

Immunoneutralization of c-Fos Using Intrathecal Antibody Electroporation Attenuates Chronic Constrictive Injury-induced Hyperalgesia and Regulates Preprodynorphin Expression in Rats

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Background: *In vivo* electroporation has been successfully used for the introduction of DNA, RNA, oligonucleotides, and proteins into cells for experimental and therapeutic purposes. The authors evaluated the efficacy of electroporation-mediated c-Fos antibody therapy for neuropathic pain *in vitro* and *in vivo*.

Methods: First, the authors studied the inhibitory effects of intrathecal c-Fos antibody electroporation on the activating protein (AP-1) promoter activity in cultured spinal neuronal cells transfected with p-AP-Luc plasmid and activated with 100 μ M glutamate. The inhibitory effect of c-Fos antibody electroporation in the regulation of AP-1 promoter activity was assessed according to the relative luciferase activity. Second, rats with chronic constrictive injury underwent electroporation treatment for neuropathic pain using c-Fos antibody. Thermal nociceptive thresholds were measured before chronic constrictive injury and then on even-numbered days, up to and including day 14, to assess and compare the therapeutic effects of intrathecal electroporation. The time course was assessed by Western blot analysis and by immunohistochemical analysis. Pronociceptive gene expression was measured by assessing prodynorphin mRNA and dynorphin peptides on days 2 and 10 after intrathecal c-Fos electroporation.

Results: Cotransfection of c-Fos antibody significantly decreased glutamate-induced AP-1 activity. Intrathecal electrotransfer of c-Fos antibody attenuated spinal dynorphin levels, as manifested by significantly elevated pain thresholds in the chronic constrictive injury-affected limbs.

Conclusion: This study shows that transfer of antibody into rat spinal cords by intrathecal electroporation is a useful method to study the function of endogenous factors of spinal-related disorders.

AN animal model of mononeuropathy—also known as the chronic constriction nerve injury (CCI) model—is produced experimentally by compressing a peripheral nerve and exhibits many of the clinical characteristics seen in patients with nerve injuries such as hyperalgesia

and allodynia.^{1,2} Sciatic nerve ligation evokes an expression of Fos protein from the *c-fos* gene in neurons in spinal cord dorsal horn regions associated with the transmission of nociceptive messages.³ Increased Fos immunoreactivity directly reflects increases in neuronal activity.^{4,5} The increased activity of the dorsal horn neurons in the spinal cord in response to sciatic nerve ligation can apparently be attributed to abnormal activity in the injured peripheral nerve and to changes in the spinal cord dorsal horn neurons secondary to the nerve injury.^{3,6,7}

Neurons in the superficial layers of the lumbar spinal dorsal horn laminae I and II are known to be involved in nociceptive processing and relaying nociceptive information to higher levels of the neuraxis.⁸ Noxious stimulation of the hind paw has been shown to result in an induction of *c-fos* expression in laminae I and II.^{9–12} The *c-fos* gene encodes proteins that act as transcription factors, binding to DNA promoter “cis-acting” elements of the activating protein (AP-1) binding site.^{11,13–15} Regulation of *c-fos* may play a key role in the production of late-onset gene products such as enkephalin and dynorphin. These changes in turn may result in prolonged sensory alterations after nociceptive stimulation.^{16,17} Because the prodynorphin gene contains an AP-1-like binding site in the 5' untranslated region,^{11,18} it is reasonable to assume that knock down of the immediate early gene can inhibit the synthesis of dynorphin, a pronociceptive opioid peptide that plays an important role in the maintenance of neuropathic pain.^{19,20}

Electroporation has been used for the intracellular deposition of macromolecules *in vitro*^{21–24} and *in vivo*.^{24–28} Thus, transfer of antibodies into intact mammalian cells permits the study of the function of various endogenous factors that form complexes with these antibodies in their natural environment²⁹ and incorporated antibodies that are capable of binding to vimentin³⁰ and of neutralizing viruses.³¹ Electroporation is thus a useful tool for studying cellular functions, because proteins such as antibodies or enzymes can be induced to enter a viable cell and interact with cytoplasmic or nuclear proteins.

In these experiments, we initially transferred antibodies into the spinal cord *in vivo* by electroporation. We assessed the effects of c-Fos antibody delivered by electroporation on the AP-1 promoter activity in glutamate-activated neuronal cells, which mimic neuronal cells

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after nerve injury. We then transferred the c-Fos antibody into the spinal cord by intrathecal electroporation with a specially designed probe and observed the nociceptive behavior induced after CCI to the sciatic nerve in rats. We demonstrate that this introduction of c-Fos antibody by electroporation *in vivo* can suppress the thermal withdrawal latency in mononeuropathic rats.

Materials and Methods

Experimental Design

We divided our experiments into three parts. In the first part, we studied the knock down effects of intrathecal c-Fos antibody electroporation on the AP-1 promoter activity in activated cultured spinal neuronal cells *in vitro*. Cultured spinal neuronal cells received p-AP1-Luc plasmid DNA with different concentrations of c-Fos antibody (0–2 $\mu\text{g}/\text{ml}$) using a single brief electric pulse of 150 V and a duration of 20 ms. Thirty-six hours later, 100 μM glutamate was added to the culture medium for 15 min to stimulate AP-1 promoter activity according to our preliminary results. The inhibitory effect of c-Fos antibody electroporation on the regulation of AP-1 promoter activity was assessed according to the relative luciferase activity. In the second part, we studied the effects of intrathecal c-Fos antibody electroporation on neuropathic pain status *in vivo*. Animals (six per group) were randomly assigned to one of five treatment groups. Depending on group designation, the animals received saline, nonspecific immunoglobulin, 0.4 μg c-Fos antibody, and 4 μg c-Fos antibody followed by electroporation or nonelectric treatment. c-Fos antibody was administered immediately after the sciatic nerve injury. Thermal nociceptive and mechanical allodynic thresholds were measured before CCI and then on even-numbered days, up to and including day 14, to assess and compare the therapeutic effects of intrathecal electroporation. The time course was assessed by Western blot analysis and by immunohistochemical analysis.

In the third part, we identified the therapeutic mechanism activated using our innovative method. We measured pronociceptive gene expression by assessing prodynorphin mRNA dynorphin peptides on days 2 and 10 after intrathecal c-Fos electroporation.

Transfer of Antibody into Cultured Spinal Neuronal Cells through In Situ Electroporation

Spinal neuronal cells were isolated from rat lumbar spinal cords, mainly using the Papain Dissociation System (Worthington Biochemical Corporation, Freehold, NJ) as described previously.³² Cells were plated on a six-well plate at 3×10^5 cells/ml in 2 ml complete medium per well, and the cells obtained 75% confluence on the day of electric pulse treatment. Cells were

washed twice with 1 ml Neurobasal medium (Gibco, Grand Island, NY) and incubated using 0.6 ml Neurobasal medium mixed with rabbit polyclonal antibody to c-Fos (0.2 $\mu\text{g}/\mu\text{l}$) or with rabbit immunoglobulin (Santa-Cruz, Santa Cruz, CA) at a series of dilution ratios ranging from 1 to 10^{-4} . The cells were then exposed to a single brief electric pulse of 150 V for 20 ms at room temperature that was delivered from a BTX 35-mm Petri Pulser (BTX; ElectroGenetics, Islip, NY). Five minutes after pulsing, 2.4 ml prewarmed complete medium was added. After a 3-h incubation, the medium was changed once to ensure good cell survival.³³

Luciferase Assay for AP-1 Promoter Activity

Spinal neuronal cells cultured for 10 days were cotransfected by electroporation with an AP-1 promoter-luciferase reporter gene construct (Stratagene, La Jolla, CA) and with c-Fos antibody. The cells were scraped harvested on appropriate days using 1 ml scraping buffer (40 mM Tris-HCl, pH 7.4, 1 mM EDTA, 180 mM NaCl), pelleted by centrifugation at 300g for 10 min, resuspended in 100 μl luciferase reporter lysis buffer (Promega, Madison, WI), and lysed using three freeze-thaw cycles. Debris was removed by centrifugation at 300g for 10 min, and the supernatant protein concentration was determined using the micro-BCA protein assay (Pierce, Rockford, IL). A luciferase assay system (Promega) was used to detect luciferase reporter gene activity.

Animals

The following studies were reviewed and approved by the Chung Gang Memorial Hospital Animal Care and Use Committee (Kaohsiung, Taiwan). A total of 198 male Sprague-Dawley rats (250–300 g) were used. All animals were analyzed behaviorally. One hundred eight animals (three per time point) were used for studying the time course of c-Fos antibody levels after intrathecal pretreatment with saline, nonspecific immunoglobulin, and c-Fos antibody with and without electroporation. Thirty animals ($n = 6$ per group) were used to assess nociceptive alternation over 21 days. Sixty animals were examined 2 and 10 days after intrathecal electroporation by reverse transcription-polymerase chain reaction analysis of preprodynorphin ($n = 6$) and enzyme-linked immunosorbent assay of spinal dynorphin ($n = 6$).

The Bennett neuropathic model was used as the basis for generation of neuropathic pain. The right sciatic nerve was exposed during isoflurane anesthesia. Proximal to the sciatic trifurcation, ≈ 7 mm of the nerve was freed of adhering tissue, and four ligatures (5–0 chromic gut) were tied loosely around it at 0.5- to 1.0-mm intervals.

The incision was closed in layers, and prophylactic penicillin (50 mg/kg) was administered intramuscularly, followed by subcutaneous injection of 5 ml glucose solution, 5%, to prevent dehydration. Sham surgery was

done by exposing the left sciatic nerve without performing ligation. Animals were maintained under standard colony conditions with food and water available *ad libitum*.

Transfer of Antibody into Spinal Cords In Vivo by Intrathecal Electroporation

An electrode catheter was implanted intrathecally using a previously described technique.^{34,35} Briefly, a double lumen catheter was constructed using polyethylene tubing (PE-5; Becton Dickinson, Baltimore, MD) and a cuprophane hollow fiber dialysis membrane. Before connecting the dialysis membrane (1 cm in length) to one of the PE-5 tubes (8 cm in length), a Nichrome wire (0.0026"; Wire Tronic, Inc., Pine Grove, CA) was inserted into the blind-looped dialysis fiber. The other PE-5 tubing (9 cm in length) served as the intrathecal antibody injection. The electrode catheter was then implanted into the lumbar (L2-L5) intrathecal space *via* a cisternal incision during isoflurane (2–2.5%) anesthesia. All animals were allowed to recover for 2 days before the start of the study. The specific c-fos antibody reacts to a peptide corresponding to amino acids 210–235, mapping onto the amino terminus of human c-Fos (Santa Cruz). This primary antibody does not react with Fos-B, Fra-1, or Fra-2 (0.4 or 4 μ g in phosphate-buffered saline added with Triton X-100, 0.2%). Nonspecific immunoglobulin or saline samples (20 μ l) were delivered through the intrathecal catheter as controls. The intrathecal electrode contacted a negative electrode clamp through the sheath. A positive electrode was placed on the animal's tail, and five 200-V shocks, with a pulse duration of 75 ms and an interval of 925 ms, were applied using a BTX pulse generator during isoflurane (2–2.5%) anesthesia.

Localization of Incorporated c-Fos Antibody in Spinal Cords of Rats Receiving Intrathecal c-Fos Antibody Electroporation

Rats were killed with intraperitoneal pentobarbital sodium (50 mg/kg) and ethyl carbamate (600 mg/kg). They were then perfused transcardially with 100 ml saline followed by 500 ml fixative containing 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). The spinal cords were removed, postfixed in the same fixative 2–4 h at 4°C, and then transferred to a solution of sucrose, 30%, in 0.1 M phosphate-buffered saline overnight at 4°C. Alternate serial transverse sections (10 μ m thick) of the spinal cord were cut using a freezing microtome. Sections were washed in phosphate-buffered saline (0.1 M, pH 7.4) for 20 min and blocked with normal goat serum, 1%, for 20 min before incubation for 12 h with antibody to NeuN (1:200; Chemicon, Temecula, CA). Fluorescein-conjugated goat antibody to mouse and rhodamine-conjugated goat antibody to rab-

bit served as the secondary antibodies (1:250; Jackson ImmunoResearch Laboratory, West Grove, PA). Immunoreactivity was detected using a Leica microscope (Leica Microsystems AG, Wetzlar, Germany). Images (at 512 \times 512 pixels) were obtained with a SPOT digital camera and processed using Adobe Photoshop 6.0 software (Adobe Systems, Mountain View, CA).

Time Course of Incorporated c-Fos Antibody in Lumbar Spinal Cords of Rats Receiving Intrathecal Electroporation

The time course of c-Fos antibody immunoreactivity was determined using Western blotting with some modifications. Total protein concentrations were determined using a BCA protein assay (Pierce). Fifteen micrograms of total protein was run by sodium dodecyl sulfate (10%)-polyacrylamide gel electrophoresis and was transferred to a polyvinylidene fluoride membrane. The blot was blocked with buffered nonfat milk, 5%, for 1 h. Rabbit β -actin primary antibody (25 μ g; SantaCruz) was incubated in buffered nonfat milk, 1%, for 1 h followed by washes and incubation in secondary antibody to rabbit immunoglobulin G (SantaCruz). The bands were detected using a nonradioactive detection system (Chemoluminescence Reagent Plus; NEN, Boston, MA). Molecular size standards, ranging from 200 to 6.5 kd, were run with the samples to ensure quantification of the molecular size of the signal. For densitometric analysis, blots were scanned using the Model GS-710 imaging densitometer (NEN), and results were expressed as the ratio of c-Fos antibody immunoreactivity to β -actin immunoreactivity.

Assessment of Lumbar Spinal Pronociceptive Gene Expression Using Reverse Transcription-Polymerase Chain Reaction Analysis

RNA was extracted using a standard TRIzol^R (Gibco) method.³⁶ After precipitation of RNA, complementary DNA synthesis was performed using oligo d(T) primers, as described by the manufacturer. Polymerase chain reaction analysis was performed using an amplification cycle profile consisting of 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min per cycle. After 30 polymerase chain reaction cycles, an additional cycle at 72°C for 7 min was performed to ensure complete DNA extension. The primers were designed for preprodynorphin and were as follows: sense, 5'-GAG GAC TTG AGA AAA CAG GCC-3'; and antisense, 5'-GGT ATT GGG GTT CTC CTG GGA-3'. Primers for β actin were designed for the rat cytoplasmic β -actin gene and were as follows: sense, 5'-ACA CCC CAG CCA TGT ACG-3'; and antisense, 5'-TGG TGG TGA AGC TGT AGC C-3'.

Quantification of Lumbar Spinal Dynorphin Peptide Using Enzyme Immunoassay

During isoflurane anesthesia, animals received intrathecal *p*-hydroxymercuribenzoate (8 nmol; Sigma, St. Louis, MO) and phosphoramidon (4 nmol; Sigma) as described previously.³⁷ Three hours later, the animals were killed, and spinal cords were removed and stored at -70°C until later use. Peptides were extracted as previously described.³⁸ Levels of dynorphin A(1-17) and dynorphin A(1-8) were determined using commercial enzyme immunoassay kits (Peninsula Laboratories, San Carlos, CA).

Behavioral Assessment of Thermal and Mechanical Stimulation

The thermal nociceptive threshold was measured before and after induction of the nerve injury or after intrathecal electroporation using a technique somewhat modified from the one originally described by Hargreaves *et al.*³⁹ in 1988. The animal was placed on a glass plate (maintained at 30°C) for 5–10 min for adaptation. Latency between the application of a focused light beam and the hind paw withdrawal response was measured to the nearest 0.1 s, with a cutoff time in the absence of a response after 20 s.

The foot withdrawal threshold in response to a mechanical stimulus was determined using a series of von Frey filaments (Stoelting, Wood Dale, IL) that ranged from 0.23 to 59.0 g. Animals were placed in a plastic cage with a metal mesh floor, allowing them to move freely. They were allowed to acclimatize to this environment before the experiment. The filaments were presented to the mid-plantar surface.

Values for withdrawal latency were then determined at daily intervals up to 14 days. Six animals per group were used, each receiving pretreatment with 4 μg c-Fos, 0.4 μg of c-Fos plus nonspecific antibody, and saline (total $n = 30$). The differences in percentage withdrawal latency between animals pretreated with c-Fos and nonspecific antibody and saline at each time point were assessed using nonparametric Mann-Whitney U tests, with $P < 0.05$ assumed as a significant difference.

Statistical Analysis

All data are expressed as mean \pm SEM. The changes in the parameters between the groups and over time were compared using two-way ANOVA for repeated measurements. If significant differences were observed, then the *post hoc* Bonferroni-Dunn test was used to detect specific differences between the groups and across time. Differences between the c-Fos electroporation group and the control group reverse transcription-polymerase chain reaction data were analyzed using the Student *t* test for unpaired observations. Significance was assumed at $P < 0.05$.

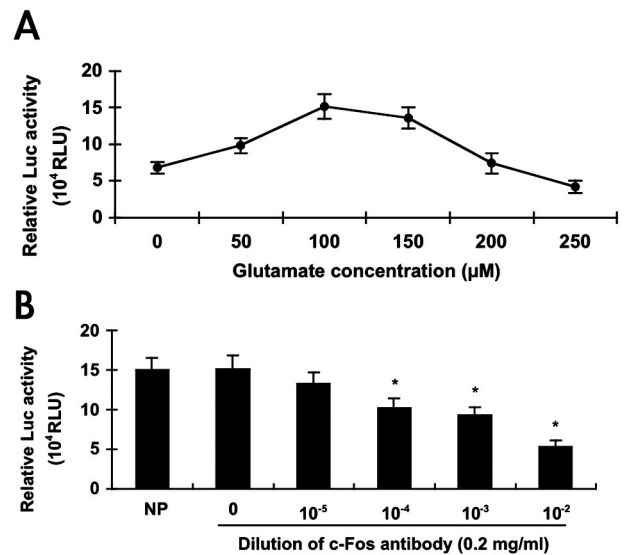


Fig. 1. (A) Effects of glutamate on the activation of activating protein (AP-1) promoter activity in cultured neuronal cells. Cultured neuronal cells transfected with the p-AP-Luc vector were incubated using 0–250 μM glutamate in Mg^{2+} -free Hanks balanced salt solution for 15 min. The glutamate exposure was terminated by changing the medium. Relative luciferase activity was determined 24 h later using a luciferase assay system. (B) Inhibitory effects of c-Fos antibody electroporation on the regulation of AP-1 promoter activity in cultured neuronal cells. AP-1 promoter activity in cultured neuronal cells cotransfected with p-AP-Luc vector and c-Fos antibody (or nonspecific [NP] antibody) and treated with 100 μM glutamate was determined after 24 h. For all groups, $n = 3$ and values are mean \pm SEM. *Different from value of NP group ($P < 0.05$). RLU = relative light units.

Results

Effects of c-Fos Antibody Electroporation on AP-1 Promoter Activity in Cultured Neuronal Cells Transfected with p-AP-Luc Vector (In Vitro Neuropathic Pain Model)

AP-1 promoter activity in cultured neuronal cells transfected with the p-AP-Luc vector was activated after exposure to glutamate, peaking at 100 μM (fig. 1A). Cultured neuronal cells exposed to high concentrations ($> 150 \mu\text{M}$) died, as shown by released lactate dehydrogenase activity (data not shown). Cotransfection with c-Fos antibody significantly decreased the effect of glutamate exposure (fig. 1B).

Distribution of Incorporated c-Fos Antibody in Spinal Cords of Rats Receiving c-Fos Antibody Electroporation

In the control group (intrathecal c-Fos antibody injection without an electrical pulse), no immunoreactivity was noted at either time point (fig. 2A). By contrast, significant immunoreactivity was detected in meningeal cells, pia mater, and lumbar dorsal roots. Some immunostained cells were located in the spinal cord near the pia mater (fig. 2B). The most intensive staining was noted 1 to 2 days after electroporation, with decreases in inten-

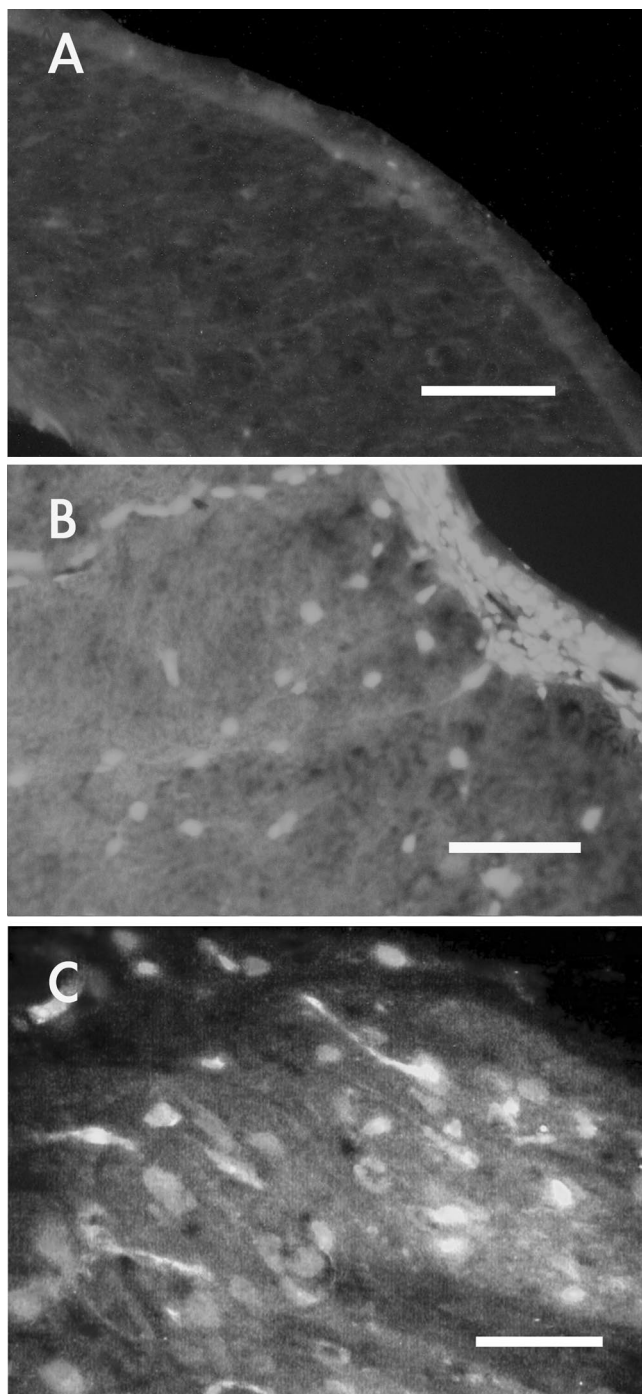


Fig. 2. Transverse section from lumbar (L4) spinal cords of rats receiving c-Fos antibody *via* electroporation (day 2). (A) Intrathecal c-Fos antibody administration without electroporation as a control (*bar* = 250 μ m). (B) Immunohistochemical analysis with antibody to immunoglobulin G revealed the cellular localization of c-Fos antibody (*bar* = 250 μ m). (C) Double localization of c-Fos antibody immunoreactivity with an antibody to NeuN (Chemicon, Temecula, CA) showed that some positive cells were located in the dorsal neuronal cells (*bar* = 125 μ m).

sity for all animals at 14 days. In some samples, immunoreactivity was also determined in the lower thoracic spinal cord at 1 or 3 days, and the degree of expression of immunoreactivity was positively related to the anti-

body titer. Intrathecal injections of 4 μ g c-Fos antibody resulted in higher levels of immunoreactivity and more intense distribution than did injections of 0.4 μ g c-Fos antibody. In two control groups, no immunoreactivity was noted at either time point. Double localization with antibody to NeuN showed that the c-Fos antibody was located in both neuronal and nonneuronal cells (fig. 2C). Compared with control rats, rats receiving c-Fos antibody electroporation had no apparent alterations in cellular morphology (data not shown).

Time Course of Incorporated c-Fos Antibody in Spinal Cords of Rats Receiving c-Fos Antibody Electroporation

The mean immunoreactivity of c-Fos antibody based on results of Western blotting is summarized in figure 3. There was an increase in the c-Fos antibody immunoreactivity from day 2 through day 14 in the rats receiving 4 μ g antibody electroporation, peaking between days 1 and 3. A minor increase in antibody immunoreactivity was also noted for rats receiving 0.4 μ g antibody electroporation from day 2 through day 6.

Effects of c-Fos Antibody Electroporation on General Behavior

We observed the effects of intrathecal electroporation of saline alone and of 0.4 and 4 μ g c-Fos antibody on the general behavior of animals (*n* = 6 rats in all cases). All

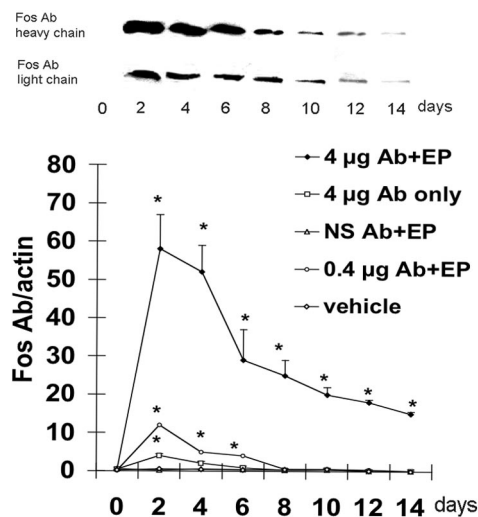


Fig. 3. Time course for c-Fos antibody (Fos Ab) immunoreactivity (from modified Western blots) after direct *in vivo* electroporation with 4 μ g c-Fos antibody (*top*). The relative c-Fos antibody immunoreactivity is expressed as a ratio of c-Fos antibody/ β actin; protein levels were determined by densitometric scanning of Western blots (*bottom*). Note the peak activity on day 2 after electroporation. 4 μ g Ab + EP = intrathecal 4 μ g c-Fos antibody *via* electroporation; 4 μ g Ab only = intrathecal 4 μ g c-Fos antibody without electroporation; NS Ab + EP = intrathecal 4 μ g nonspecific antibody *via* electroporation; 0.4 μ g Ab + EP = intrathecal 0.4 μ g c-Fos antibody *via* electroporation; vehicle = saline only. For all groups, *n* = 3 and values are mean \pm SEM. *Different from value for vehicle group (*P* < 0.05).

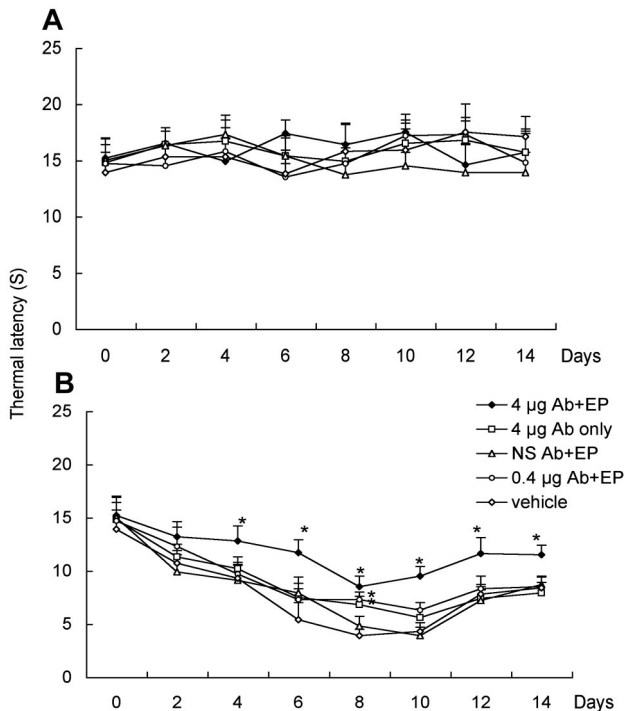


Fig. 4. Time course of withdrawal latency in relation to noxious thermal stimulation in the contralateral limb with sham operation (A) and in the hind paw with induction of chronic constriction nerve injury (B). 4 µg Ab + EP = intrathecal 4 µg c-Fos antibody *via* electroporation; 4 µg Ab only = intrathecal 4 µg c-Fos antibody without electroporation; NS Ab + EP = intrathecal 4 µg nonspecific antibody *via* electroporation; 0.4 µg Ab + EP = intrathecal 0.4 µg c-Fos antibody *via* electroporation; vehicle = saline only. For all groups, n = 6 and values are mean ± SEM. *Different from value for vehicle group ($P < 0.05$).

of the animals receiving intrathecal electroporation during isoflurane anesthesia survived for as long as the rats that did not receive electric shocks. The animals had minor lower extremity twitches while receiving the electric pulses, but these ceased as soon as the pulses stopped. No significant relationships were found in the duration or degree of response, food intake, and body weight among animals treated or not treated with electric shocks.

Effects of c-Fos Antibody Electroporation on CCI-induced Nociceptive Behavior

In a separate set of 30 animals divided into five groups of six, the effect of c-Fos antibody electroporation on CCI-induced nociceptive behavior was examined. Two groups received injections of c-Fos antibody at doses of 4 and 0.4 µg followed by electroporation, whereas another two groups received injections of nonspecific antibody followed by electroporation or 4 µg c-Fos antibody without electroporation. A fifth control group received injections of saline alone. There were no significant differences in the contralateral sham-operated paw withdrawal latencies (non-CCI side) among these five groups (fig. 4A). Induction of CCI resulted in an immediate

decrease in the mean percentage withdrawal latency (*i.e.*, hyperalgesic state). Comparison of latency values obtained after treatment with either nonspecific antibody or saline revealed no significant differences (fig. 4B). However, when compared with values obtained after treatment with c-Fos antibody electroporation, significantly longer withdrawal latencies were observed from day 2 through day 14 for 4 µg c-Fos antibody electroporation-treated animals and at day 8 for animals treated with 0.4 µg c-Fos antibody electroporation (fig. 4B).

Effects of c-Fos Antibody Electroporation on CCI-induced Allodynic Behavior

Among these five groups, no alternation in mechanical allodynia was demonstrated for the contralateral sham-operated limb (fig. 5A). By contrast, mechanical withdrawal latency was significantly increased for CCI rats at 2, 4, 6, and 8 days after lesion, comparing rats treated with intrathecal (4 µg) c-Fos antibody electroporation and control rats. Rats receiving 0.4 µg c-Fos antibody electroporation and those receiving 4 µg c-Fos antibody without electroporation also showed increased mechanical latency at days 2 and 3, respectively (fig. 5B).

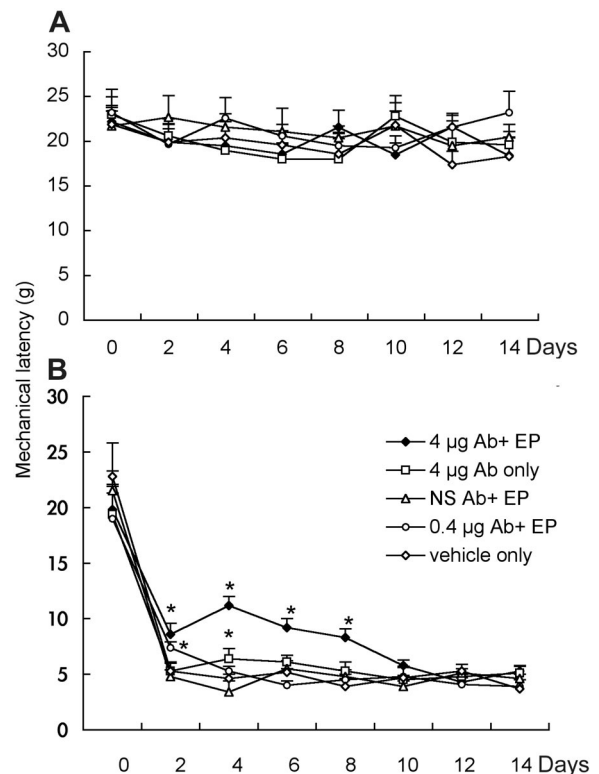


Fig. 5. Time course of withdrawal latency in relation to mechanical stimulation in the contralateral limb with sham operation (A) and in the hind paw with induction of chronic constriction nerve injury (B). 4 µg Ab + EP = intrathecal 4 µg c-Fos antibody *via* electroporation; 4 µg Ab only = intrathecal 4 µg c-Fos antibody without electroporation; NS Ab + EP = intrathecal 4 µg nonspecific antibody *via* electroporation; 0.4 µg Ab + EP = intrathecal 0.4 µg c-Fos antibody *via* electroporation; vehicle = saline only. For all groups, n = 6 and values are mean ± SEM. *Different from value for vehicle group ($P < 0.05$).

Down-regulation of Spinal Prodynorphin Expression in Rats Receiving Intrathecal c-Fos Antibody Electroporation

Given the proposed effects of c-Fos antibody in transcriptionally inhibited animals, we analyzed the abundance of prodynorphin transcripts in the lumbar spinal cords of c-Fos antibody electroporation-treated rats and controls by reverse transcription-polymerase chain reaction analysis. In control rats, basal expression of prodynorphin mRNA was detected. Similar levels of spinal prodynorphin mRNA were seen in CCI rats and CCI rats receiving c-Fos electroporation at day 2 after lesion or at days 2 or 10 after sham surgery, whereas spinal dynorphin was significantly increased in CCI rats at day 10 after lesion. CCI rats receiving c-Fos electroporation at days 10 after lesion showed markedly decreased prodynorphin expression compared with CCI rats (fig. 6A).

Decrease in Lumbar Spinal Dynorphin Levels in Rats Receiving Intrathecal c-Fos Electroporation

To assess whether the decreased basal level of spinal prodynorphin mRNA is accompanied by a concurrent decrease in dynorphin peptide content, the amounts of dynorphin A(1-17) and dynorphin A(1-8) in the spinal cord were determined by enzyme immunoassay. The level of dynorphin A(1-17) but not dynorphin A(1-8) was greater in the spinal cords of CCI rats than in those of CCI rats receiving c-Fos antibody electroporation at day 10 after lesion (fig. 6B). No significant differences were found among the sham-operated rats, CCI rats, and CCI rats receiving c-Fos electroporation at days 2 or sham-operated rats at days 10 after lesion. We conclude that depressed prodynorphin mRNA levels reflect decreased synthesis of dynorphin in the treated spinal cord. Taken together, our data suggest that c-Fos antibody electroporation has a significant effect on repressing expression of the prodynorphin gene in the spinal cord.

Discussion

Electroporation has been successfully used to introduce large molecules into eukaryotic and prokaryotic cells for the transformation and expression of various gene products. Because it is a physical method, electroporation has few biologic side effects and is free of chemical toxicity.²⁹ Transfer of antibodies *in vitro* has previously been reported several times. Results from the first part of our experiments suggest that electroporation can introduce antibodies into living mammalian cells in a very efficient manner. Luciferase activity and Western blotting analysis showed that the incorporated immunoglobulin was functionally intact. The level of luciferase increased in cells stimulated by glutamate, and this incremental protein was simultaneously neutralized using

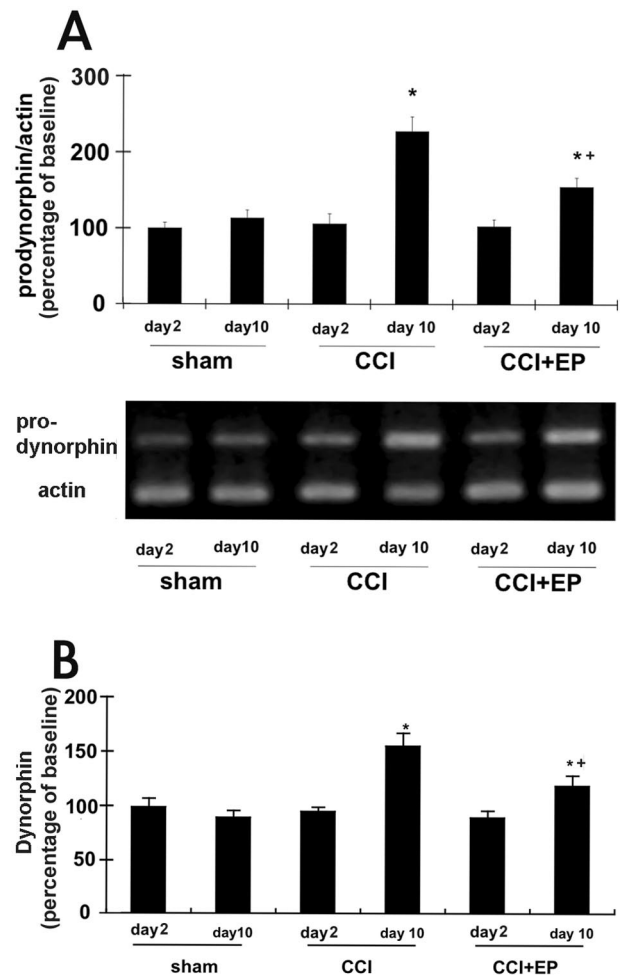


Fig. 6. (A) Effects of intrathecal c-Fos electroporation (EP) on spinal prodynorphin expression. Prodynorphin mRNA in lumbar spinal cords was assessed by semiquantitative reverse transcription-polymerase chain reaction analysis. β -Actin mRNA served as the control. Graphs represent the relative abundance of prodynorphin-specific reverse transcription-polymerase chain reaction products. Values obtained for each group at day 2 showed no significant difference. (B) Effects of intrathecal c-Fos electroporation on spinal dynorphin peptide synthesis. Values obtained for each group at day 2 showed no significant difference. Dynorphin peptide in the lumbar spinal cord was assessed using enzyme-linked immunosorbent assay. For all groups, $n = 5$ and values are mean \pm SEM. *Different from day 2 value within groups ($P < 0.05$). †Different from day 10 value for chronic constriction nerve injury (CCI) group.

incorporated antibodies that inhibit AP-1 promoter activity. We also demonstrated that the rate of AP-1 activity inhibition increased in tandem with the increment in c-Fos antibody in a dose-dependent manner. These results further support the hypothesis that c-Fos is an AP-1 promoter binding protein that plays an important role in neuronal activation. High concentrations of c-Fos antibody can inhibit AP-1 activity almost completely, which indicates that c-Fos is one of the key factors in this signal transduction pathway. Electroporation with c-Fos antibody is thus a feasible method for inhibiting glutamate-induced excitation in neuronal cells.

In the second group of experiments, we initially transferred the antibody into spinal cords *in vivo* using a specially designed probe. We previously reported the advantages of gene transfection using intrathecal electroporation.³⁴ Clearly, electroporation can introduce antibodies into the spinal cord very effectively and quickly, and it took less than 10 min to complete the procedure. The incorporated antibodies were located mostly in the meningeal cells and superficial layers of the spinal cord.

Western blotting showed that the incorporated immunoglobulin was structurally intact and thus retained normal function. The level of c-Fos antibody peaked on the second day and was still detectable on day 14 after transfer. Thermal and mechanical withdrawal latencies were significantly increased on days 6 through 14 and days 2 through 8 after CCI to the sciatic nerve, respectively. We also found that the effects of antihyperalgesia increased along with the increments in c-Fos antibody in a dose-dependent manner. These results further suggest that c-Fos may play an important role in neuropathic pain. High concentrations of c-Fos antibody reduce the magnitude of neuropathic pain, which indicates that c-Fos is one of the key factors in this signal transduction pathway.

In the third group of experiments, the sham-treated rats exhibited a significant up-regulation in spinal dynorphin on day 10 after CCI, which is similar to phenomena previously observed in sciatic nerve-injured rats.⁴⁰ This overexpression of spinal dynorphin corresponded with the presence of sustained neuropathic pain in the CCI rats. This was because an accelerated recovery rate and a decreased sensitivity to noxious thermal stimuli were observed by day 10 after injury in c-Fos antibody electroporation-treated CCI rats with decreased spinal dynorphin.

The demonstrated increases in expression of spinal dynorphin at day 10 after CCI supports that dynorphin is involved in the mechanisms of c-Fos electroporation in relation to CCI-induced pain, rather than other products of the AP-1-related gene. It should be emphasized that the potential reduction of proopiomelanocortin products such as β endorphin in these intrathecal c-Fos antibody electroporation-treated rats would be expected to increase sensitivity to pain, rather than promote a decrease in sensitivity to noxious stimuli as observed in the current experiments. These effects, together with the activity of intrathecal dynorphin, decrease the effect of decreased spinal dynorphin caused by c-Fos antibody electroporation. The data strongly suggest that, unlike dynorphin, a potential decrease in the expression of proopiomelanocortin products does not play a role in the observed consequences of CCI.

However, both sham-treated and c-Fos antibody electroporation-treated CCI rats demonstrated similar pre-dynorphin mRNA and dynorphin levels on day 2, suggesting that the initiation of the postinjury state does not

depend on the action of prodynorphin products. It should also be noted that the reversal by c-Fos antibody electroporation was not greater than preinjury baseline levels, indicating blockade of a pronociceptive effect rather than production of true "analgesia."

This activity of c-Fos underscores the likely importance of repetitive discharge originating from the injured or adjacent primary afferent neurons in the initiation of CCI-induced neuropathic pain.³ Ectopic discharges are known to occur and to peak within 1 day after sciatic nerve injury in rats but to decrease over time to one half the initial discharge rate by day 5 after injury.^{41,42} Although the discharge rate of the injured nerve fibers depends on the fiber type, it is clear that the discharge rate diminishes with time after nerve injury.^{43,44} However, the observed signs of neuropathic pain remain essentially unchanged in magnitude for several weeks.^{1,2} These findings suggest that the mechanisms required to maintain the presence of the neuropathic pain state extend beyond afferent input to the central nervous system. Thus, intrathecal c-Fos antibody electroporation, which inhibits the Fos protein function and downstream prodynorphin gene expression, may provide a reasonable treatment for neuropathic pain.

From these results, it seems that electroporation of antibodies into spinal cords *in vivo* provides an efficient and powerful tool for inhibiting certain cellular functions. Using intrathecal antibody electroporation, we are now able to study the immediate effects of the functional absence of key regulators of neuropathic pain. The introduction of antibodies into the spinal cord *in vivo* has become an important experimental option for the investigation of the biologic functions of proteins within the spinal cord. It could be widely used in the study of the cell cycle,⁴⁵ apoptosis, signal transduction, and other cell activities. The immediate, complete, and even reversible inhibition of protein functions involved in pain opens new perspectives for the study of spinal-related disorders.

References

1. Bennett GJ, Xie YK: A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 1988; 33:87-107
2. Attal N, Jazat F, Kayser V, Guibaud G: Further evidence for "pain-related" behaviours in a model of unilateral peripheral mononeuropathy. *Pain* 1990; 41:235-51
3. Kajander KC, Madsen AM, Iadarola MJ, Draisci G, Wakisaka S: Fos-like immunoreactivity increases in the lumbar spinal cord following a chronic constriction injury to the sciatic nerve of rat. *Neurosci Lett* 1996; 206:9-12
4. Morgan JI, Cohen DR, Hempstead JL, Curran T: Mapping patterns of c-fos expression in the central nervous system after seizure. *Science* 1987; 237:192-7
5. Handa RJ, Nunley KM, Bollnow MR: Induction of c-fos mRNA in the brain and anterior pituitary gland by a novel environment. *Neuroreport* 1993; 4:1079-82
6. Simpson RK Jr, Huang W: Glycine receptor reduction within segmental gray matter in a rat model of neuropathic pain. *Neurol Res* 1998; 20:161-8
7. Abram SE, Yaksh TL: Systemic lidocaine blocks nerve injury-induced hyperalgesia and nociceptor-driven spinal sensitization in the rat. *ANESTHESIOLOGY* 1994; 80:383-91
8. Besson JM, Chaouch A: Peripheral and spinal mechanisms of nociception. *Physiol Rev* 1987; 67:67-186

9. Yashpal K, Mason P, McKenna JE, Sharma SK, Henry JL, Coderre TJ: Comparison of the effects of treatment with intrathecal lidocaine given before and after formalin on both nociception and Fos expression in the spinal cord dorsal horn. *ANESTHESIOLOGY* 1998; 88:157-64
10. Hunt SP, Pini A, Evan G: Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. *Nature* 1987; 328:632-4
11. Naranjo JR, Mellstrom B, Achaval M, Sassone-Cors P: Molecular pathways of pain: Fos/Jun-mediated activation of a noncanonical AP-1 site in the prodynorphin gene. *Neuron* 1991; 6:607-17
12. Noguchi K, Kowalski K, Traub R, Solodkin A, Iadarola MJ, Ruda MA: Dynorphin expression and Fos-like immunoreactivity following inflammation induced hyperalgesia are colocalized in spinal cord neurons. *Mol Brain Res* 1991; 10:227-33
13. Morgan JI, Curran T: Stimulus-transcription coupling in the neurons: Role of cellular immediate-early genes. *Trends Neurosci* 1989; 12:459-62
14. Sonnenberg JL, Rauscher FJ 3rd, Morgan JI, Curran T: Regulation of proenkephalin by Fos and Jun. *Science* 1989; 246:1622-5
15. Goodman RH: Regulation of neuropeptide gene expression. *Annu Rev Neurosci* 1990; 13:111-27
16. Hunter JC, Woodburn VL, Durieux C, Pettersson EK, Poat JA, Hughes J: c-fos antisense oligodeoxynucleotide increases formalin-induced nociception and regulates prodynorphin expression. *Neuroscience* 1995; 65:485-92
17. Hou WY, Shyu BC, Chen TM, Lee JW, Shieh JY, Sun WZ: Intrathecally administered c-fos antisense oligodeoxynucleotide decreases formalin-induced nociceptive behavior in adult rats. *Eur J Pharmacol* 1997; 329:17-26
18. Civelli O, Douglass J, Goldstein A, Herber E: Sequence and expression of the rat prodynorphin gene. *Proc Natl Acad Sci USA* 1985; 82:4291-5
19. Wang Z, Gardell LR, Ossipov MH, Vanderah TW, Brennan MB, Hochgeschwender U, Hruby VJ, Malan TP Jr, Lai J, Porreca F: Pronociceptive actions of dynorphin maintain chronic neuropathic pain. *J Neurosci* 2001; 21:1779-86
20. Vanderah TW, Gardell LR, Burgess SE, Ibrahim M, Dogrul A, Zhong CM, Zhang ET, Malan TP Jr, Ossipov MH, Lai J, Porreca F: Dynorphin promotes abnormal pain and spinal opioid antinociceptive tolerance. *J Neurosci* 2000; 20:7074-9
21. Potter H, Weir L, Leder P: Enhancer-dependent expression of human kappa immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. *Proc Natl Acad Sci USA* 1984; 81:7161-5
22. Fakhrai H, Dorigo O, Shawler DL, Lin H, Mercola D, Black KL, Royston I, Sobol RE: Eradication of established intracranial rat gliomas by transforming growth factor beta antisense gene therapy. *Proc Natl Acad Sci USA* 1996; 93:2909-14
23. Nomura M, Nakata Y, Inoue T, Uzawa A, Itamura S, Nerome K, Akashi M, Suzuki G: In vivo induction of cytotoxic T lymphocytes specific for a single epitope introduced into an unrelated molecule. *J Immunol Methods* 1996; 193:41-9
24. Heller R, Jaroszeski M, Atkin A, Moradpour D, Gilbert R, Wands J, Nicolau C: In vivo gene electroinjection and expression in rat liver. *FEBS Lett* 1996; 389:225-8
25. Heller R: The development of electroporation. *Science* 2002; 295:277
26. Fewell JG, MacLaughlin F, Mehta V, Gondo M, Nicol F, Wilson E, Smith LC: Gene therapy for the treatment of hemophilia B using PINC-formulated plasmid delivered to muscle with electroporation. *Mol Ther* 2001; 3:574-83
27. Roscilli G, Rinaudo CD, Cimino M, Sporeno E, Lamartina S, Ciliberto G, Toniatti C: Long-term and tight control of gene expression in mouse skeletal muscle by a new hybrid human transcription factor. *Mol Ther* 2002; 6:653-63
28. Nishi T, Yoshizato K, Yamashiro S, Takeshima H, Sato K, Hamada K, Kitamura I, Yoshimura T, Saya H, Kuratsu J, Ushio Y: High-efficiency in vivo gene transfer using intraarterial plasmid DNA injection following in vivo electroporation. *Cancer Res* 1996; 56:1050-5
29. Chakrabarti R, Wylie DE, Schuster SM: Transfer of monoclonal antibodies into mammalian cells by electroporation. *J Biol Chem* 1989; 264:15494-500
30. Glogauer M, McCulloch CA: Introduction of large molecules into viable fibroblasts by electroporation: Optimization offloading and identification of labeled cellular compartments. *Exp Cell Res* 1992; 200:227-34
31. Baron S, Poast J, Rizzo D, McFarland E, Kieff E: Electroporation of antibodies, DNA, and other macromolecules into cells: A highly efficient method. *J Immunol Methods* 2000; 242:115-26
32. Lin CR, Wu PC, Shih HC, Cheng JT, Lu CY, Chou AK, Yang LC: Intrathecal spinal progenitor cell transplantation for the treatment of neuropathic pain. *Cell Transplant* 2002; 11:17-24
33. Berglund DL, Starkey JR: Introduction of antibody into viable cells using electroporation. *Cytometry* 1991; 12:64-7
34. Lin CR, Yang LC, Lee TH, Lee CT, Huang HT, Sun WZ, Cheng JT: Electroporation-mediated pain-killer gene therapy for mononeuropathic rats. *Gene Ther* 2002; 9:1247-53
35. Lin CR, Tai MH, Cheng JT, Chou AK, Wang JJ, Tan PH, Marsala M, Yang LC: Electroporation for direct spinal gene transfer in rats. *Neurosci Lett* 2002; 317:1-4
36. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162:156-9
37. Tan-No K, Taira A, Sakurada T, Inoue M, Sakurada S, Tadano T, Sato T, Sakurada C, Nylander I, Silberring J, Terenius L, Kisara K: Inhibition of dynorphin-converting enzymes prolongs the antinociceptive effect of intrathecally administered dynorphin in the mouse formalin test. *Eur J Pharmacol* 1996; 314:61-7
38. Malan TP, Ossipov MH, Gardell LR, Ibrahim M, Bian D, Lai J, Porreca F: Extraterritorial neuropathic pain correlates with multisegmental elevation of spinal dynorphin in nerve-injured rats. *Pain* 2000; 86:185-94
39. Hargreaves K, Dubner R, Brown F, Flores C, Joris J: A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 1988; 32:77-88
40. Draisci G, Kajander KC, Dubner R, Bennett GJ, Iadarola MJ: Up-regulation of opioid gene expression in spinal cord evoked by experimental nerve injuries and inflammation. *Brain Res* 1991; 560:186-92
41. Chen Y, Devor M: Ectopic mechanosensitivity in injured sensory axons arises from the site of spontaneous electrogenesis. *Eur J Pain* 1998; 2:165-78
42. Han HC, Lee DH, Chung JM: Characteristics of ectopic discharges in a rat neuropathic pain model. *Pain* 2000; 84:253-61
43. Liu CN, Wall PD, Ben-Dor E, Michaelis M, Amir R, Devor M: Tactile allodynia in the absence of C-fiber activation: Altered firing properties of DRG neurons following spinal nerve injury. *Pain* 2000; 85:503-21
44. Liu X, Eschenfelder S, Blenk KH, Janig W, Habler H: Spontaneous activity of axotomized afferent neurons after L5 spinal nerve injury in rats. *Pain* 2000; 84:309-18
45. Lukas J, Bartek J, Strauss M: Efficient transfer of antibodies into mammalian cells by electroporation. *J Immunol Methods* 1994; 170:255-9