal laser-scanning microscope. Positive immunoreactivity was quantified by counting cells positive for Iba1 and GFAP in the ipsilateral side of the superficial dorsal horn, including laminae I and II. Only those cells with clearly observable nuclei (stained with DAPI) in the focal plane were counted. To quantify microglia proliferation, we counted microglia in the 5-bromo-2'-deoxy-uridine (BrdU) kit (Roche Applied Science, Indianapolis, IN, USA) and incubated overnight at 4°C with one of the following antibodies: anti-p-p38 antibody (anti-rabbit, 1:1000; Cell Signaling Technology); anti-p-ERK [extracellular signal-regulated kinase 1/2 antibody (anti-rabbit, 1:1000; Cell Signaling Technology); anti-p-JNK antibody (anti-rabbit, 1:1000; Cell Signaling Technology); anti-IL-1β antibody (anti-rabbit, 1:1000; Santa Cruz Biotechnology); anti-TNF-α antibody (anti-rabbit, 1:1000; Abcam plc); anti-IL-6 antibody (anti-goat, 1:1000; Santa Cruz Biotechnology); anti-μ-opioid receptor antibody (anti-rabbit, 1:1000; Sigma Aldrich, St. Louis, MO); anti-β-opioid receptor antibody (anti-rabbit, 1:1000; Sigma Aldrich); and anti-β-actin antibody (anti-rabbit, 1:1000; Abcam plc). After 3 washes in 0.1 M PBS containing 0.05% Tween 20, the membranes were incubated for 1 hour at room temperature in the secondary IgG/horseradish peroxidase complex antibody (anti-rabbit 1:1000, anti-goat 1:1000; both from Santa Cruz Biotechnology). After 3 washes in 0.1 M PBS containing 0.05% Tween 20, a commercial detection kit (ECL Advance Western Blot Detection kit; GE Healthcare, Amersham, Buckinghamshire, UK) was used for 5 minutes. Immunoblot analysis images were captured on Image Quant LAS 4000 mini chemiluminescence imaging analyzer (GE Healthcare Life Science, NJ, USA) and band intensities were quantified using Image Quant TL software (GE Healthcare Life Science). The intensity of each band was expressed relative to that of β-actin. Data are expressed as fold change in band intensity normalized to sham control ± SD. Prestained Standards (Nakalai Tesque, Kyoto, Japan) were used as molecular weight controls.

2.8. Flow cytometric analysis

Immediately after deep anesthesia, mice were intracardially perfused with ice-cold PBS, and the spinal cords were harvested. The L4-5 spinal dorsal horns were dissected out carefully and dissociated with collagenase (175 U/mL; Sigma Aldrich) for 1 hour at 37°C. The cells were washed in Dulbecco’s modified Eagle’s medium (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum, and filtered through a 40-μm nylon cell strainer (BD Biosciences, San Jose, CA, USA) under centrifugation to remove tissue debris in order to obtain a single-cell suspension, based on the procedures described previously [49].

From this point on, the cell number was counted in each sample to maintain a cell density of 1.0 × 10^6 cells/100 μL. Cells were incubated for 1 hour on ice with the following fluorescent antibodies:
allophycocyanin rat anti-rat CD45, 0.25 μg/1 mL; Pacific Blue rat anti-Ly-6 G/Ly-6 C, 1.0 μg/1 mL (equivalent to Gr-1) (both from BioLegend, San Diego, CA, USA), and PerCP-Cy 5.5 rat anti-CD11b, 0.25 μg/1 mL (BD Pharmingen, San Jose, CA, USA). The following isotype control antibodies were used for flow cytometry: allophycocyanin rat IgG2b, Pacific Blue rat IgG2b (both from BioLegend), and PerCP-Cy 5.5 rat IgG2b (BD Pharmingen).

For intracellular staining [43,60], the cells were resuspended in 1% buffered formalin (Fixation Buffer; Santa Cruz Biotechnology), and permeabilized with methanol (Permeabilization Buffer; Santa Cruz Biotechnology), followed by re-suspension in ice-cold PBS and incubation for 1 hour with rabbit anti-p-p38 antibody (1:1000; Cell Signaling Technology) secondarily conjugated to phycoerythrin (PE) (1:200; Santa Cruz Biotechnology). Samples with cells alone (ie, no antibody staining) were used as negative controls to eliminate background autofluorescence, and samples containing cells incubated with a single added antibody were used as positive control to eliminate potential problems of dual immunolabeling. These were used to set up the cytometer alignment and to remove any spectral overlap.

Flow cytometry was performed using a fluorescence-activated cell-sorting (FACS) device (FACS CantoTM II; BD Biosciences) with forward scatter to further eliminate any cellular debris from analysis. In each test, a minimum of 250,000 cells was analyzed and the data processed (BD FACSDiva software; BD Biosciences) based on the protocol described in our previous studies [13,63]. The different cells in the suspension were classified according to the combination of the expressed antigens, as stated in a previous report, where a profile of CD11b-positive/CD45-positive/Gr-1-negative immunoreactivity identified microglia [51]. The level of p-p38 immunopositivity in microglia was quantified.

2.9. Study protocols

2.9.1. Protocol of early TENS treatment

Mice were randomly divided into 3 groups. Mice of the first group (TENS group, n = 36) were subjected to SNI and then to TENS under anesthesia from the first day after SNI surgery. Mice of the second group (SNI group, n = 26) underwent SNI and TENS electrodes were also placed under anesthesia, but no stimulation was applied. Mice of the third group (Sham surgery group, n = 26) underwent sham surgery and TENS electrode placement under anesthesia, also without any stimulation.

Behavior assessment of the mice in all 3 groups was carried out the day before surgery and every day after surgery. TENS treatment was applied from days 1 to 7.

2.9.2. Protocol of late TENS treatment

To assess the analgesic effects of late TENS treatment, TENS stimulation was started from late phase (ie, >7 days after SNI surgery). Mice of the TENS-1w group (n = 6) received TENS from days 7 to day 13 (1 week) after surgery, while those of the TENS-2w group (n = 6) received TENS from day 14 to day 20 (1 week) after surgery.

2.10. Drug administration

To examine whether the effect of TENS is mediated through antagonism of spinal mu-opioid receptor (μOR) by opioid antagonists, mice of the early TENS group were administered daily naloxone hydrochloride (2 mg/kg, Sigma Aldrich) or saline vehicle intraperitoneally 15 minutes before daily TENS treatment, based on the procedure described previously [11].

2.11. Statistical analysis

All values are expressed as mean ± SD. Differences between groups were examined for statistical significance using either paired t-tests or one-way factorial analysis of variance. A p value of ≤0.05 was considered significant (Bonferroni's post hoc test).

All statistical analyses were conducted using the SPSS software (version 11.0 for Windows; SPSS, Chicago, IL).

3. Results

3.1. Effects of early and late TENS on mechanical and thermal hyperalgesia in SNI

Mice of the SNI group developed marked mechanical and thermal hyperalgesia from days 1 to 7 after surgery compared to the baseline response. Following subsequent daily TENS applications, mechanical and thermal hyperalgesia gradually decreased in the TENS group from days 2 to 7 (Fig. 1A, B). Mechanical hyperalgesia was significantly milder in the TENS group from days 3 to 7 compared with the SNI group (P < 0.05, Fig. 1A). Thermal hyperalgesia of the TENS group was also reduced on days 6 and 7, compared with the SNI group (P < 0.05, Fig. 1B). Mechanical and thermal threshold values of the sham group did not change throughout the study period. In comparison to the effect of early TENS, mice of the TENS-1w (Fig. 1C, D) and TENS-2w groups (Fig. 1E, F) showed no alleviation of mechanical and thermal hyperalgesia.

3.2. Effect of TENS on Iba1 immunoreactivity in spinal cord dorsal horn

Analysis of Iba1 immunoreactivity was performed to identify microglial activation in the lumbar SC-DH across the treatment groups (Fig. 2). In the SNI group, the expression of Iba1 was increased in both the superficial and deep dorsal horns, compared with the sham group (Fig. 2B). Semi-quantification of high-magnification immunofluorescence images showed a significant increase in the number of microglia in the superficial spinal dorsal horn of the SNI group, compared with the sham group (P < 0.05, Fig. 2M). Microglia in the SNI group exhibited marked cellular hypertrophy and retraction of processes compared with the sham group (Fig. 2G). Mice of the TENS group showed low expression of Iba1, together with a significant reduction in the number of microglia in the superficial spinal dorsal horn, compared with the SNI group (P < 0.05, Fig. 2C, M). Furthermore, the morphology of microglia in the TENS group was similar to that of the sham group (Fig. 2I). Unlike the changes seen in mice of the early TENS group, the TENS-1w (Supplemental Fig. 1) and TENS-2w (Supplemental Fig. 2) groups showed no suppression of Iba1 immunoreactivity.

To examine whether TENS suppresses microglial proliferation after SNI, we examined microglial proliferation using BrdU. In the SNI group, the number of BrdU-positive/Iba1-positive cells in the superficial dorsal horns was higher in the SNI group than in the sham group (Fig. 2K). Semi-quantification of high-magnification immunofluorescence images showed a significant increase in the number of BrdU-positive/Iba1-positive cells in the superficial spinal dorsal horn of the SNI group, compared with the sham group (P < 0.05, Fig. 2N). In contrast, the number of BrdU-positive/Iba1-positive cells in the TENS group was significantly lower than in the SNI group (P < 0.05, Fig. 2L, N).

3.3. Effect of TENS on GFAP immunoreactivity in spinal cord dorsal horn

GFAP immunoreactivity was analyzed to identify activation of astrocytes in the lumbar SC-DH (Fig. 3). Mice of the SNI group showed increased GFAP immunoreactivity in both the superficial and deep dorsal horns of the lumbar SC, compared with the sham group (Fig. 3A, B). Semi-quantitative analysis confirmed a
significant increase in the number of astrocytes in the superficial spinal dorsal horn in the SNI group, compared with the sham group ($P < 0.05$, Fig. 3C, J). Furthermore, astrocytes of the SNI group exhibited marked hypertrophy compared with the sham group (Fig. 3E, G). Mice of the TENS group showed low GFAP expression and a significant reduction in the number of astrocytes in the superficial spinal dorsal horn, compared with the SNI group ($P < 0.05$, Fig. 3J). Application of TENS resulted in reduction of astrocyte cell size (Fig. 3I). Unlike the changes seen in mice of the early TENS group, the TENS-1w (Supplemental Fig. 1) and TENS-2w (Supplemental Fig. 2) groups showed no reduction in GFAP immunoreactivity.

3.4. Effect of early TENS on p-p38 in microglia in spinal cord dorsal horn

Figure 4 shows representative examples of immunofluorescence confocal images following immunolocalization of p-p38. SNI increased p-p38 immunoreactivity in the SC-DH at day 8 after injury (Fig. 4A). Early TENS completely reduced the SNI-induced increase of spinal p-p38 immunoreactivity ($P < 0.05$, Fig. 4A, C). Double immunofluorescence showed that p-p38 was colocalized with CD11b-positive microglia (Fig. 4B), but not with GFAP-positive astrocytes (Supplemental Fig. 3) in the spinal dorsal horn in all groups. Early TENS reduced the number of microglia.
colocalized with p-p38 in the spinal dorsal horn, compared with SNI group (Fig. 4B). Flow cytometry was used to quantify the number of p-p38-positive cells among the microglia (Fig. 5). The percentage of p-p38-positive cells within the CD11b positive/CD45 positive/Gr-1 negative (microglia) population was significantly lower in the TENS group than the SNI group (P < 0.05, Fig. 5A, B).

3.5. Effects of early TENS on PKC-γ and p-CREB in spinal cord dorsal horn

We also examined the effect of early TENS on PKC-γ and p-CREB in ipsilateral spinal dorsal horn neurons. SNI increased PKC-γ expression in ipsilateral spinal dorsal horn neurons at day 8, compared with the sham group (Fig. 6A). Early TENS reduced the SNI-induced increase in PKC-γ expression. Semi-quantitative analysis confirmed that TENS reduced the SNI-induced increase in the number of p-CREB-positive cells in the superficial spinal dorsal horn compared with the SNI group (P < 0.05, Fig. 6B).

3.6. Effects of early TENS on protein levels of MAP kinases, proinflammatory cytokines, and opioid receptors in the spinal cord

We also examined the effect of early TENS on the protein levels of MAP kinases (p-p38, p-ERK1/2, p-JNK), proinflammatory cytokines (IL-1β, TNF-α, IL-6), and opioid receptors (μOR and δOR) (Fig. 7). Early TENS significantly reduced the band intensities of p-p38, p-ERK1/2, and p-JNK, compared with the SNI group (P < 0.05, Fig. 7A). Early TENS also...
significantly reduced the band intensities of IL-1β, TNF-α, and IL-6, compared with the SNI group (P < 0.05, Fig. 7B). These results indicate that early application of TENS reduces the levels of MAP kinases and proinflammatory cytokines present in the spinal cord. On the other hand, early TENS significantly increased the band intensities of μOR and δOR, compared with the SNI group (P < 0.05, Fig. 7C). These results indicate that early application of TENS maintains the presence of spinal opioid receptors, which is known to decrease following SNI surgery.

3.7. Effects of naloxone on hyperalgesia (mechanical and thermal) and spinal glial activation in early TENS group

In the TENS-saline group, daily TENS application gradually decreased mechanical and thermal hyperalgesia from days 2 to 7 (Fig. 8A, B). In contrast, the reduction of mechanical and thermal hyperalgesia by daily TENS from early phase was blocked by daily administration of naloxone (2 mg/kg, intraperitoneally) immediately before application of TENS. Iba1 and GFAP were downregulated in mice of the TENS-saline group, while upregulation of these proteins was noted in the superficial spinal dorsal horns in the TENS-naloxone group, compared with the TENS-saline group (P < 0.05, Fig. 8C). Semi-quantitative analysis showed that early TENS significantly reduced the number of astrocytes in the superficial spinal dorsal horn compared with the SNI group (Fig. 3J). Data are mean ± SD of 5 mice per group. *P < 0.05.

4. Discussion

The major findings of the present study are: 1) Application of TENS immediately after SNI improved mechanical and thermal hyperalgesia induced by SNI. In contrast, application of TENS at 1 and 2 weeks after SNI did not improve mechanical or thermal hyperalgesia; 2) early, but not late, TENS suppressed SNI-induced microglia and astrocyte activation; 3) at a subcellular level, early TENS decreased p-p38 within microglia compared with SNI group, and reduced the protein levels of both PKC-γ and p-CREB in the superficial spinal dorsal horn neurons, compared with SNI group; 4) the effects of early TENS were likely mediated through downregulation of MAP kinases and proinflammatory cytokines, as well as increased protein expression of opioid receptors; 5) the inhibitory effects of early TENS on hyperalgesia (mechanical and thermal) and glial activation was blocked by daily naloxone administration immediately before application of TENS.

Generally, TENS is defined as the application of electrical stimulation to the skin for pain relief. TENS is a noninvasive, low-cost, safe, easy-to-use, and effective treatment for individuals with a variety of pain conditions [10,55]. Clinically, TENS is effective in at least some patients with neuropathic pain [6,18,26]. Also, studies in animal models of neuropathic pain reported that TENS reduces hyperalgesia. Irrespective of the methodology used for TENS application (frequency, intensity, placement of electrode and duration of application), TENS inhibits mechanical [44,58,59] and thermal [21,56,58] hyperalgesia; representing the response of spinal dorsal horn neurons to mechanical stimulation [33] and C-fiber responses of spinal dorsal horn neurons [15]. In agreement with the above studies, we demonstrated here the ameliorative effects of TENS on mechanical and thermal hyperalgesia in mice with neuropathic pain. Interestingly, these effects occurred only when TENS was applied on the first day after injury, but not at 1 or 2 weeks after injury. This finding is similar to those of previous studies, which showed a better outcome after early SC stimulation (after 24 hours of neuropathic pain onset) than late SC stimulation.
Peripheral nerve injury provokes changes in spinal microglia, including increased proliferation, hypertrophy, and overexpression of neuropeptides, cytokines, chemokines, neurotransmitters, nucleotides, and various receptors, which, in turn, lead to sustained changes in the dorsal horn environment and pain sensation (after 16 days) [65], and significant reduction of thermal hyperalgesia after early TENS (next day postsurgery), but not after late TENS (day 3 postsurgery) [56]. Our results also indicated that early application of TENS reduces hyperalgesia and may prevent early changes in the nervous system that can elicit neuropathic pain.

Fig. 4. Effects of spared nerve injury (SNI) with and without early transcutaneous electrical nerve stimulation (TENS) on p-p38 immunoreactivity in lumbar spinal cord dorsal horn. SNI induced p-p38 upregulation in the spinal cord dorsal horn at day 8 after injury. TENS suppressed p-p38 immunoreactivity (scale bar = 100 μm) (A). Double immunofluorescence for p-p38/CD11b colocalization in the medial superficial dorsal horn of SNI mice with or without early TENS treatment (scale bar = 20 μm) (B). Early TENS reduced p-p38 that colocalized with CD11b. Semi-quantitative analysis showed significantly lower number of p-p38-positive cells in the superficial spinal dorsal horn of the TENS group compared with the SNI group (C). Data are mean ± SD of 5 mice per group. *P < 0.05.
At least some of the signaling information transferred between activated microglia and neurons is mediated by these chemicals and receptors [4]. Microglia play a role in the initiation of neuropathic pain in the early induction phase [52], and microglia proliferation was reported to correlate with the development of neuropathic pain [11]. Thus, microglia are considered a suitable target for treatment of neuropathic pain. In this study, early, but not late, TENS suppressed microglial activation and proliferation in the SC-DH. In this regard, activation of astrocytes is also involved in neuropathic pain, especially during the extension of the chronic phase of pain [62]. Our data showed that suppression of aberrant astrocytic activation in SC-DH also occurred only after early, but not late, TENS. This result also supports our hypothesis that early TENS seems to prevent the development of neuropathic pain.

The development and maintenance of neuropathic pain involves the activation of MAP kinase in glial cells [22]. MAP kinases are a family of molecules that play a critical role in cell signaling and gene expression. This family consists of 3 major members: ERK (including ERK1/2), p38, and c-JNK. The p38 pathway is phosphorylated by cellular stress and proinflammatory cytokines. P-p38 regulates the synthesis of numerous proinflammatory cytokines via transcriptional regulation [24,31]. Nerve injury results in increased p-p38 levels in spinal hyperactive microglia [67], and intrathecal injection of p38 inhibitors in the early postinjury phase suppressed pain hypersensitivity [25]. These results suggest that p-p38 is important for the development of neuropathic pain and that inhibition of p-p38 may prevent neuropathic pain. The present results demonstrated that early TENS reduced...
p-p38 expression in activated microglia, suggesting that early TENS reduces the development of neuropathic pain by preventing p-p38 activation in the spinal dorsal horn. Previous studies also suggested that both p-ERK1/2 and p-JNK pathways seem to play important roles in the development and maintenance of pain after peripheral nerve injury [7,35,72,73]. Our results also showed that early TENS reduced p-ERK1/2 and p-JNK expression, suggesting that the analgesic effects of early TENS seem to be mediated at least in part by inhibition of p-ERK1/2 and p-JNK.

Early TENS also prevented nerve injury-induced increases in PKC-γ and p-CREB protein levels in SC-DH neurons. Overexpression of PKC-γ is generally associated with neuropathic pain behavior [37,38,42]. It is possible that early TENS prevented the development of neuropathic pain through the blockade of injury-induced increase in PKC-γ. Both long-term potentiation and central sensitization involve activation of the N-methyl-d-aspartate receptor followed by subsequent activation of certain proteins such as PKC-γ. Stimulation of these cascades can lead to phosphorylation of transcription factors and changes in gene transcription [23]. The transcription factor CREB is considered to play an important role in the formation of long-term memory, largely due to the demonstration of CREB activation in the late phases of both long-term facilitation and long-term potentiation [3]. Recent studies on central sensitization following peripheral insults have also highlighted the role of activated CREB in nociceptive hyperreactivity [23,45]. Interestingly, p-CREB is also upregulated during the time course of peripheral neuropathic pain [34]. Considered together with the above results, the present findings suggest that

Fig. 6. Early transcutaneous electrical nerve stimulation (TENS) reversed spared nerve injury (SNI)-induced changes in protein kinase C (PKC-γ) expression and phosphorylated anti-phospho-cyclic AMP response element-binding protein (p-CREB) expression in ipsilateral spinal dorsal horn neurons. Mice of the SNI group show increased PKC-γ immunoreactivity in the ipsilateral side of spinal dorsal horn (scale bar; upper panel = 100 μm and lower panel = 50 μm) (A). Early TENS reduced PKC-γ expression in neurons. Semi-quantitative analysis showed that TENS significantly reduced the number of PKC-γ-positive cells in the superficial spinal dorsal horn compared with the SNI group (B) (n = 3, each). In the SNI group, p-CREB immunoreactivity was increased in the ipsilateral side of spinal dorsal horn (scale bar; upper panel = 100 μm and lower panel = 50 μm) (C). TENS reduced p-CREB expression in neurons. Semi-quantitative analysis showed significantly low number of p-CREB-positive cells in the superficial spinal dorsal horn in the TENS group compared with the SNI group (D) (n = 3, each). Data are mean ± SD. *P < 0.05.
early TENS suppresses the activation of these molecular mechanisms in neuropathic pain following peripheral nerve injury. The spinal mechanism by which TENS produces analgesia is not clear. Previous reports suggested that the analgesic effect of TENS involves changes in endogenous opioids [53,70], serotonin receptors [47], muscarinic receptors [48], and inhibitory neurotransmitters (GABA and glycine) [36,57] in the SC. Among these, we focused in this study on endogenous opioid receptors. Our immunoblot results showed that early TENS increased the protein levels of spinal μ- and δORs, compared with the SNI group. Animal experiments have demonstrated the stimulatory effects of TENS on the release of endogenous opioids [53,70]. In humans, TENS increased endogenous opioid concentrations in the lumbar cerebrospinal fluid [14,19]. Opioids are believed to inhibit synaptic transmission in SC-DH neurons [29,30]. Opioid receptors are localized in the dorsal horn, both presynaptically on primary afferent fibers and postsynaptically on dorsal horn neurons, where presynaptic activation of opioid receptors results in reduction of the release of excitatory neurotransmitters, glutamate and substance P, and therefore prevent glial activation. On the other hand, it has been reported that downregulation of spinal cord μORs following peripheral nerve injury is related to pain hypersensitivity in neuropathic pain animal models [1,46,71] and may contribute to opioid-insensitive mechanisms [30]. Considered together, our results indicate that early TENS both upregulates endogenous opioid after nerve injury and also results in maintenance of opioid receptors in mice with neuropathic pain.

Immunoblot analysis showed that early application of TENS after SNI resulted in downregulation of proinflammatory cytokines. The pathology of neuropathic pain involves release of proinflammatory cytokines from activated glial cells [9,50,69]. Furthermore, acute opioid analgesia is potentiated by blockade of key proinflammatory cytokines [27]. Therefore, early TENS may reduce the release of proinflammatory cytokines by activation of opioid receptors, although further research is needed to validate such conjecture.

In conclusion, the present study demonstrated that early TENS attenuated SNI-induced hyperalgesia. This effect was mediated by suppression of glial cell activation, activation of p-p38, PKC-γ, and p-CREB expression in neurons, downregulation of proinflammatory cytokines, and upregulation of SC endogenous opioid and preservation of opioid receptors. The results also showed that late TENS was ineffective in attenuating pain behavior or glial cell activation. The findings suggest that the outcome of TENS treatment should be more successful when used as early after nerve injury as possible.
FIG. 8. Effects of early transcutaneous electrical nerve stimulation (TENS) with and without daily administration of naloxone (2 mg/kg, intraperitoneally), μOR antagonist, on hyperalgesia and glial activation after spared nerve injury (SNI). The TENS-saline group showed gradual attenuation of mechanical (A) and thermal hyperalgesia (B) (n = 5, each). In contrast, naloxone blocked the early TENS-induced inhibitory effect on mechanical and thermal hyperalgesia. Mice of the TENS-saline group showed downregulation of both Iba1 (C) and glial fibrillary acidic protein (GFAP) (F) at day 8 after SNI within the ipsilateral side of the dorsal horn. In contrast, mice of the TENS-naloxone group showed no such downregulation of Iba1 (C) and GFAP (E). Scale bars: (C, E) = 100 μm. Semi-quantitative analysis confirmed that the expression levels of both Iba1 (D) and GFAP (F) were significantly higher in the TENS-naloxone group than the TENS-saline group (n = 5, each). Data are mean ± SD. *P < 0.05.

Conflict of interest statement

The authors declare no financial interest or any relationship with any of the commercial companies or institutions mentioned in this article.

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Appendix A. Supplementary data

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References


