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Down-regulation of GFRα-1 expression by antisense oligodeoxynucleotide attenuates electroacupuncture analgesia on heat hyperalgesia in a rat model of neuropathic pain

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Abstract

Glial cell line-derived neurotrophic factor (GDNF) has been proved to play an important role in the modulation of nociceptive transmission especially during neuropathic pain. It was reported that electroacupuncture (EA) had potent analgesic effect on neuropathic pain and our previous studies indicated that EA could activate endogenous GDNF signaling system (GDNF and its receptor GFRα-1) in dorsal root ganglions (DRGs) of neuropathic pain rats. In order to investigate whether GDNF signaling system was involved in EA analgesia on neuropathic pain, which was induced by chronic constriction injury (CCI) of the sciatic nerve in rats, antisense oligodeoxynucleotide (ODN) specifically against GFRα-1 was used in the present study to result in down-regulation of GFRα-1 expression. The results showed that: (1) cumulative EA had potent analgesic effect on neuropathic pain in rats; (2) the expression of GFRα-1 in DRGs was down-regulated by intrathecal delivery of antisense ODN, but not by normal saline (NS) or mismatch ODN; (3) EA analgesia was significantly attenuated by antisense ODN treatment. The present study demonstrated that endogenous GDNF signaling system was involved in EA analgesia on neuropathic pain in rats, which would deepen our realization of the mechanism of EA analgesia.

Keywords: GDNF; GFRα-1; Electroacupuncture; Neuropathic pain; Antisense; Analgesia

1. Introduction

GDNF was originally purified from a rat glioma cell-line supernatant as a trophic factor for embryonic midbrain dopamine neurons [24], and was later found to have potent survival-promoting effects on various types of neurons including primary sensory neurons [4,8,22,28]. The biological action of GDNF is mediated by a two-component receptor complex consisting of a glycosylphosphatidylinositol-linked cell surface molecule, the GDNF family receptor GFRα-1 (originally named GDNFR-α), which acts as a ligand binding domain and the receptor protein tyrosine kinase Ret, which acts as the signal transducing domain. GDNF is thought to bind preferentially to GFRα-1 and GDNF fails to exert its biological action in the absence of GFRα-1. Besides its potent survival-promoting effects on diverse groups of neurons, GDNF has been proved by previous studies to play an important role in the modulation of nociceptive signals especially during neuropathic pain [4,6,13,36].

Injury to the nervous system occasionally leads to persistent neuropathic pain, which is characterized by the combination of spontaneous burning pain, hyperalgesia and allodynia. Such pain is often intense and refractory to conventional analgesic therapy. Acupuncture, which has been practiced for over 2000 years in China, has long been used to relieve pain. Acupuncture is one of the most promising supplementary medical treatments in pain management because it is known to be effective in certain painful conditions, it is easy to apply, the cost is low and side effects are minimum. In clinic, acupuncture is an established adjuvant analgesic modality for the treatment of chronic pain. Electrical stimulation of acupuncture points, which is called electroacupuncture (EA), is widely used both in clinic and in basic experimental studies. It has been shown that EA had potent analgesic effect in neuropathic pain patients [7,9,17,31] and rat models [10,16,19], and it was also well known that EA analgesia

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was mediated by endogenous pain modulating system such as the opioid system. However, the mechanism of EA analgesia on neuropathic pain has not been fully understood, since EA has comprehensive modulating effects on the nervous system. It was reported that endogenous GDNF in the rat's brain might be involved in the therapeutic effect of EA on Parkinson's disease in rats lesioned by medial forebrain bundle transection [23]. Our previous studies showed that EA could activate endogenous GDNF and GFRα-1 signaling system of rats with neuropathic pain induced by CCI of the sciatic nerve, and both mRNA and protein levels of GDNF and GFRα-1 in DRGs of neuropathic pain rats were enhanced by EA treatment [11]. However, whether endogenous GDNF system was involved in EA analgesia on neuropathic pain remains to be determined. To test whether a signaling protein molecule participates in a given physiological or pathological activity, the most concise and convincing way is to apply the specific antagonist to its receptor. Unfortunately, the specific antagonist for the receptor of GDNF has not been available. However, antisense oligonucleotide strategy, an approach successfully used for many years [1,2,34], could be a reliable alternative. It was found that intrathecally injected antisense ODN could be uptaken by DRG cells and the expression of endogenous molecules in DRG could be successfully down-regulated by intrathecal antisense ODN treatment [15,20]. Therefore, the present study used antisense strategy to down-regulate the expression of GFRα-1 and to investigate the role of GDNF signaling system in EA analgesia on neuropathic pain of rats.

2. Materials and methods

2.1. Experimental animals and induction of neuropathic pain

Experiments were performed on adult male Sprague–Dawley rats (Experimental Animal Center, Shanghai Medical College of Fudan University, China) weighing 200–220 g. Prior to experimental manipulation, rats were allowed to acclimate for 1 week and maintained on a 12-h light:12-h dark cycle with free access to food and water. In order to minimize the number of animals used and their suffering, all rats in the study were used 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 [41].

The hyperalgesic state was induced by CCI of the sciatic nerve with four loose ligatures as previously described [5]. Briefly, under isoflurane anesthesia, the left sciatic nerve was exposed at the level of middle of the thigh by blunt dissection through biceps femoris and four 4-0 chronic gut sutures were each tied loosely with a square knot around the sciatic nerve. In every animal, an identical dissection was performed on the right side, except that the sciatic nerve was not ligated. All animals postoperatively displayed normal feeding and drinking. In order to assess the neuropathic pain, rats were tested for thermal hyperalgesia on the fourth day after CCI.

2.2. EA administration

For EA treatment, rats were placed in wood holders. The rat was bound moderately to the holder so that the movement of the rat's body was restrained while the limbs could move freely. Rats were allowed to acclimate for 30 min before EA treatment. According to our previous study [11,25], 'Huan-Tiao' and 'Yang-Ling-Quan' acupoints were selected. A pair of stainless steel needles of 0.3 mm diameter were inserted with a depth of 5 mm into the contralateral (in a respect of performed CCI) 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). The two needles were connected with the output terminals of an electroacupuncture apparatus (Model G 6805-2, Shanghai Medical Electronic Apparatus Company, China). Alternating strings of dense-sparse frequencies (60 Hz for 1.05 s and 2 Hz for 2.85 s alternately) were selected. The intensity was adjusted to induce slight twitch of the hindlimb ($\leq$1 mA, 12 V), with the intensity lasting for 30 min. EA was administered once every other day from the seventh day after CCI surgery until the end of the experiment.

2.3. Behavioral test

The paw withdrawal latency (PWL) to radiant heat was examined as previously decribed [5] for evidence of heat hyperalgesia in animals using the Model 336 combination unit for paw stimulation (IITC/Life Science Instruments, USA). The rats were placed beneath an inverted, clear plastic cage upon an elevated floor of window glass. After an adaptation period of 30 min, radiant heat (50 W, 8 V bulb) was applied to the plantar surface of each paw until the animal lifted its paw from the glass. The time from onset of radiant heat application to withdrawal of the rat's hindpaw was defined as the PWL. The heat was maintained at a constant intensity, and a cut-off time of 20 s was imposed on the stimulus duration to prevent tissue damage.

2.4. Antisense oligodeoxynucleotide

Down-regulation of GFRα-1 was obtained by intrathecal (i.t.) delivery of antisense ODN specifically complementary to a segment of the sequence of GFRα-1 mRNA. The sequence of antisense ODN was: 5′-TAGGAACATGGTGCC-3′ [37]. Another 15-mer ODN with four mismatched bases (5′-TAGAGACTAGGTGCC-3′) was used as the control. These two kinds of ODN were fully phosphorothioated.

2.5. ODN administration

Both antisense and mismatch ODNs were used at a dose of 30 μg dissolved in 5 μl of nuclease-free NS and each i.t. injection of ODN was followed by 9 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 [38] for ODN administration. The animals were allowed to recover from the implantation surgery for 3 days prior to any experimentation, and monitored daily after surgery for signs of motor deficiency. Rats that showed any neurological deficits resulting from the surgical procedure were excluded from the experiments. Location of the distal end of the intrathecal catheter was verified at the end of every experiment by injection of Pontamine Sky Blue via the i.t. catheter.

2.6. Western blot analysis

Given an overdose of urethane (1.5 g/kg, i.p.), rats were sacrificed and the L4–L6 DRGs were collected in dry ice and stored at −70 °C until assayed. Each assay sample consisted of the pooled unilateral L4–L6 DRGs from one rat. 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 phenylmethylsulfonylfluoride) at 4 °C. Supernatant after 12,000 r.p.m. centrifugation for 10 min was used for Western blotting. Samples (30 μg of total protein) were dissolved with equal volume of loading buffer (0.1 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 100 V to Immun-Blot PVDF membrane for 1 h at 4 °C. Membranes were blocked in TBST containing 5% non-fat dried milk overnight at 4 °C before incubation for 2 h at room temperature with anti-GFRα-1 polyclonal antibody (1:1000, Sigma, USA) diluted in TBST containing 5% BSA. Blots were washed extensively in TBST and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Amersham) in TBST/1.25% BSA for 1 h at room temperature. The signal was detected by an enhanced chemiluminescence method

(ECL kit, Amersham), and exposed to Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA). The intensity of the selected bands was captured and analyzed using GeneSnap Image Analysis Software (Syngene, UK).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Rats were sacrificed with an overdose of urethane (1.5 g/kg, i.p.) and the L4–L6 DRGs were collected in dry ice. Total RNA extraction was performed using the Trizol reagent, following the instructions of the manufacturer. RNA was further purified using the RNeasy kit according to the RNA clean-up protocol, and eluted in 20 μl of RNase-free distilled H_2O. The amount of RNA was measured spectrophotometrically. Total RNA (1 μg) was used for the synthesis of the first strand of cDNA using the SuperScript reverse transcriptase. Briefly, RNA, oligo (dT)18 primers (0.5 μg/μl) were first denatured for 5 min at 65 °C, chilled on ice for 1 min and then incubated for 50 min at 42 °C, 15 min at 70 °C in 20 μl of a reaction mixture containing 10 × first-strand buffer, 10 mM dNTP mix, 0.1 M DTT and 50 units of SuperScript II reverse transcriptase. The sequences of primers for GFRa-1 were as follows: forward: 5′-ATTGGCACAGTCATGACTCCCAAC-3′ 1178–1201, reverse: 5′-GAGGAGCAGCCATTGATTTTGTGG-3′ 1599–1622 (U59486) [35]; β-actin forward: 5′-CACCATGTACCCTGGCATTG-3′ reverse: 5′-TAACGCAACTAAGTCATAGT-3′. The primers were synthesized and purified by Shanghai Institute of Biochemistry, Chinese Academy of Science. One microliter of cDNA was added to 49 μl of PCR mix containing 5× PCR buffer, 18 pmol/l concentrations of each primer, 2.5 mM of dNTP and three units of Taq DNA polymerase. PCR reaction was performed as follows: 12 min at 94 °C to activate the Taq polymerase, followed by 30 cycles of 45 s at 94 °C, 45 s at 60 °C and 1 min at 72 °C. A final elongation step at 72 °C for 10 min completed the PCR reaction. Each PCR production (10 μl) was electrophoresed in 1% 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 GFRα-1 bands to that of β-actin band.

2.8. Immunohistochemsitry

Rats were given an overdose of urethane (1.5 g/kg, i.p.) and perfused through the ascending aorta with 200 ml of normal saline followed by 300 ml 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The L4/5/6 DRGs were then removed, postfixed in the fixative solution for 4 h at 4 °C and immersed in 30% sucrose in PB for 24–48 h 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.

GFRα-1 immunohistochemistry was performed as previously described [40]. Briefly, following three 15-min rinses in 0.01 M PBS, the sections were preincubated for 30 min at room temperature in a blocking solution of 3% normal goat serum in 0.01 M PBS with 0.3% Triton-X 100 (NGST) and then incubated in rabbit anti-GFRα-1 polyclonal antibody (1:1000, Sigma, USA) diluted in 1% NGST at 4 °C for 48 h. The incubated sections were washed three times in 1% NGST and incubated in biotinylated goat anti-rabbit immunoglobulin G (IgG) (1:200, Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The sections were then washed three times in 1% NGST and incubated for 1 h in avidin–biotin–peroxidase complex (1:200, Vector Laboratories) at room temperature. Finally, the sections were washed three times in 0.01 M PBS, and 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.

To test the specificity of the primary antibody, controls were performed, including the substitution of normal rabbit sera for the primary antibody and omission of the primary antibody. None of these controls showed any sign of immunohistochemical reaction.

For quantification, images of positive staining in the DRG sections were captured and analyzed using Leica Q500IW image analysis system. The total number of neurons and the number of neurons positive for GFRα-1 immunoreactivity were counted for each section and percentages of immunoreactive neurons were calculated. For each animal, nine sections were taken (three of each L4/5/6 DRG) from the ipsilateral DRGs and the mean of the percentages of immunoreactive neurons was calculated. The investigator responsible for image analysis was blind to the experimental condition of each rat.

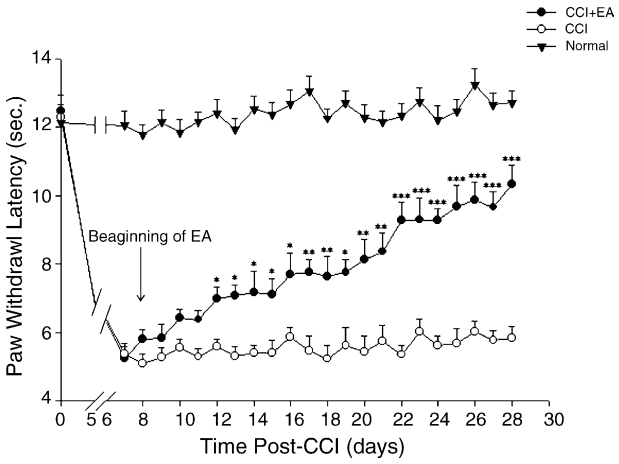

Fig. 1. Analgesic effects of EA on CCI-induced heat hyperalgesia in rats. EA was administered once every other day from the seventh day after surgery until the end of the experiment. Values are mean ± S.E.M. (CCI group and CCI + EA group $n = 12$; normal group $n = 6$) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. CCI group.

2.9. Data analysis

Data are presented as mean ± S.E.M. and analyzed by SPSS 10.0. Repeated measures analysis of variance (ANOVA) followed by S–N–K test was used for post hoc analysis for differences between groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of EA on heat hyperalgesia of neuropathic pain rats

As reported previously [5], CCI to the sciatic nerve produced severe heat hyperalgesia in the ipsilateral hindpaws. From the seventh day after surgery, the rats with neuropathic pain were randomly divided into two groups, and EA was administered in one group from the seventh day after surgery, once every other day, until the end of the experiment. On the days of EA treatment, PWL was examined before EA treatment to avoid the disturbance of immediate influence of EA on pain behavior. Another group of rats without CCI or EA treatment served as normal group. As shown in Fig. 1, ipsilateral PWL of the EA treatment group increased gradually compared with those of CCI group, and the difference was statistically significant ($P < 0.05$ to <0.001, $n = 12$) after three times of EA treatment (from the 12th day after surgery).

3.2. Effect of antisense ODN on GFRα-1 expression

To determine the down-regulation of GFRα-1 expression, rats were sacrificed after 3 days delivery of ODNs and ipsilateral L4/5/6 DRGs from different groups were removed and assayed using Western blot analysis, RT-PCR and immunohistochemistry. A single band (∼58 kDa, coincident with the known

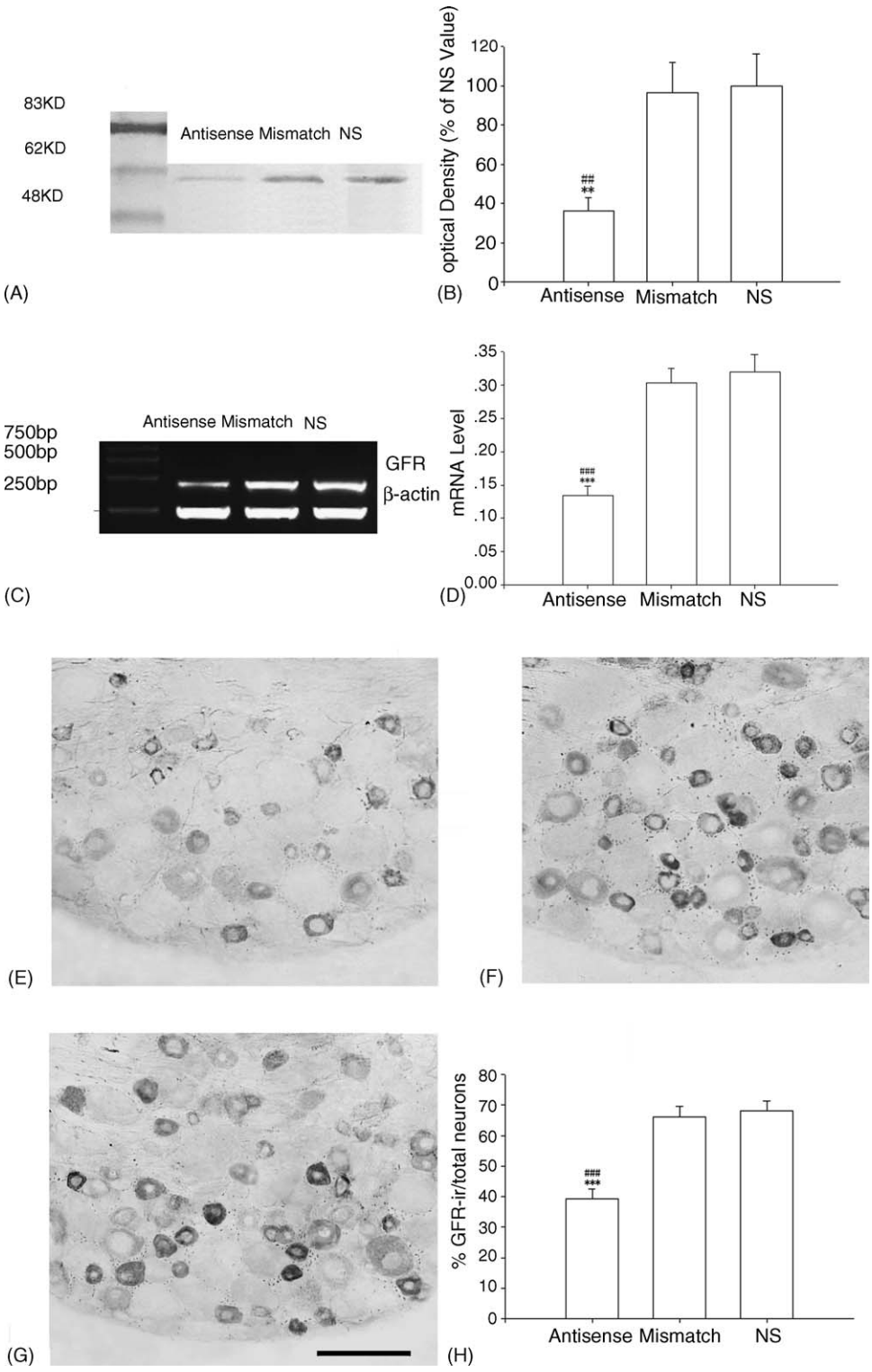

Fig. 2. The down-regulation of GFRα-1 expression by intrathecal delivery of antisense ODN. Western blot analysis detected a protein band of ∼58 kDa, coincident with the known molecular weight of GFRα-1 (A). PCR products of expected size were acquired corresponding to GFRα-1 (C). Images were shown for GFRα-1 immunostaining in the ipsilateral L5 DRG of antisense ODN treatment group (E), mismatch ODN treatment group (F) and NS treatment group (G). The results were quantified and demonstrated. The optical densities of immunoblot bands were expressed as a percentage to that of the NS group sample (100%) (B). The mRNA levels of different group were expressed as a ratio to that of corresponding β-actin (D). GFRα-1-immunoreactive (GFRα-1-ir) neurons were quantified as described in Methods and Materials. The number of GFRα-1-ir neurons was expressed as a percentage of GFRα-1-ir neurons to total neurons (H). Values are mean ± S.E.M. ($n = 6$ in each group) $^{**}P < 0.01$, $^{***}P < 0.001$ vs. NS group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. mismatch group. Scale bar = 100 μm.

molecular weight of GFRα-1) was detectable in western blot analysis as immunoreactive GFRα-1 (Fig. 2A). Samples treated without first antibody did not obtain any detectable band at the same position (data not shown). Density analysis showed that expression of GFRα-1 protein in DRGs of the antisense ODN group was significantly inhibited compared with that of NS group or mismatch ODN group ($P < 0.01$, $n = 6$, Fig. 2B). RT-PCR analysis obtained an expected 444-bp product for GFRα-1 mRNA (Fig. 2C). Semi-quantitive analysis showed that mRNA level of GFRα-1 in DRGs of antisense ODN group was significantly decreased compared with that of NS group or mismatch ODN group ($P < 0.001$, $n = 6$, Fig. 2D). Immunostaining of GFRα-1 in DRG sections is shown (Fig. 2E–G) and the number of GFRα-1-immunoreactive (GFRα-1-ir) neurons was counted. As shown in Fig. 2H, the number of GFRα-1-ir neurons in DRGs was significantly reduced by antisense ODN treatment ($P < 0.001$, $n = 6$). The results of the above different analyses conformably showed that the expression of GFRα-1 in DRGs was significantly knocked down by antisense ODN treatment.

3.3. Effect of antisense ODN on EA analgesia

Thirty-six rats were randomly divided into three groups and i.t. catheters were inserted to the subarachnoid space of lumbar enlargement on the fourth day after CCI surgery. On the seventh day, the rats received i.t. delivery of antisense ODN, mismatch ODN and NS, respectively. ODN was delivered at a dose of 30 μg per injection (once daily) for 8 days. EA was administered once every other day from the seventh day after CCI surgery for 3 weeks. Besides these above groups, six rats received CCI surgery without EA or ODN treatment serving as CCI model group. PWL of rats was measured as described above. The ipsilateral PWL of rats of all the three EA treatment groups was enhanced gradually with EA treatment accumulated, while the ipsilateral PWL of the antisense ODN group increased slowly compared with that of the mismatch ODN group or the NS group, and the difference was statistically significant from the 12th day to the 19th day (from 5 days after ODN delivery beginning, to 4 days after the end of ODN delivery) ($P < 0.05$ to <0.01, Fig. 3).

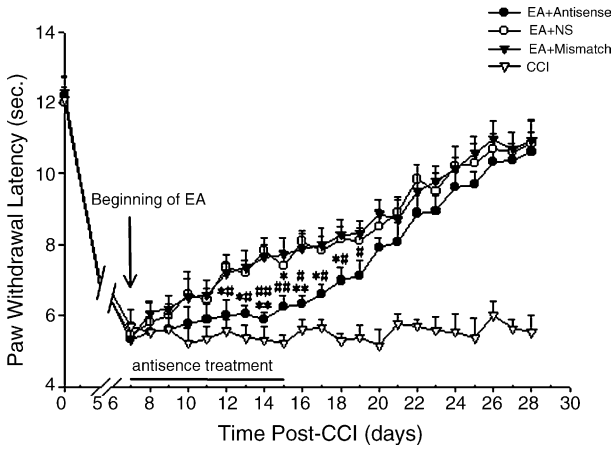

Fig. 3. Effects of antisense ODN against GFRα-1 on EA analgesia. Antisense ODN was delivered at a dose of 30 μg per injection (once daily) for 8 days since the seventh day after CCI surgery. EA was administered once every other day from the seventh day after surgery until the end of the experiment. Values are mean ± S.E.M. (CCI group $n = 6$; the other three groups $n = 12$) $^{*}P < 0.05$, $^{**}P < 0.01$ vs. EA + NS group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. EA + mismatch group.

4. Discussion

The present study proved that EA had potent analgesic effect on neuropathic pain in rats, consistent with a series of previous studies [10,16,19]. However, the mechanism of EA analgesia has not been fully understood. EA has modulating effect on the release and expression of various endogenous bioactive substances in the nervous system including monoamines, opioids, oxytocin and so on, which are important in the transmission and modulation of nociception [12,33,39]. This is considered as one of the underlying mechanisms of acupuncture analgesia. Our previous studies revealed that EA treatment resulted in an up-regulation of the expression of endogenous GDNF and GFRα-1 in DRGs of neuropathic pain rats and the up-regulation was persistent along with EA treatment, which suggested a close relationship between GDNF signaling system and EA analgesia on neuropathic pain [11].

GDNF has been recently proved to play an important role in the modulation of nociceptive signals especially during neuropathic pain. In the peripheral nervous system, a distinct subgroup of small DRG cells, which is believed to play nociceptive function, expresses GDNF receptor components and GDNF has been shown to protect these neurons after nerve injury [3,4,27]. GDNF was found to coexist with pain-related substances, such as substance P (SP) and calcitonin gene-related peptide (CGRP) and GDNF could exert mighty influence on their expression and function [4,29,30,32]. More importantly, a series of studies provided compelling evidence that intrathecally administered GDNF as well as HSV-mediated expression of GDNF could exert potent analgesic effect on neuropathic pain in rats [6,13,36]. Thus, we could hypothesize that GDNF might be involved in EA analgesia on neuropathic pain. It was proved by the present study that EA analgesic effect was attenuated when antisense ODN specifically against GFRα-1 was intrathecally administered and resulted in 'down-regulation' of GFRα-1 expression.

GDNF might be involved in EA analgesia on neuropathic pain through various pathways. Previous studies proved that GDNF protected against multiple phenotypic changes induced by peripheral nerve injury. It was shown that GDNF could prevent A-fibre sprouting into lamina II, and the latter was suggested as an underlying mechanism of neuropathic pain [4]. GDNF was also demonstrated to partially reverse axotomy-induced increase in a sodium channel subunit, $Na_v1.3$, resulting in partial reversal of axotomy-induced changes in repriming kinetics of the sodium current. The ability of exogenous GDNF to block the expression of $Na_v1.3$ in the injured nerve was proposed to be a critical mechanism of GDNF's anti-allodynic and anti-hyperalgesic effects [6,21]. It was proved that GDNF could suppress the expression of NPY that was increased during neuropathic pain and the GDNF-induced suppression of NPY production might also be causal to GDNF's ability to block tactile hypersensitivity [36]. Interestingly, GDNF was reported to promote the release of endogenous

somatostatin (SOM) from adult primary sensory neurons [26], and SOM might be an endogenous non-opioid neuropeptide that had analgesic effects in rodents and humans.

The present study found that the expression of GFRα-1 in the spinal cord was also down regulated by antisense ODN treatment (data not shown). Since the expression of GDNF could also be detected in the superficial layer of the spinal dorsal horn [14,18], it may be hard to determine the definite acting site of GDNF. Most possibly, GDNF might play a role both peripherally and centrally.

The present study for the first time addressed the involvement of GDNF signaling system in EA analgesia on neuropathic pain in rats. This could deepen our understanding of the mechanism of EA analgesia and provide a rational basis for enhancing EA analgesic effect by potentiating the function of GDNF signaling system.

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