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Involvement of Spinal Neurotrophin-3 in Electroacupuncture Analgesia and Inhibition of Spinal Glial Activation in Rat Model of Monoarthritis

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Abstract: Although electroacupuncture (EA) has been proven to effectively relieve pain associated with arthritis, the underlying mechanism of EA analgesia requires further investigation. Here, the involvement of spinal neurotrophin-3 (NT-3) in EA's analgesic effects on complete Freund's adjuvant (CFA)-induced inflammatory pain was examined. The present study demonstrated that: 1) repeated EA stimulation of ipsilateral GB30 and GB34 acupoints remarkably suppressed CFA-induced hyperalgesia; 2) EA treatment markedly enhanced the upregulation of spinal NT-3 mRNA and protein levels following CFA injection; 3) antisense oligodeoxynucleotides (ODN) specifically against NT-3 intrathecally administered during EA treatment for 7 days significantly attenuated the EA analgesia; and 4) the suppressed expression of spinal GFAP (astrocytic marker), OX-42 (microglial marker) as well as proinflammatory cytokines, interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α by EA treatment was significantly attenuated following NT-3 antisense ODN delivery. These results suggested that endogenous NT-3 may be involved in the analgesic effect of EA on inflammatory pain in rats, mediated through the inhibition of spinal glial activity as well as proinflammatory cytokine production.

***Perspective:** The present study may initiate a discussion on the possible roles of NT-3/glia/cytokines in the therapeutic effects of acupuncture and provide insight on the mechanism underlie the analgesic effects of acupuncture on pain associated with arthritis.*

***Key words:** Monoarthritis, electroacupuncture, neurotrophin-3, glia, proinflammatory cytokines.*

Arthritis is a painful and disabling disease that affects millions of patients.[30] Currently, pharmacological management of arthritis is often ineffective and may cause unwanted and dangerous side effects if taken continuously.[4] Alternatively, acupuncture, an important part of traditional Chinese medicine, has been demonstrated to be an effective strategy for pain relief by numerous clinical observations and experimental studies.[3,19,24] Electroacupuncture (EA), an acupuncture therapy, has been shown to effectively treat human rheumatoid arthritis and osteoarthritis with satisfactory results.[3] In spite of the known modulatory effect of EA on the expression and release of various endogenous bioactive substances including opioids, monoamines, and oxytocin,[12,18] the precise mechanisms of EA analgesia, especially in pathological conditions, are not fully understood.

Of the various neuroactive substances modulated by EA, neurotrophin-3 (NT-3), a neurotrophin, is considered important because of its role in modulating nociceptive signaling in pathological conditions.[11,27,31] NT-3 is widely distributed throughout both the peripheral and central nervous system (PNS and CNS, respectively). In the CNS, NT-3 is observed in the forebrain, hippocampus, brain stem, and spinal cord. NT-3 activates the tyrosine kinase

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receptors (Trk) A and TrkB in addition to its own cognate TrkC receptor.[13] NT-3 can prevent the development and maintenance of thermal hyperalgesia and generally antagonizes nerve growth factor (NGF) in neuropathic pain conditions.[31] Additionally, NT-3 delivered by exogenous administration has been reported to alleviate the mechanical hyperalgesia caused by intramuscular acid injection in transgenic mice.[11] These studies demonstrate the beneficial actions of exogenous NT-3 administration on painful states.

Furthermore, it has been demonstrated that EA increases spinal NT-3 expression in bilateral partial dorsal rhizotomized cats.[6] After adjacent dorsal root ganglion (DRG) removal, NT-3 immunoreactivity in the spared DRG significantly increases on the EA side, suggesting that EA upregulated endogenous NT-3 in DRG neurons.[26]

There is growing recognition that spinal glia contribute to the development and maintenance of central sensitization in chronic pain.[28] Glia can contribute to pain processing by releasing a number of glial and neuronal signaling molecules, including proinflammatory cytokines interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α.[5] Recently, it was reported that EA could inhibit complete Freund's adjuvant (CFA)-induced glia activation and the upregulation of the proinflammatory cytokines.[17] In addition, NT-3 can be produced by astrocytes[16] and microglia.[10] Pretreatment with NT-3 was shown to reduce the production of inducible nitric oxide synthase (iNOS), NO, and proinflammatory cytokines in primary microglia stimulated with LPS.[21,22] Therefore, it is possible that NT-3 regulates glial activation as well as the production of proinflammatory cytokines.

Using the CFA-induced monoarthritic rat model, the present study was intended to: 1) examine the expression of NT-3 in the spinal cord during EA treatment in monoarthritic rats; 2) observe the analgesic effects of intrathecally administered antisense oligodeoxynucleotide (ODN) targeted specifically against NT-3 on EA in monoarthritic rats; and 3) determine the EA-induced changes in the expression of glial and inflammatory cytokines after NT-3 knock-down.

Methods

Animals

Experiments were performed on adult male Sprague Dawley rats weighing 200 to 220 g, supplied by the Experimental Animal Center, Chinese Academy of Sciences, Shanghai. Prior to experimental manipulation, rats were allowed to acclimate for 1 week in groups of 4 rats per cage, and maintained under controlled conditions (22 $\pm$ 1°C, 6 am to 6 pm, alternate light-dark cycles) with food pellets and water ad libitum. All experiments were conducted strictly in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the guidelines of the International Association for the Study of Pain.[33] All efforts were made to minimize the number of animals used and their suffering.

Induction of Monoarthritis (MA)

Monoarthritis was induced by an intra-articular injection of CFA (Sigma, St. Louis, MO; 1 mg/mL) by a method developed previously.[14] Briefly, the sterilized left foot of the anesthetized rat was held and the fossa of the lateral malleolus of the fibula was located. A 28-gauge needle was inserted vertically to penetrate the skin, and turned distally to insert into the articular cavity from the gap between the tibiofibular and tarsus bone until a distinct loss of resistance was felt. A volume of 50 μl CFA was then injected.

Behavioral Test

The paw withdrawal latency (PWL) to radiant heat was examined as previously described.[14] Briefly, rats were placed into an inverted, clear plastic cage upon an elevated floor of window glass. After an accommodation period of 30 minutes, using IITC Model 390 Paw Stimulator Analgesia Meter (Life Science Instruments, Woodland Hills, CA), a constant intensity radiant heat source (50-W, 8-V bulb) was aimed at the ankle joint until the rat lifted its paw. The time from onset of radiant heat application to paw withdrawal was defined as PWL. The intensity of radiant heat was adjusted to elicit the response around 12 seconds in normal rats, and a cutoff time was set at 20 seconds in order to avoid tissue injury. Both hind paws were tested independently with a 15-minute interval between tests. All the experiments were carried out every other day at the same time of the day between 8 am and 12 pm to avoid diurnal variation in behavioral tests.

Electroacupuncture Treatment

The detailed EA procedure has been described previously.[14] In brief, during EA treatment, the trunk of the rat was kept motionless while the head and 4 limbs kept freedom of movement in a specially designed holder. Rats were allowed to acclimate for 30 minutes before EA treatment. The skin cleaned with alcohol swabs, a pair of stainless steel needles of 0.3-mm diameter were inserted into the ipsilateral acupoints Huan-Tiao (GB-30, located near the hip joint, on the inferior borders of muscle gluteus maximus and muscle piriformis; the inferior gluteal cutaneous nerve, the inferior nerve; deeper, the sciatic nerve) and Yang-Ling-Quan (GB-34, located near the knee joint, anterior and inferior to the small head of the fibula, in muscle peroneus longus and brevis, where the common peroneal nerve bifurcates into the superficial and deep peroneal nerves) at a depth of 7 and 5 mm, respectively. The 2 needles were connected with the output terminals of an EA apparatus (Model G-6805-1A, Shanghai Huayi Medical Electronic Apparatus Company, Shanghai, China). Alternating strains of dense-sparse frequencies (60 Hz for 1.05 seconds and 4 Hz for 2.85 seconds alternately, bidirectional asymmetric pulse, 0.6 ms pulse width) were selected. The intensity of stimulation was approximately 1 mA, and mild muscle twitching was observed. The stimulation lasted for 30 minutes each time.

GB30 and GB34 were chosen based on Traditional Chinese Medicine (TCM) meridian theory and their

successful use in previous studies in the treatment of inflammatory pain and arthritis in both clinical and basic researches.[3,17,23]

ODN Administration

The oligonucleotides used in this study were NT-3 antisense, 5′-CAT CAC CTT GTT CAC-3′ and NT-3 sense, 5′-GTG AAC AAG GTG ATG-3′, as previously used.[29] The oligonucleotides were fully phosphoroexamine and HPLC purified. To test the effectiveness of NT-3 antisense ODN to the expression of NT-3, 12.5 μl of 100 mM of either antisense or sense ODN was administered intrathecally (i.t.) once daily for 3 days. Then the rats were overdosed with sodium pentobarbital and samples of spinal cord were removed. The tissue was weighed and stored at -80°C until processed. RT-PCR and ELISA were used to test the effectiveness of antisense ODN to the expression of NT-3.

Both antisense and sense ODNs were used at a dose of 12.5 μl of 100 μM of nuclease-free normal saline (NS) and each it injection of ODN was followed by 5 μl NS flush.

Chronically indwelling i.t. catheters were implanted into the subarachnoid space of lumbar enlargement of rats according to the method described previously.[8] Briefly, an intrathecal catheter (PE-10 tube) was inserted through the gap between the L4 and L5 vertebrae and extended to the subarachnoid space of the lumbar enlargement (L4 and L5 segments) under sodium pentobarbital (40 mg/kg, i.p.) anesthesia. The catheter was filled with sterile NS (approximately 4 mL), and the outer end was plugged. The animals were monitored daily after surgery for signs of motor deficiency, and rats that showed any neurological deficit resulting from the surgical procedure were excluded from the experiments. Location of the distal end of the intrathecal catheter was verified by injection of pontamine sky blue via the it catheter.

Polymerase Chain Reaction (PCR)

Real-Time Quantitative PCR

The mRNA changes of NT-3 expression after CFA injection and EA treatment were examined by real-time RT-PCR. Rats were sacrificed with an overdose of sodium pentobarbital (50 mg/kg, i.p.) and the lumbar spinal cord was collected in dry ice. A real-time RT-PCR method was used to quantify the relative expression of NT-3 mRNA. Briefly, total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed with oligo (dT) and M-MLV reverse transcriptase (Promega, Madison, WI). PCR reactions were performed in the presence of the oligonucleotide primers for NT-3 (F: 5′-GACGTC CCTGGAAATAGTCATAC-3′; R: 5′-GCCACGGAGATAAGC AAGAA-3′) and quantified by Sybergreen I (Molecular Probes, USA). And the GAPDH (F: 5′-AAGAAGGTGGT GAAGCAGGC-3′; R: 5-′TCCACCACCCTGTTGCTGTA-3′) housekeeping gene was chosen as an internal control. End point PCR products were initially assessed on ethidium bromide-stained agarose gels that gave a single band of the expected size for each assay. The lightcycler software Version 4.0 (Roche, Germany) was used for instrument control, automated data collection, and data analysis. Relative quantification of the mRNA expression levels of target genes was calculated. All samples were run in duplicate and the average values were used for the relative quantification of the mRNA expression.

RT-PCR

The mRNA changes of the expression of NT-3 after ODN administration as well as the expression of GFAP, CD11b, IL-1β, IL-6, and TNF-α were examined by RT-PCR. Rats were sacrificed after the EA treatment and ODN administration.

The RNA extraction and the reverse transcription procedure were similar as the real-time RT-PCR. Primers used (Sangon, Shanghai, China) are shown in Table 1. cDNA (1 μl) was amplified with Taq DNA polymerase in a 25-μl reaction mixture (TIANGEN Biotech, Beijing, China). PCR reaction was performed as follows: 5 minutes at 95°C to activate the Taq polymerase, followed by 30 cycles of 30 seconds at 95°C, 45 seconds at 57°C (IL-6/β- actin), 58°C (NT-3/GFAP), 59°C (IL-1β/TNF-α/CD11b), and 45 seconds at 72°C. A final elongation step at 72°C for 10 minutes completed the PCR reaction. Each PCR production (10 μl) was electrophoresed in 2% agarose gel, visualized by ethidium bromide staining, and scanned with ultraviolet transilluminator (GDS 8000, Gene Tools from Syngene Software, UK). The PCR quantitative method takes advantage of the fact that β-actin was employed as internal standard in the same condition. All the results were expressed as ratios of the intensity of the expected bands to that of β-actin band.

Immunohistochemistry

Sixteen deeply anaesthetized rats were perfused via the aorta with 200 mL of NS followed by 200 mL 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). Of the rats, 4 rats in the normal group and the MA 4 d, MA 14 d, and EA 14 d group each were used to determine changes in NT-3 expression after CFA injection and EA treatment. The lumbar spinal cords were removed, postfixed in the fixative solution for 4 hours at 4°C, and immersed in 30% sucrose in PB for 24 hours at 4°C for cryoprotection. Frozen sections (30 μm) were cut and collected in cryoprotectant solution (0.05 M PB, 30% sucrose, 30% ethylene glycol) and then stored at -20°C until use.

Free-floating tissue sections were processed for NT-3 immunohistochemistry by the avidin-biotin method. Sections were brought to room temperature and rinsed in 0.01 M PBS followed by blocking in 0.3% H_2O_2 for 10 minutes. Following 3, 15-minute rinses in 0.01 M PBS, the sections were preincubated for 30 minutes at room temperature in a blocking solution of 3% normal goat serum in 0.01 M PBS with 0.3% Triton-X 100 (NGST). The sections were then incubated in rabbit anti-NT-3 (1:1000, Chemicon International, Temecula, CA) and diluted in 1% NGST at 4°C for 24 hours. The incubated sections were washed 3 times in 0.01 M PBS and incubated in biotinylated goat anti-rabbit immunoglobulin G (IgG) (1:200, Jackson Immunoresearch Laboratories,

Table 1. Primers Used for RT-PCR: (+), forward primer; (−), reverse primer

Gene	*Sequence (5′—3′)*	*Annealing Temperature*	*Product*
NT-3	(+) TGCAGAGCATAAGAGTCACC	58°C	265 bp
	(−) AAGTCAGTGCTCGGACGTAG		
GFAP	(+) TGAGGCAGAAGCTCCAAGATGAAA	58°C	726 bp
	(−) CTGGTTTCTCGGATCTGG		
CD11b	(+) AGAGTGTGATCCAGCTTGGTGAAA	59°C	574 bp
	(−) AGTTTTTGTCCTTCCATTCAG		
IL-1β	(+) ATGAGAGCATCCAGCTTCAAATC	59°C	328 bp
	(−) GCTTATGTTCTGTCCATTGAGGT		
IL-6	(+) GACAAAGCCAGAGTCCTTCA	57°C	256 bp
	(−) ACTAGGTTTGCCGAGTAGAC		
TNF-α	(+) CGAGATGTGGAACTGGCACA	59°C	225 bp
	(−) CTACGGGCTTGTCACTCGA		
β-actin	(+) TCAGGTCATCACTATCGGCAAT	57°C	432 bp
	(−) AAAGAAAGGGTGTAAAACGCA		

West Grove, PA) for 1 hour at room temperature, washed 3 times in 0.01 M PBS, and incubated for 1 hour in avidin-biotin-peroxidase complex (1:100, Vector Laboratories, Burlingame, CA) at room temperature. Finally, the sections were washed 3 times in 0.01 M PBS, and the immunoreactive products were visualized by catalysis of 3, 3-diaminobenzidine (DAB) by horseradish peroxidase in the presence of 0.01% H_2O_2. The sections were then mounted, dehydrated, and covered. The sections were observed using Leica Q500IW image analysis system.

Control experiments were carried out to determine the amount of nonspecific binding by omitting the primary antiserum from the incubation media. Preincubation of primary antibody with the blocking peptide overnight reduced the labeling to background levels.

Western Blot Analysis

The lumbar spinal cord were collected and stored as PCR. Each sample was weighed and homogenized in 1.5 mL of sample buffer (0.01 M Tris-HCl buffer (pH 7.6) containing 0.25 M sucrose, 0.1 M NaCl, 1 mM EDTA, and 1 mM phenylmethyl sulfonylfluoride) at 4°C. Supernatant after 12,000 rpm. centrifugation for 10 minutes was used for western blot. Samples (30 μg of total protein) were dissolved with equal volume of loading buffer (0.01 M Tris–HCl buffer (pH 6.8) containing 0.2 M DTT, 4% SDS, 20% glycerol, and 0.1% bromophenol blue), separated on 10% SDS-PAGE and then electrotransferred at 300 mA to Immun-Blot PVDF membrane for 1 hour. Membranes were blocked in Tris-buffered saline with Tween 20 (TBST) containing 5% nonfat dried milk overnight at 4°C before incubation for 1 hour at room temperature with mouse anti-GFAP monoclonal antibody (1:1000, Sigma, St. Louis, MO), rabbit anti-Iba-1 antibody (1:1000, Wako, Osaka, Japan), or anti-rat-GAPDH-HRP antibody (1:1000, Kangcheng, Shanghai, China), diluted in TBST containing 5% BSA. Blots were washed extensively in TBST and incubated with goat anti-mouse IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1000, Vector Laboratories, Burlingame, CA) in TBST/1.25% BSA for 1 hour at room temperature. The signal was detected by an enhanced chemiluminescence method (ECL kit, Pierce, Rockford, IL), and exposed to Kodak X-OMAT film (Eastman Kodak, Rochester, NY). The intensity of the selected bands was captured and analyzed using GeneSnap Image Analysis Software (Syngene, UK).

Enzyme-Linked Immunosorbent Assay (ELISA)

Concentrations of NT-3, IL-1β, IL-6, and TNF-α in the spinal cord were detected by ELISA. Rats were sacrificed and the lumbar segments of spinal cord were collected and stored at −80°C until sonication. Total protein was dissociated mechanically from tissue using an ultrasonic cell disruptor, and then centrifuged at 3,000 × g for 15 minutes. Supernatant was removed and stored at −20°C until analysis. NT-3, IL-1β, IL-6, and TNF-α protein were quantified using an enzyme-linked immunosorbent assay kit according to the manufacturer's protocol (RapidBio Lab, Calabasas, CA). Measurement was completed using an enzyme-linked immunosorbent assay with an absorbency maximum at 450 nm.

Experimental Design

Experiment 1: Effects of Repeated EA on MA-Induced Thermal Hypersensitivity and NT-3 Expression

After baseline behavioral assessments were completed, rats received an intra-articular injection of 50 μl CFA (day 0). Behaviors were reassessed at 6 hours, 4 days, and 14 days after CFA injection to confirm the development of thermal hypersensitivity in the MA rats. Repeated EA was performed every other day beginning on the 4th day following CFA injection, and the anti-hyperalgesic effects were tested the day after EA treatment to avoid the disturbance of immediate influence of EA on pain behavior. Upon completion of testing on day 14, half of the rats were perfused with fixative, and the lumbar spinal cords were removed for immunohistochemistry analysis. The remaining rats were sacrificed for PCR analyses. Some other rats were induced with monoarthritis, and sacrificed at 6 hours and 4 days (n = 4 per

group) for PCR, ELISA, and immunohistochemistry. Also, another 4 rats were given EA and sacrificed on day 14 for ELISA.

Experiment 2: Effects of i.t. NT-3 Antisense ODN on Thermal Hypersensitivity, Glial Activation and Proinflammatory Cytokine Production in MA Rats With EA Treatment

The rats received an intra-articular injection of CFA, and it catheters were inserted into the subarachnoid space of lumbar enlargement. Three days after CFA injection, the rats were administered either antisense ODN, sense ODN, or NS once daily for 7 days. Meanwhile, EA was given once every other day beginning on the 3rd day after CFA injection until the end of the experiment. The anti-hyperalgesic effects were tested on the day after EA treatment to avoid disturbing the immediate influence of EA on pain behavior. The pain threshold was measured before the CFA injection and every other day after the CFA injection. On the 10th day, the rats were sacrificed, and the spinal cords were removed and stored at −80°C until processed.

All of the testing (including behavior, RT-PCR, immunohistochemistry, western blot, and ELISA) was conducted by experimenters who were blind to the experimental conditions.

Statistical Analysis

Experimental data are presented as mean ± S.E.M. and analyzed by statistical software SPSS 16.0. Repeated measures analysis of variance (ANOVA) followed by Student-Newman-Keuls test was used for post hoc analysis for differences between groups. $P < 0.05$ was considered statistically significant in all cases.

Results

Effects of Repeated EA on the Thermal Hyperalgesia of MA Rats

Before CFA injection, the basal withdrawal latencies of all rats were not distinctly different (Fig 1). The PWL in normal rats remained stable over the observation period, while the ipsilateral PWL markedly decreased at 6 hours, 4 days, and 14 days in MA rats compared with normal rats ($F_{9,70} = 44.287$, $P < 0.01$). EA was applied to GB-30 and GB-34 for 30 minutes every other day beginning on the 4th day following CFA injection. On day 14, 5 treatments with EA markedly increased the PWL ($P < 0.01$), indicating a therapeutic anti-hyperalgesic effect of repeated EA on pain in MA rats. There were no apparent changes in PWL on the contralateral side in all groups tested (data not shown).

Changes in the Expression of NT-3 in the Lumbar Spinal Cord During EA Treatment in MA Rats

The expression of NT-3 mRNA in the rat lumbar spinal cord was examined using real-time PCR. Low levels of NT-3 mRNA were present in the spinal cords of normal animals (Fig 2A). Intra-articular CFA injection produced a marked elevation in the spinal NT-3 mRNA, which was observed at 6 hours and 4 days postinjection compared with the normal group ($P < 0.05$). Repeated EA treatment for 10 days markedly enhanced the mRNA expression of NT-3 in comparison with the MA 14 d group ($F_{4,15} = 44.834$, $P < 0.05$).

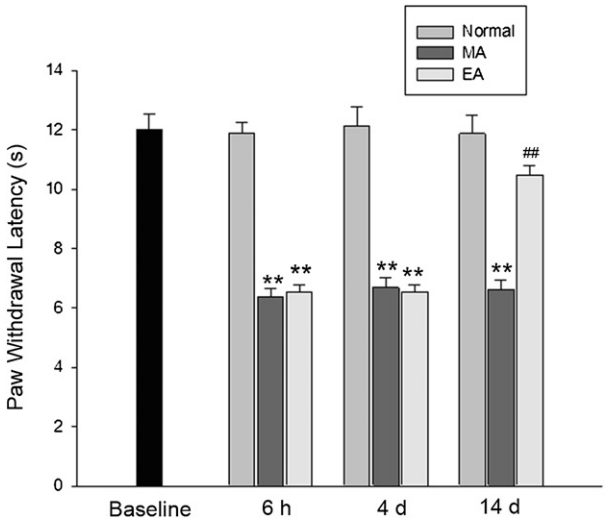

Figure 1. The anti-hyperalgesic effects of EA on pain in monoarthritic rats. EA treatments were administered beginning on the 4th day and continuing to the 12th day (once every other day), and each treatment lasted for 30 minutes. Data are presented as the mean ± S.E.M. (n = 8 per group). $^{**}P < 0.01$ versus normal group; $^{\#\#}P < 0.01$ versus MA group. MA, monoarthritis; EA, electroacupuncture.

The results of the ELISA showed that the expression of spinal NT-3 increased remarkably during inflammatory pain and was further enhanced by repeated EA treatment ($F_{4,15} = 21.797$, $P < 0.05$, Fig 2B). Similarly, immunohistochemistry revealed dense immunoreactivity for NT-3 in the spinal dorsal horn after CFA administration (Fig 2C). Mild expression of NT-3-like immunoreactivity (NT-3-LI) was observed in the nerve-fiber-like structures of the superficial layers of spinal dorsal horn in normal rats. On day 14, following 5 EA administrations, dense NT-3-LI was observed.

Effects of Antisense ODN Treatment on NT-3 Expression and Pain Threshold of Normal Rats

To test the effects of NT-3 antisense ODN on the synthesis of NT-3, rats were sacrificed after 3 days of ODN administration, and their spinal cords were removed and assayed using RT-PCR and ELISAs. RT-PCR analysis showed an expected 269-bp product for NT-3 mRNA (Fig 3A). Semiquantitative analysis showed that mRNA levels of NT-3 in the spinal cords of rats receiving antisense ODN were significantly decreased compared with mRNA levels of NT-3 in rats receiving NS or sense ODN ($F_{2,9} = 21.817$, $P < 0.01$, Fig 3B). In parallel with the results of RT-PCR, the ELISA showed an inhibition of the expression of NT-3 protein in rats of the antisense group in comparison with rats of the NS group ($F_{1,4} = 55.002$, $P < 0.01$,

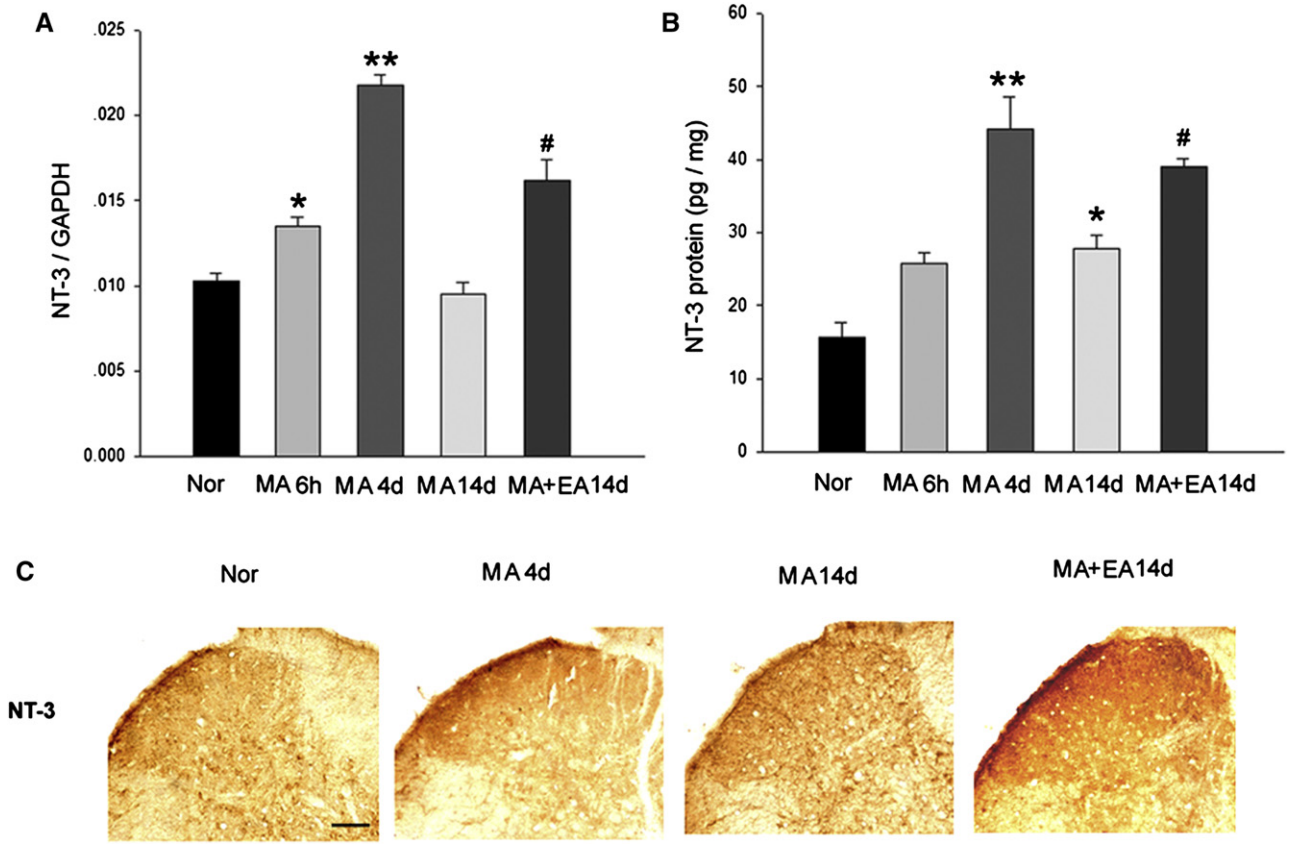

Figure 2. Changes in NT-3 expression in the rat lumbar spinal cord in CFA-induced inflammatory pain without or following EA treatment as detected by real-time PCR **(A)**, ELISA **(B)**, and immunoreactivity **(C)**. Data are presented as the mean ± S.E.M. (n = 4 per group at each time point). $^{*}P < 0.05$, $^{**}P < 0.01$ versus normal group; $^{\#}P < 0.05$ versus MA 14d group. Scale bar = 100 μm. MA, monoarthritis; CFA, complete Freund's adjuvant; EA, electroacupuncture; NT-3, neurotrophin-3.

Fig 3C). These results obtained using different techniques indicate that the expression of NT-3 in the spinal cord was significantly knocked down by antisense ODN treatment.

In addition, NT-3 antisense ODN showed no significant effect on the normal thermal thresholds as no significant alteration in the thermal sensitivity was observed when NT-3 antisense ODN was infused into normal rats for 3 days ($P > 0.05$, Fig 3D). A similar lack of effect on thermal thresholds was observed in normal rats receiving vehicle control infusions.

Effects of Knockdown of NT-3 on EA Analgesia in MA Rats

To address whether the NT-3 signaling system is involved in EA's analgesic effects on inflammatory pain, EA was administered once every other day beginning on the 3rd day after the CFA injection. Antisense ODN was given daily during EA treatment for 7 days beginning on the 3rd day after the CFA injection. EA treatment for 4 times markedly increased the ipsilateral PWL compared with the NS group ($P < 0.05$, Fig 3E). NT-3 antisense ODN administration during EA treatment partial reversal the PWLs compared with the EA group ($F_{4,39} = 46.684$, $P < 0.05$). The contralateral PWL did not significantly change following CFA injection or antisense administration (data not shown).

Effects of NT-3 Knock-Down on the Expression of GFAP, Iba-1, and Proinflammatory Cytokines During EA Treatment in MA Rats

To further test the mechanism underlying the involvement of NT-3 in the EA analgesia and illustrate whether NT-3 knock-down affect the glial activation and proinflammatory cytokine production in MA rats with EA treatment, RT-PCR, western blot, and ELISA were used to examine the expression of GFAP, Iba-1, IL-1β, IL-6, and TNF-α in the spinal cord.

Changes in the Expression of GFAP After NT-3 Antisense ODN Administration in MA Rats With EA Analgesia

RT-PCR and western blot were used to examine the expression of GFAP in the spinal cord. As shown in Fig 4A, an expected 726-bp PCR product of GFAP was obtained using RT-PCR. Repeated EA treatment markedly decreased the MA-induced upregulation of GFAP mRNA levels compared with the sense group ($P < 0.05$). NT-3 antisense ODN treatment in combination with EA alleviated the downregulation of GFAP mRNA in comparison with EA group ($F_{3,12} = 32.209$, $P < 0.01$, Fig 4B).

With a single band (~50 kDa, coincident with the known molecular weight of GFAP), western blot analysis

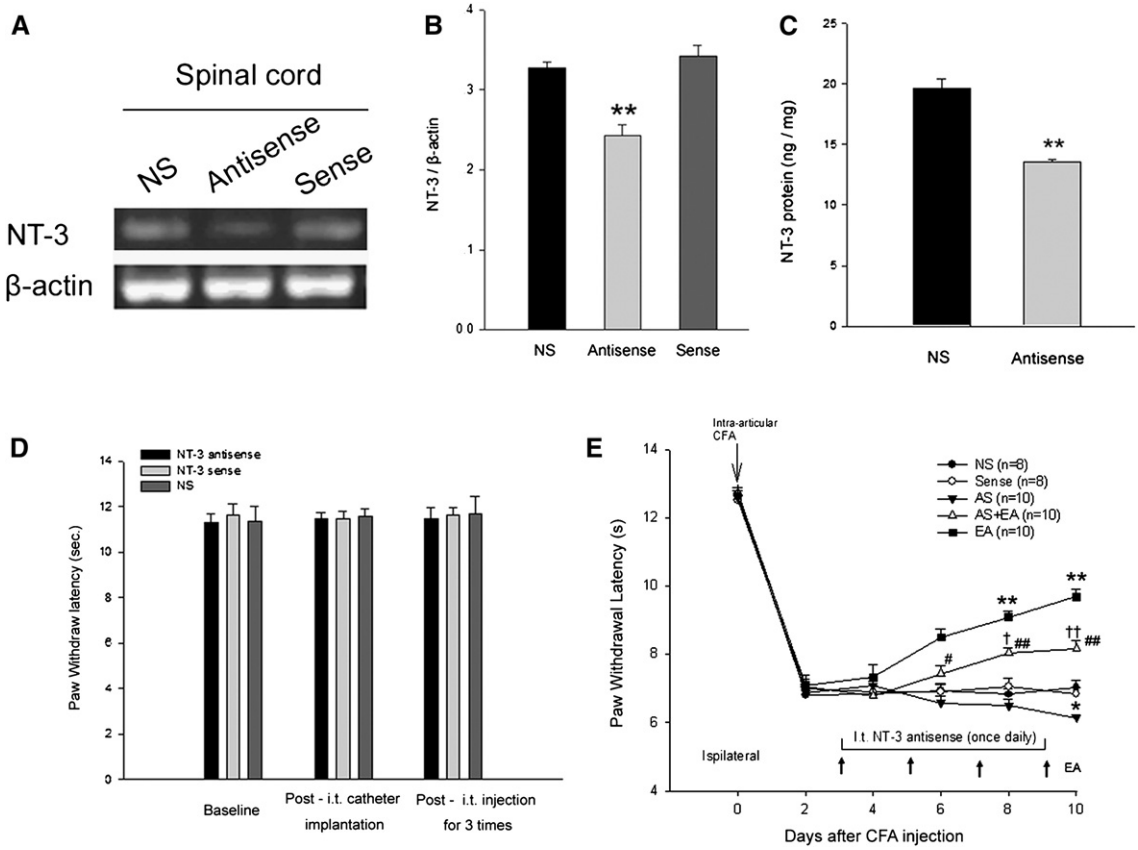

Figure 3. Down-regulation of NT-3 expression in the spinal cord of normal rats by intrathecal delivery of antisense ODN as detected by RT-PCR **(A, B)** and ELISA **(C)**. The effects of NT-3 antisense ODN treatment on the pain threshold in normal rats **(D)** and EA analgesia on the ipsilateral side of monoarthritic rats **(E)**. NT-3 antisense (AS) ODN was delivered at a dose of 12.5 μl/100 μM per injection per rat once daily for 7 days beginning on the 3rd post-CFA injection day. EA was administered once every other day beginning on the 3rd day and continuing through the end of the experiment. The mRNA levels of different groups are expressed as a ratio to levels of β-actin **(B)**. Data are presented as the mean $\pm$ S.E.M. $^{**}P < 0.01$, versus NS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus AS group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, versus EA group. CFA, complete Freund's adjuvant; EA, electroacupuncture; AS, antisense; NT-3, neurotrophin-3.

revealed a significant decrease in the expression of GFAP following EA treatment ($P < 0.05$). This downregulation was reversed by NT-3 antisense ODN administration ($F_{3,12} = 110.061$, $P < 0.01$, Figs 4C and 4D).

Changes in the Expression of Iba-1 After NT-3 Antisense ODN Administration in MA Rats With EA Analgesia

Similar to GFAP, an expected 572-bp product for CD11b mRNA was obtained, and semiquantitative analysis revealed that CD11b mRNA levels were significantly decreased after EA treatment (Figs 5A and 5B). And the decreased mRNA level of CD11b was attenuated by the combined treatment of EA and NT-3 antisense ODN ($P < 0.01$, $F_{3,12} = 77.212$).

A single protein band of characteristic size (~18 kDa) for Iba-1 was detected in the western blot by the Iba-1-specific primary antibody (Fig 5C). Western blot analysis showed the same trend observed using RT-PCR, and the decrease of Iba-1 protein by EA treatment was significantly attenuated after antisense delivery ($P < 0.01$, $F_{3,12} = 75.994$, Fig 5D).

Changes in the Expression of IL-1β, IL-6 and TNF-α After NT-3 Antisense ODN Administration in MA Rats With EA Analgesia

Using RT-PCR and ELISA, mRNA (Figs 6A–6D) and protein (Figs 6E–6G) levels of spinal IL-1β, IL-6, and TNF-α were assessed. EA treatment produced a marked down-regulation in the levels of spinal proinflammatory cytokines compared to the sense group ($P < 0.05$). NT-3 antisense ODN delivery during EA treatment attenuated the decreased expression of proinflammatory cytokines in comparison with the EA group (RT-PCR: IL-1β: $F_{3,12} = 31.161$; IL-6: $F_{3,12} = 52.939$; TNF-α: $F_{3,12} = 36.118$; ELISA: IL-1β: $F_{4,15} = 33.167$; IL-6: $F_{4,15} = 22.486$; TNF-α: $F_{4,15} = 34.164$, $P < 0.05$).

Discussion

Acupuncture and EA have been accepted worldwide as complementary and alternative medicines for the treatment of acute and chronic pain.[32] Several processes have been proposed to explain the effects of EA on pain. Studies have revealed that endogenous opioid

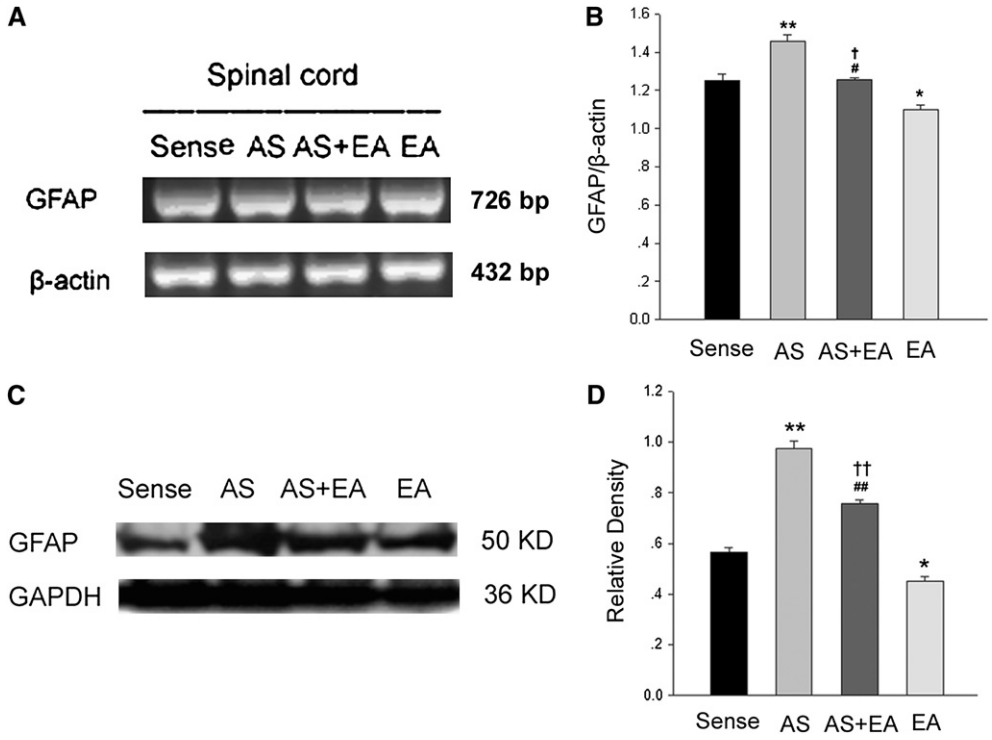

Figure 4. Effects of repeated intrathecal injections of NT-3 antisense ODN on GFAP expression in the spinal dorsal horn during EA treatment, as detected by RT-PCR **(A, B)** and western blot **(C, D)**. NT-3 antisense ODN was delivered at a dose of 12.5 μl/100 μM per injection per rat once daily for 7 days beginning on the 3rd day following CFA injection. EA was administered once every other day beginning on the 3rd day and continuing through the end of the experiment. The mRNA levels of different groups were expressed as a ratio to levels of β-actin **(B)**. Images of protein bands are shown for GFAP and GAPDH in the spinal dorsal horn **(C)**. The GFAP protein levels in different groups were expressed as a ratio to that of corresponding GAPDH **(D)**. Data are presented as the mean $\pm$ S.E.M. (n = 4 in each group). $^{*}P < 0.05$, $^{**}P < 0.01$ versus Sense group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus AS group; $^{\dagger}P < 0.05$ versus EA group. CFA, complete Freund's adjuvant; EA, electroacupuncture; AS, antisense; NT-3, neurotrophin-3.

peptides in the CNS play an essential role in mediating the analgesic effects of EA.[12] In recent years, it has been demonstrated that neurotrophins and cytokines are released in response to various stimuli, such as electronic stimulation or inflammation. This crosstalk between the PNS and the CNS is involved in the pathophysiology of many human diseases and may contribute to the effects of acupuncture. Based on the knowledge of neurotrophins and cytokines, the neurotrophin/cytokine hypothesis for the mechanism of acupuncture has been proposed.[7,9]

In the present study, the expression of NT-3 in the spinal cord was found to be increased at 6 hours, 4 days, and 14 days post-CFA injection, indicating a relationship between NT-3 and the hyperalgesia in inflammatory pain. Also, the NT-3 increased in both mRNA and protein levels, which suggests that NT-3 could be locally produced in the spinal cord in response to inflammatory pain. Moreover, it is well known that DRG neurons project to the spinal cord, and it is thus possible that NT-3 is transported in an anterograde direction. A previous study showed that NT-3 is transported from the DRG to the spinal cord in the dorsal rhizotomy model.[25] Together, these previous findings may explain why we observed increased expression of NT-3 in the spinal cord following injections of CFA.

However, the upregulation of an active substance does not necessarily mean that it is proalgesic or analgesic, because in pathological conditions, proalgesic as well as some analgesic substances will be released, and an imbalance between these 2 substances will lead to pain. By downregulating the expression of NT-3, the biological activities of NT-3 can be better elucidated. Using an antisense oligonucleotide strategy, which acts through downregulating the expression of cell-surface receptors and attenuating cellular responsiveness,[1] we found that the expression of endogenous molecules in the spinal cord could be successfully knocked down by intrathecal antisense ODN treatment.[2] Thus, to confirm the role of NT-3 in hyperalgesia in rats with inflammatory pain, we used an antisense ODN to knock down the expression of NT-3. Our experimental data show that the thermal hyperalgesia of rats with inflammatory pain was significantly aggravated after antisense treatment compared with NS treatment and sense treatment, suggesting that NT-3 may play an anti-hyperalgesic role in inflammatory pain.

Increases of endogenous analgesic substances during pain are important to maintain homeostasis. Thus, although NT-3 is known to be an antihyperalgesic agent, it may be upregulated following exposure to the nociceptive stimulus. This result is in accordance with studies that demonstrated that the expression of antihyperalgesic agents, such as glial cell line-derived neurotrophic factor (GDNF), somatostatin (SOM), cannabinoid receptor 2 (CB2), and interleukin (IL)-10, is significantly increased in

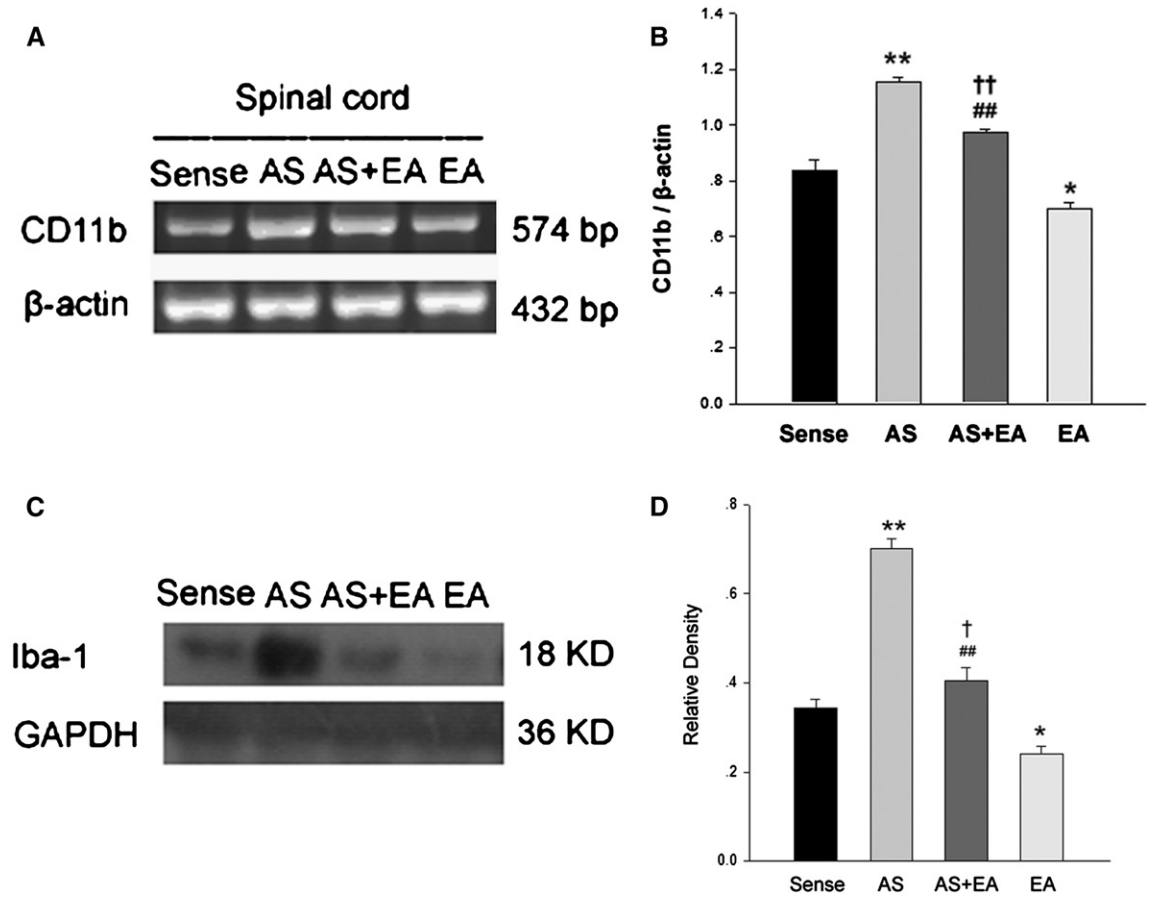

Figure 5. Effects of repeated intrathecal injections of NT-3 antisense ODN on Iba-1 expression in the spinal dorsal horn during EA treatment as detected by RT-PCR **(A, B)** and western blot **(C, D)**. NT-3 antisense ODN was delivered at a dose of 12.5 μl/100 μM per injection per rat once daily for 7 days beginning on the 3rd day following the CFA injection. EA was administered once every other day from the 3rd day and continuing through the end of the experiment. The mRNA levels of different groups were expressed as a ratio to levels of β-actin **(B)**. Images of protein bands are shown for Iba-1 and GAPDH in the spinal dorsal horn **(C)**. Iba-1 protein levels in different groups were expressed as a ratio to that of corresponding GAPDH **(D)**. Data are presented as the mean $\pm$ S.E.M. (n = 4 per group). $^{*}P < 0.05$, $P < 0.01$ versus Sense group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus AS group; $^{\dagger\dagger}P < 0.01$ versus EA group. CFA, complete Freund's adjuvant; EA, electroacupuncture; AS, antisense; NT-3, neurotrophin-3.

rats with inflammatory or neuropathic pain. And on the 4th day postinjection, although NT-3 was upregulated to play the protective role, it is still weak to keep the balance. So the animals remain hyperalgesic at 4 days.

Following 5 times of EA treatment, the expression of NT-3 in the spinal dorsal horn of rats was found markedly enhanced at 14 days post-CFA injection, indicating a relationship between NT-3 and EA analgesic effects in inflammatory pain. Consistently, it has been shown that the number of NT-3-positive neurons on the acupunctured side of the spinal lamina II of cats was significantly greater than the number on the nonacupunctured side. Following the expression of NT-3 that was knocked down by antisense ODN, we found that EA's analgesic effects in monoarthritic rats were attenuated by NT-3 antisense treatment, which indicates the involvement of NT-3 in EA analgesia.

The relationship between EA and glia as key contributors to pathological and chronic pain mechanisms[15] has gained increasing attention in the recent years. Accumulating evidence suggests that acupuncture inhibits the activation of glia and decreases the levels of pro-inflammatory cytokines, and that the anti-neuroinflammatory effects of EA may be considered one of the mechanisms of its anti-arthritic pain effects.[17]

Furthermore, it has been demonstrated that neurotrophins are produced from astrocytes and microglia and that neurotrophins receptors (such as TrkA, TrkB, TrkC, and p75NTR) are expressed in glia. In addition, neurotrophins are believed to modulate neuron-glia interactions at a vertebrate synapse.[20] Using LPS-activated BV2 cells, it was found that reduced expression of inducible NO synthetase by NT-3 pretreatment was mediated by MAP kinase and PI3 kinase signaling pathways.[21,22] These findings suggest that the regulatory mechanisms associated with NT-3 that are produced by active healthy neurons may attenuate microglia-induced inflammation.

In parallel with these published reports, the present study showed that knockdown of NT-3 markedly increased the expression of GFAP, OX-42, and the proinflammatory cytokines IL-1β, IL-6, and TNF-α in the spinal dorsal horn during inflammatory pain. These data indicate that the anti-hyperalgesic role of NT-3 in inflammatory pain may be mediated through the inhibition of glial activity as well as through reducing the levels of proinflammatory cytokines. In addition, we

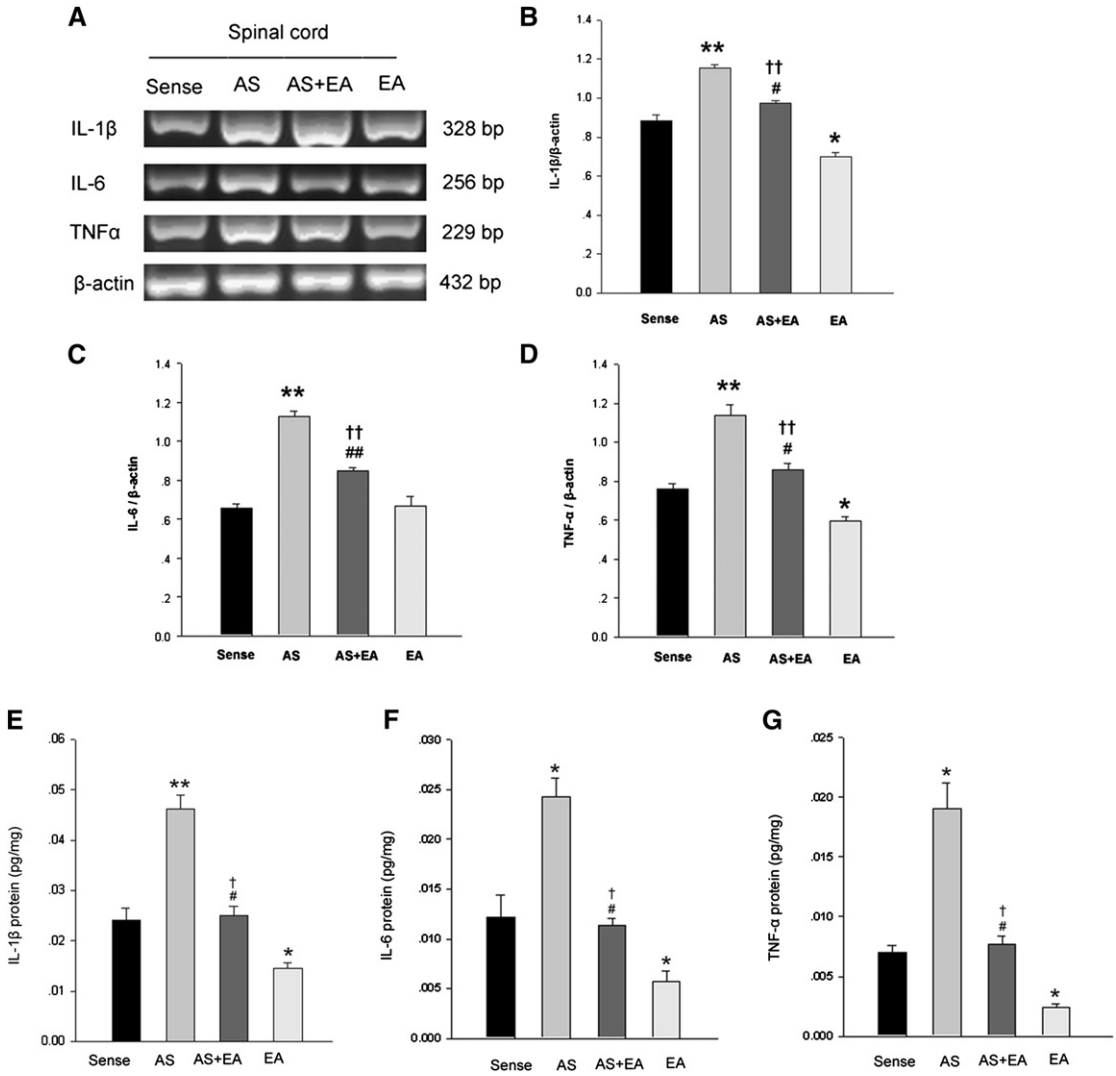

Figure 6. Effects of repeated intrathecal injections of NT-3 antisense ODN on IL-1β (A, B, E), IL-6 **(A, C, F)**, and TNF-α **(A, D, G)** expression in the spinal dorsal horn during EA treatment as detected by RT-PCR **(A–D)** and ELISA **(E–G)**. NT-3 antisense ODN was delivered at a dose of 12.5 μl/100 μM per injection per rat once daily for 7 days beginning on the 3rd day following the CFA injection. EA was administered once every other day for the duration of the experiment. The mRNA levels of different groups are expressed as ratio levels of β-actin **(B, C, D)**. Data are presented as the mean $\pm$ S.E.M. (n = 4 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ versus Sense group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus AS group; $^{\dagger\dagger}P < 0.01$ versus EA group. CFA, complete Freund's adjuvant; EA, electroacupuncture; AS, antisense; NT-3, neurotrophin-3.

found that the downregulation of GFAP, OX-42, and proinflammatory cytokine expression in the spinal cord following EA treatment was significantly attenuated by NT-3 antisense ODN treatment, thus providing further evidence that the involvement of NT-3 in the analgesic effects of EA is mediated through the inhibition of glial activity as well as through reducing the levels of proinflammatory cytokines. However, it is possible that the NT-3 antisense ODN and EA had opposite and independent effects. Thus, the mechanism of EA requires further investigation of additional endogenous mechanisms.

Taken together, these data indicate that endogenous NT-3 may have an anti-hyperalgesic role in the inflammatory pain of rats and may be involved in mediating the analgesic effects of EA. Furthermore, the current results suggest that this effect may be through the inhibition of glial activity as well as through reducing the production of proinflammatory cytokines. The present study may initiate a discussion on the possible roles of NT-3/glia/cytokines in the therapeutic effects of acupuncture and provide insight on the mechanism by which acupuncture relieves arthritic pain.

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