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Down-regulation of GFRα-1 expression by antisense oligodeoxynucleotide aggravates thermal hyperalgesia in a rat model of neuropathic pain

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Abstract

Glial cell line-derived neurotrophic factor (GDNF) has been hypothesized to play an important role in the modulation of nociceptive signals especially during neuropathic pain. The present study examined the expression of GDNF and GFRα-1 (the high-affinity receptor of GDNF) in dorsal root ganglions (DRG) in a rat model of neuropathic pain induced by chronic constriction injury (CCI) to the sciatic nerve. In order to address the role of GDNF and GFRα-1 in neuropathic pain, antisense oligodeoxynucleotide (ODN) specifically against GFRα-1 was intrathecally administered to result in down-regulation of GFRα-1 expression. The results showed that both the protein and mRNA levels of GDNF and GFRα-1 were significantly increased after CCI, while the thermal hyperalgesia of neuropathic pain rats could be significantly aggravated by antisense ODN treatment, but not by normal saline (NS) or mismatch ODN treatment. The present study demonstrated that endogenous GDNF and GFRα-1 might play an anti-hyperalgesic role in neuropathic pain of rats. In addition, we found a down-regulation of somatostatin (SOM) in DRG and spinal dorsal horn after expression of GFRα-1 was knocked down, which suggested the possible relationship between the anti-hyperalgesic effect of GDNF and GFRα-1 on neuropathic pain and endogenous SOM.

Keywords: GDNF; GFRα-1; Neuropathic pain; Antisense oligodeoxynucleotide; Somatostatin

1. Introduction

Neuropathic pain arises as a debilitating consequence of injury to the nervous system, which is characterized by combination of spontaneous burning pain, hyperalgesia and allodynia. Such kind of pain is often intense and refractory to conventional analgesic therapy. Previous studies on rodents showed that neuroplastic changes of a number of bioactive substances following nerve injury have been implicated in the manifestation of neuropathic pain, such as neurotransmitters as well as their receptors, ion channels, neurotrophic factors and cytokines. Some contribute to the genesis or the maintenance of neuropathic pain, such as the up-regulation of neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP). On the other hand, some may have anti-hyperalgesic effect during neuropathic pain, such as the up-regulation of galanin (GAL) and enkaphalin (ENK). These key molecules associated with signal processing of nociception have been suggested as potential targets for new analgesics.

GDNF, a member of transforming growth factor-β (TGF-β) superfamily that also includes neurturin (NTN), persephin (PSP), and artemin, was originally purified from a rat glioma cell-line B49 supernatant as a trophic factor for embryonic midbrain dopamine neurons (Lin et al., 1993), and was later found to have potent survival-promoting effects on various types of neurons including primary sensory neurons (Buj-Bello et al., 1995; Li et al., 1995; Mount et al., 1995). The biological action of GDNF is mediated by a two-component receptor

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complex consisting of the GDNF family receptor GFRα-1 (Jing et al., 1996; Treanor et al., 1996) and the receptor protein tyrosine kinase Ret (Durbec et al., 1996; Trupp et al., 1996). GFRα-1 is a glycosylphosphatidylinositol-linked cell surface molecule and acts as a ligand-binding domain. Ret acts as the signal transducing domain. GDNF is thought to bind preferentially to GFRα-1 and GDNF fails to exert its biological effect in the absence of GFRα-1. In DRG, a distinct subgroup of small DRG neurons, which is believed to play nociceptive function, expresses GFRα-1 and Ret, and GDNF has been shown to protect these neurons after nerve injury (Akkina et al., 2001; Bennett et al., 1998; Molliver et al., 1997). These neurons project the superficial layers of the spinal cord, where, it has been postulated that they may be involved in nociception. In fact, GDNF has been proved by previous studies to play an important role in the modulation of nociceptive signals especially during neuropathic pain (Bennett et al., 1998; Boucher et al., 2000; Hao et al., 2003; Wang et al., 2003). Our previous studies indicated that endogenous GDNF and GFRα-1 system might be involved in electroacupuncture (EA, electrical stimulation of acupoints) analgesia on neuropathic pain in rats (Dong et al., 2005). However, the role of endogenous GDNF and GFRα-1 system in neuropathic pain still remains unclear.

Interestingly, GDNF may have potent effects on the function of somatostatin (SOM), an endogenous analgesic non-opioid neuropeptide. In DRG, a subgroup of nociceptive sensory neurons expresses SOM. SOM is released from the central terminals of these sensory neurons into the spinal cord following noxious stimulation of peripheral nerves and produces an inhibitory effect on nociceptive neurons (Murase et al., 1982; Sandkuhler et al., 1990). In vivo, SOM is synthesized as preprosomatostatin (ppSOM), a precursor peptide that is cleaved to release active SOM. GDNF has been reported to increase the number of neurons in the DRG that can express SOM, and also increase the release of SOM from the central terminals of sensory neurons into the dorsal horn (Charbel et al., 2001).

Therefore, the aim of the present study was to (1) examine the expression changes of GDNF and GFRα-1 in DRG of neuropathic pain rats following CCI; (2) observe the effect of intrathecally administered antisense ODN specifically against GFRα-1 on thermal hyperalgesia of neuropathic pain rats; and (3) determine the expression change of SOM after GFRα-1 was knocked down by antisense treatment.

2. 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. Rats were allowed to acclimate for 1 week prior to experimental manipulation and maintained on a 12:12-h light–dark cycle with free access to food and water. The experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

The hyperalgesic state was induced by CCI of the sciatic nerve with four loose ligatures as previously described (Bennett and Xie, 1988). 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 4th day after CCI.

2.2. Experimental protocols

The expression of GDNF and GFRα-1 was examined by Western blot, RT-PCR and immunohistochemistry analyses. The time points of analysis were selected as 1 day, 3 days, 7 days, 14 days, 21 days and 28 days after CCI surgery. At each time point, six animals of each group were used for every analysis.

Two sets of experiments were conducted. In experiment 1, 36 rats were randomly divided into three groups. One group received intrathecal antisense ODN treatment 3 days before CCI surgery (30 μg per injection per rat, once daily), and the other two groups received mismatch ODN (30 μg per injection per rat, once daily) and normal saline (NS), respectively. On the 4th day, all the rats received CCI surgery and ODNs delivery continued for another 5 days (8 days delivery in all). The paw withdraw latency (PWL) of the rats was examined on the 3rd day after surgery. In experiment 2, 33 rats with thermal hyperalgesia were selected and randomly divided into three groups and intrathecal catheters were implanted into rats. On the 7th day after CCI surgery, three groups of rats received intrathecal delivery of antisense ODN, mismatch ODN and NS, respectively. ODNs were delivered at a dose of 30 μg per injection per rat, once daily for 5 days.

To determine the knockdown of GFRα-1 expression, rats were sacrificed after 3 days delivery of ODN and L4/5/6 DRG from different groups were removed and assayed using Western blot analysis, RT-PCR and immunohistochemistry. Six animals of each group were used for every analysis.

Expression of ppSOM mRNA and SOM peptide in DRG and spinal dorsal horn after GFRα-1 was knocked down by antisense ODN treatment was examined using RT-PCR and immunohistochemistry, respectively. Rats were sacrificed after 3 days delivery of ODN. Six animals of each group were used for either analysis.

2.3. Behavioral test

The paw withdrawal latency (PWL) to radiant heat was examined as previously described (Bennett and Xie, 1988) for evidence of thermal 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 heat was maintained at a constant intensity. The time from onset of radiant heat application to withdrawal of the rat's hindpaw was defined as the PWL. Both hindpaws were tested independently with a 10-min interval between trials. The cut-off time of 20 s was imposed on the stimulus duration to prevent tissue damage. During the days of ODN treatment, PWL was examined before ODN delivery to avoid the disturbance of intrathecal injection on pain behavior.

2.4. Immunohistochemistry

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 (NS) followed by 300 ml 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The L4/5/6 DRG and L4/5/6 segments of the spinal cord were then removed, post-fixed 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, and 30% ethylene glycol) and then stored at −20 °C until use.

Free-floating tissue sections were processed for GDNF, GFRα-1 and SOM immunohistochemistry by the avidin–biotin method. 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 different primary antibodies (rabbit anti-GDNF polyclonal antibody (1:200, Santa Cruz, Inc., USA), rabbit anti-GFRα-1 polyclonal antibody (1:1000, Sigma, USA), rabbit anti-SOM polyclonal antibody (1:1000, Santa Cruz, Inc., 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 with positive immunoreactivity were counted for each section and percentages of immunoreactive neurons were calculated. The total number of neurons is calculated by neurons' profile. For each animal, nine sections were taken (three of each L4/5/6 DRG) from the unilateral DRG and the mean of the percentages of immunoreactive neurons was calculated. For quantification of spinal sections, the total density of positive signals in spinal dorsal horn per section was measured. For each animal, 10 sections were randomly taken from the L4 to L6 spinal cord segments and the mean value was counted. The investigator responsible for image analysis was blind to the experimental condition of each rat.

2.5. Western blot analysis

Rats were sacrificed with an overdose of urethane (1.5 g/kg, i.p.) and the L4/5/6 DRG were collected in dry ice and stored at −70 °C until assayed. Each assay sample, containing the pooled unilateral L4–L6 DRG from one rat, 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 phenylmethylsulfonyl fluoride) at 4 °C. Supernatant after 12 000 r.p.m. centrifugation at 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-GDNF polyclonal antibody (1:1000, Santa Cruz, Inc., USA) or 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, U.K.).

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

Rats were sacrificed with an overdose of urethane (1.5 g/kg, i.p.) and the L4/5/6 DRG 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. RNA and oligo (dT)18 primers (0.5 μg/μl) were first denatured for 5 min at 65 °C, then 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 U of SuperScript II reverse transcriptase. The sequences of primers were as follows: GDNF antisense: 5′-TTTGTCGTACATTGTCTCGG-3′, sense: 5′-GACTCTAAGATGAAGTTATGG-3′ (L15305); GFRα-1 antisense: 5′-ATTGGCACAGTCATGACTCCCAAC-3′, sense: 5′-GAGGAGCAGCCATTGATTTTGTGG-3′ (U59486) (Stover et al., 2000); ppSOM antisense: 5′-CTAACAGGATGTGAATGTCTTC-3′, sense: 5′-ATGCTGTCCTGCCGTCTCCAGT-3′ (J00787) (Elliott et al., 1998); and β-actin antisense: 5′-TAACGCAACTAAGTCATAGT-3′, sense: 5′-CACCATGTACCCTGGCATTG-3′. The primers were synthesized and purified by Shanghai Institute of Biochemistry, Chinese Academy of Science. One microlitre 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 3 U of Pfu 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 the primer specific annealing temperature (60 °C for GFRα-1, 58 °C for GDNF, and 58 °C for ppSOM), 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, U.K.). The PCR semi-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 GDNF/GFRα-1/ppSOM bands to that of β-actin band.

2.7. ODN and intrathecal administration

Knockdown 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′ (Wiesenhofer et al., 2003). Another 15-mer ODN with four mismatched bases (5′-TAGAGACTAGGTGCC-3′) was used as the control. These two kinds of ODN were fully phosphorothioated. They were used at a dose of 30 μg dissolved in 5 μl of nuclease-free NS per injection per rat 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 (Yaksh and Rudy, 1976; Lai et al., 2002) for ODN administration. 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 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 deficit 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.

2.8. 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. Expression change of GDNF in the ipsilateral DRG after CCI

GDNF labeling was found in some of the L4–L6 DRG cells. Most of these cells were of small to intermediate size. Quantification analysis revealed that the number of GDNF-immunoreactive (GDNF-ir) neurons in ipsilateral DRG was enhanced significantly 1 day after CCI and the enhancement maintained until 4 weeks later. The climax of expression change happened 14 days after CCI (Fig. 1A–D). This result was further validated by Western blot analysis. A single protein band of the expected size (~35 kDa) for GDNF was detected by Western blot with the GDNF-specific primary antibody (Fig. 1E). In addition, no band was detected when the primary antibody was omitted (data not shown). These results showed that expression of GDNF in the ipsilateral DRG was increased after CCI surgery and reached the climax 14 days after CCI (Fig. 1F). As shown in Fig. 1G, an expected 482-bp PCR product was obtained by RT-PCR analysis. Semi-quantification showed that GDNF mRNA level in ipsilateral DRG was increased after CCI (Fig. 1H). There was no significant change in GDNF protein or mRNA levels of the contralateral side (data not shown).

3.2. Expression change of GFRα-1 in the ipsilateral DRG after CCI

In DRG, cells of all size showed GFRα-1 labeling. Quantification analysis revealed that the number of GFRα-1-immunoreactive (GFRα-1-ir) neurons in ipsilateral DRG was enhanced significantly 1 day after CCI and the enhancement maintained until 4 weeks later (Fig. 2A–D). This result was further validated by Western blot analysis. With a single band (~58 kDa, coincident with the known molecular weight of GFRα-1) detectable as immunoreactive GFRα-1, Western blot analysis demonstrated an increased expression of GFRα-1 after CCI surgery (Fig. 2E, F). RT-PCR analysis obtained an expected 444-bp product for GFRα-1 mRNA (Fig. 2G) and the results showed that GFRα-1 mRNA level in ipsilateral DRG was increased after CCI (Fig. 2H). There was no significant change in GFRα-1 protein or mRNA levels of the contralateral side (data not shown).

3.3. Effect of antisense ODN treatment on the expression of GFRα-1 in DRG

Immunostaining of GFRα-1 in DRG sections was shown in Fig. 3A–C and the number of GFRα-1-ir neurons was counted. As shown in Fig. 3D, the number of GFRα-1-ir neurons in DRGs was significantly reduced by antisense ODN treatment. A single band (~58 kDa) was detectable in Western blot analysis as immunoreactive GFRα-1 (Fig. 3E). Density analysis showed that the 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 (Fig. 3F). RT-PCR analysis obtained an expected 444-bp product for GFRα-1 mRNA (Fig. 3G). Semi-quantitative 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 (Fig. 3H). These results of the above different analyses conformably showed that the expression of GFRα-1 in DRG was significantly knocked down by antisense ODN treatment.

3.4. Effect of antisense ODN treatment on CCI-induced thermal hyperalgesia in rats

In experiment 1 (see Section 2.2), the ipsilateral PWL of the antisense ODN treatment group was decreased significantly compared with those of the mismatch ODN group and NS group. This effect lasted till 9th day after surgery (5 days after the end of ODN delivery) (Fig. 4A). There is no obvious difference in the contralateral PWL between three groups (data not shown).

In experiment 2 (see Section 2.2), the ipsilateral PWL of the antisense ODN treatment group was decreased significantly compared with those of the mismatch group and NS group from the 4th day after ODN delivery to 3 days after the end of ODN delivery (Fig. 4B). There is no obvious difference in the contralateral PWL between three groups (data not shown).

Identical administration of antisense ODN or mismatch ODN (30 μg per injection per rat, once daily for 5 days) to normal rats led to no significant change in PWL (data no shown).

3.5. Expression changes of SOM and ppSOM mRNA after GFRα-1 was knocked down by antisense ODN treatment

The change of ppSOM mRNA level was examined using RT-PCR. As shown in Fig. 5A, an expected 351-bp product for ppSOM mRNA was obtained and semi-quantitative analysis revealed that ppSOM mRNA level was significantly decreased after GFRα-1 expression was knocked down by antisense ODN treatment (Fig. 5B). Analysis of SOM immunoreaction in DRG sections showed a decrease in the number of SOM-immunoreactive (SOM-ir) neurons after GFRα-1 expression was knocked down (Fig. 5C–F). As shown in Fig. 5G–I, SOM-ir signals were mainly limited to the superficial layers of spinal dorsal horn and density analysis revealed that 'knock-down' of GFRα-1 expression resulted in a significant decrease of SOM expression in spinal dorsal horn (Fig. 5J).

4. Discussion

The present study found an increased expression of GDNF and its receptor GFRα-1 in the DRG of neuropathic pain rats at both protein and mRNA levels, consistent with most previous studies that the expression of GDNF and GFRα-1 was increased after nerve injury (Hammarberg et al., 1996; Hoke et al., 2000; Widenfalk et al., 2001; Nomura et al., 2002). In

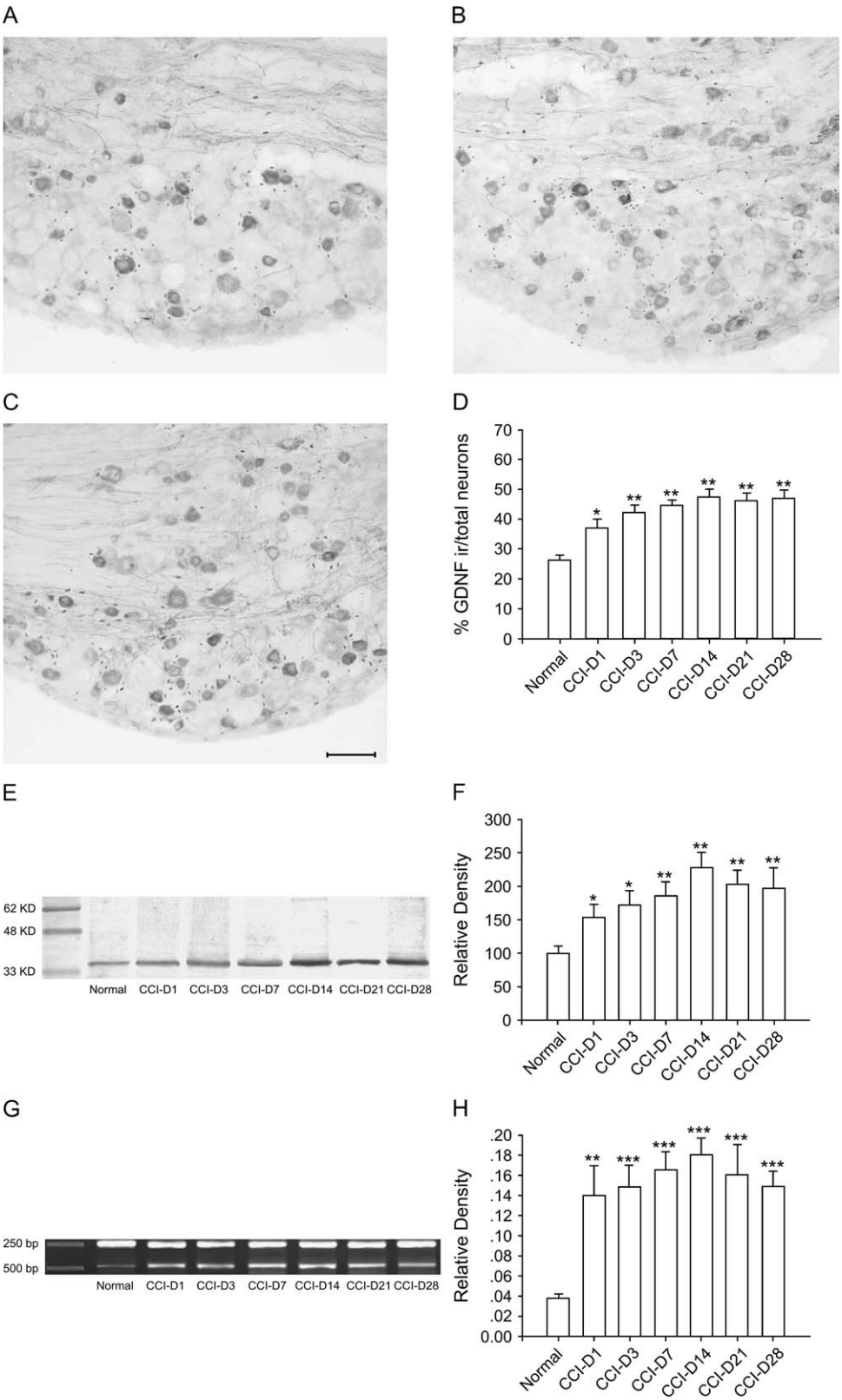

Fig. 1. Expression change of GDNF in ipsilateral DRG after CCI-induced neuropathic pain in rats. Images were shown for GDNF immunostaining in the ipsilateral L5 DRG of normal group (A), 1 day after CCI (B) and 14 days after CCI (C). The number of GDNF-ir neurons was expressed as a percentage of GDNF-ir neurons to total neurons (D). Western blot analysis detected expected size protein band of GDNF (E). The optical density was expressed as a percentage to that of the normal group sample (100%) (F). Expected size PCR products of GDNF were acquired (G) and the mRNA level was expressed as a ratio to that of corresponding β-actin (H). Data were represented as mean ± S.E.M. ($n = 6$ in each group at each time point). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. normal group. Scale bar = 100 μm.

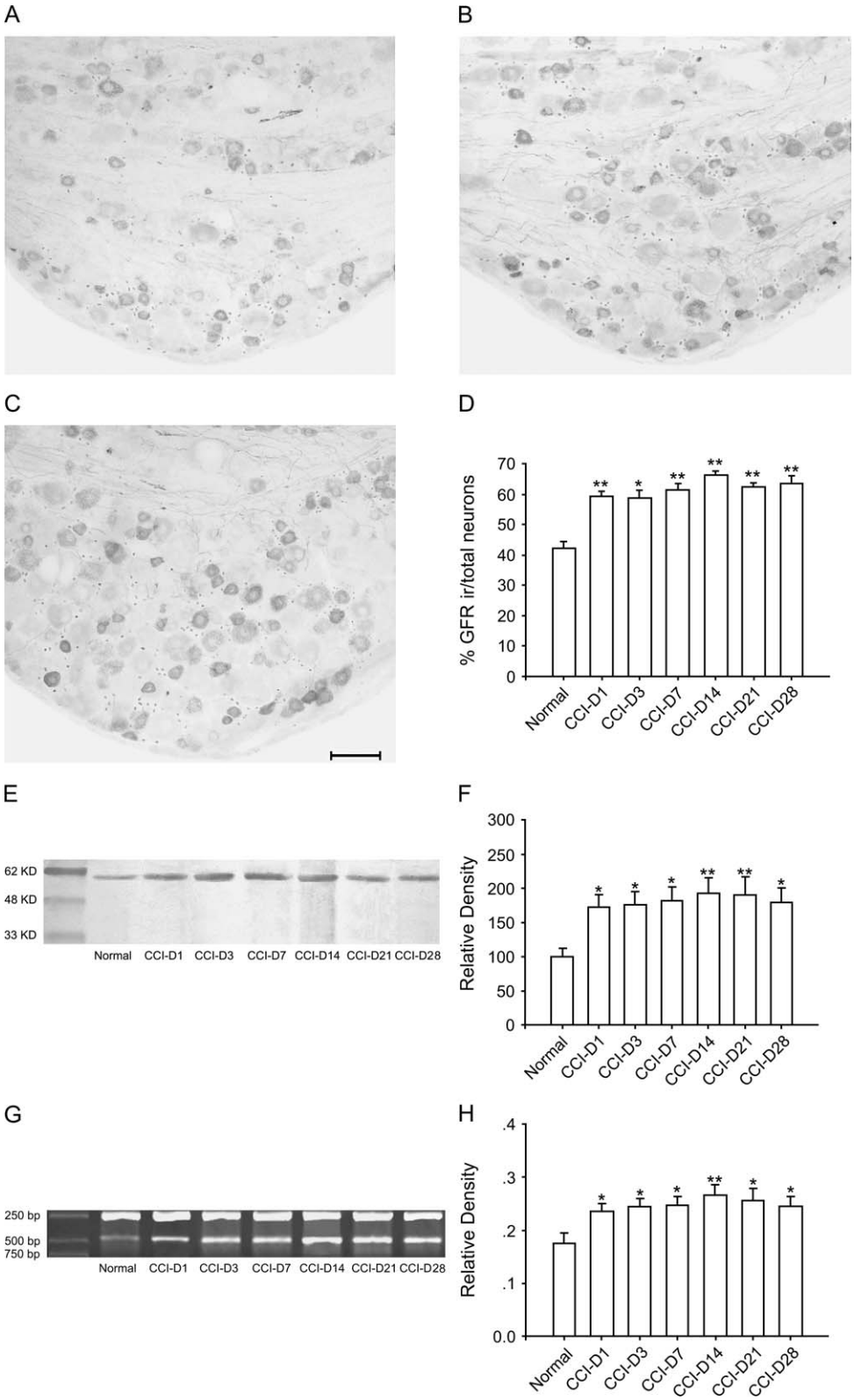

Fig. 2. Expression change of GFRα-1 in ipsilateral DRG after CCI-induced neuropathic pain in rats. Images were shown for GFRα-1 immunostaining in the ipsilateral L5 DRG of normal group (A), 1 day after CCI (B) and 14 days after CCI (C). The number of GFRα-1-ir neurons was expressed as a percentage of GFRα-1-ir neurons to total neurons (D). Western blot analysis detected expected size protein band of GFRα-1 (E). The optical density was expressed as a percentage to that of the normal group sample (100%) (F). Expected size PCR products of GFRα-1 were acquired (G) and the mRNA level was expressed as a ratio to that of corresponding β-actin (H). Data were represented as mean ± S.E.M. ($n = 6$ in each group at each time point). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. normal group. Scale bar = 100 μm.

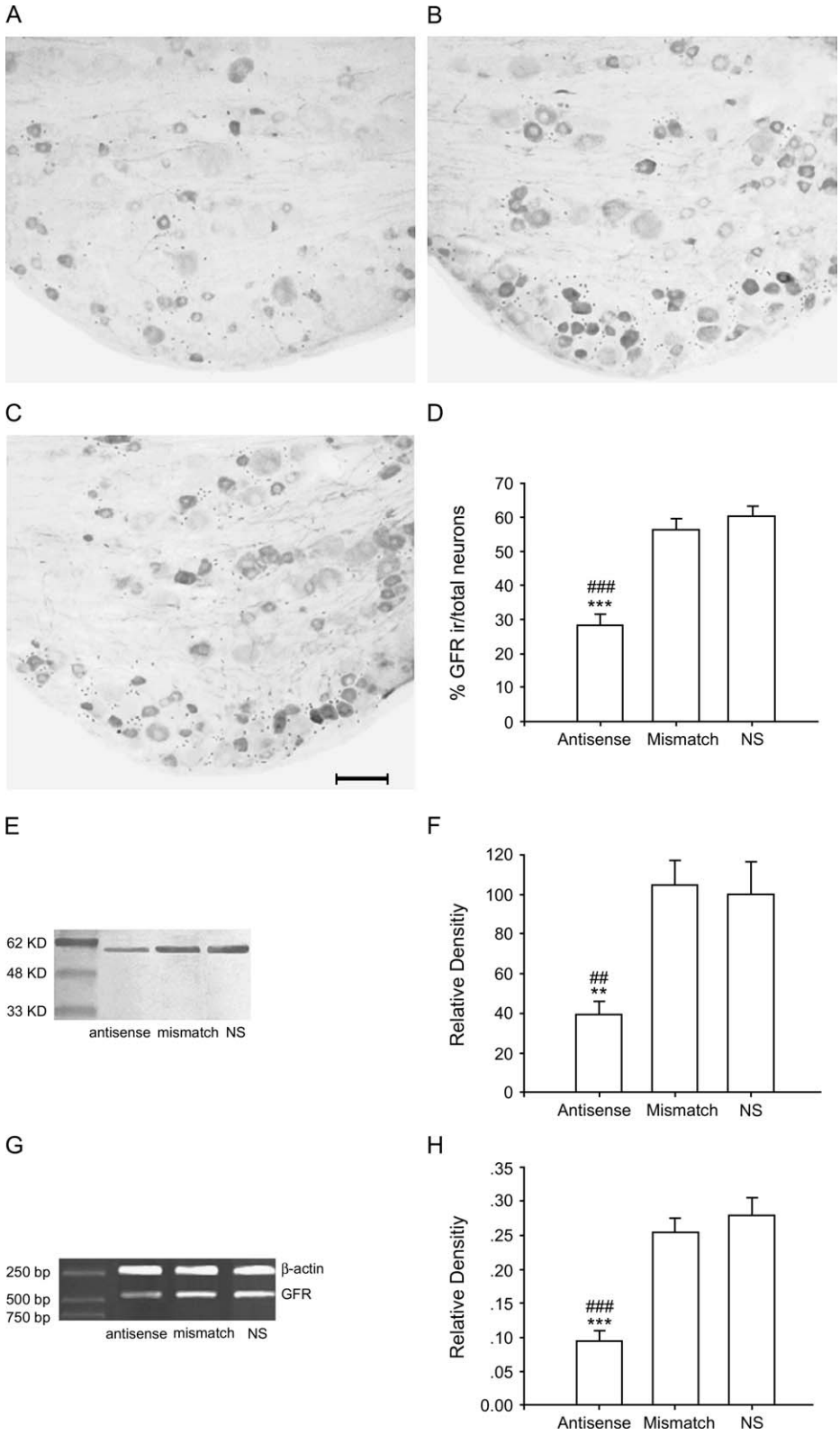

Fig. 3. Knockdown of GFRα-1 expression by intrathecal delivery of antisense ODN. Images were shown for GFRα-1 immunostaining in the ipsilateral L5 DRG of antisense ODN treatment group (A), mismatch ODN treatment group (B) and NS treatment group (C). The number of GFRα-1-ir neurons was expressed as a percentage of GFRα-1-ir neurons to total neurons (D). Western blot analysis detected a protein band of ~58 kDa, coincident with the known molecular weight of GFRα-1 (E). The optical densities of immunoblot bands were expressed as a percentage to that of the NS group sample (100%) (F). PCR products of expected size were acquired corresponding to GFRα-1 (G). The mRNA levels of different group were expressed as a ratio to that of corresponding β-actin (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.

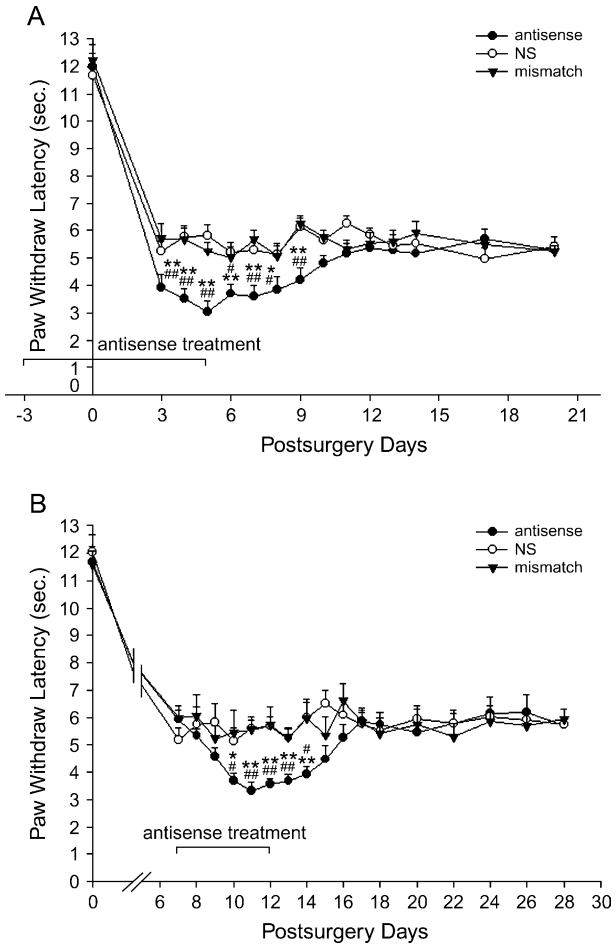

Fig. 4. Effect of antisense ODN treatment on CCI-induced thermal hyperalgesia in rats. In A, ODN was delivered at a dose of 30 μg per injection per rat once daily for 8 days (from 3 days before CCI surgery till 5 days after CCI surgery). In B, ODN was delivered at a dose of 30 μg per injection per rat once daily for 5 days since the 7th day after CCI surgery. Values are mean ± S.E.M. ($n = 12$ in each group in A; $n = 11$ in each group in B). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. NS group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. mismatch group.

contrast, another laboratory reported that expression of GDNF was decreased during neuropathic pain (Nagano et al., 2003; Takahashi et al., 2003). The difference might be associated with diversities between individual manipulators during experimental procedures especially CCI surgery operation, which might lead to different extent of injury to the sciatic nerve. Therefore, further investigation should be necessary.

In DRG, there is a lack or undetectable level of GDNF mRNA in the same neurons that contain substantial amounts of GDNF protein, while adjacent glial cells express GDNF mRNA and GDNF mRNA was readily up-regulated to substantial levels in Schwann cells after nerve injury. According to classical neurotrophic theory, a neuronal survival factor should be secreted by target cells, then be internalized, retrogradely transported and activate a signal transduction pathway that results in neuronal survival. However, GDNF was found to be bi-directionally transported in the peripheral axon (Russell et al., 2000). Previous studies discovered a high expression of GDNF mRNA in peripheral tissues and a retrograde transport of GDNF that was consistent with classical neurotrophic theory (Leitner et al., 1999). On the other hand, It was proved that glia-derived GDNF, which was produced by glial cells, could be internalized by DRG neurons and anterogradely transported in their axons (Rind and von Bartheld, 2002). Therefore, the increased expression of GDNF in DRG neurons found in the present study might come from three sources: (1) the glial cells of DRG, as shown in our results that the expression of GDNF mRNA in DRG was increased during neuropathic pain; (2) Schwann cells in the sciatic nerve (we also found an increase of GDNF mRNA in the sciatic nerve (data not shown)); and (3) the peripheral target tissues of the sciatic nerve.

The up-regulation of GDNF and GFRα-1 expression was persistent along with neuropathic pain, which suggested a close relationship between GDNF and GFRα-1 system and neuropathic pain. The most concise and convincing way to test whether a signaling protein molecule participates in a given physiological or pathological activity 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 (Agrawal and Zhao, 1988; Sproat, 1995; Akhtar and Agrawal, 1997), 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 knocked down by intrathecal antisense ODN treatment (Honore et al., 2002; Lai et al., 2002). Therefore, in order to confirm the role of GDNF system in neuropathic pain, antisense ODN was used in the present study to result in a 'knock-down' of GDNF high-affinity receptor GFRα-1. Our preliminary data showed that 3-day continuous injection of antisense ODN at a dose described in the present study could induce significant down-regulation of GFRα-1. Therefore, in experiment 1, antisense ODN was administered 3 days before CCI surgery to address the role of GDNF system in the genesis of neuropathic pain. In experiment 2, rats received antisense ODN delivery after neuropathic pain was firmly established to determine the role of GDNF system in the maintenance of neuropathic pain. In both conditions, thermal hyperalgesia of neuropathic pain rats was significantly aggravated after antisense ODN treatment compared with NS and mismatch ODN group, indicating that the up-regulation of GDNF and GFRα-1 system might play an anti-hyperalgesic role in neuropathic pain. Furthermore, a series of studies provided compelling evidence that intrathecally administered GDNF as well as elevated expression of GDNF could exert potent analgesic effect on neuropathic pain of rats (Boucher et al., 2000; Hao et al., 2003; Wang et al., 2003) and these studies also gave confirmation to the conclusion of the present study.

Some of the GDNF-dependent sensory neurons of DRG express somatostatin, an endogenous non-opioid neuropeptide that has an inhibitory effect on the nerve system. SOM depresses the firing of dorsal horn neurons activated by noxious stimulation, produces analgesia in animals and humans, and is released in the spinal cord following noxious stimulation of peripheral nerves (Murase et al., 1982; Sandkuhler et al.,

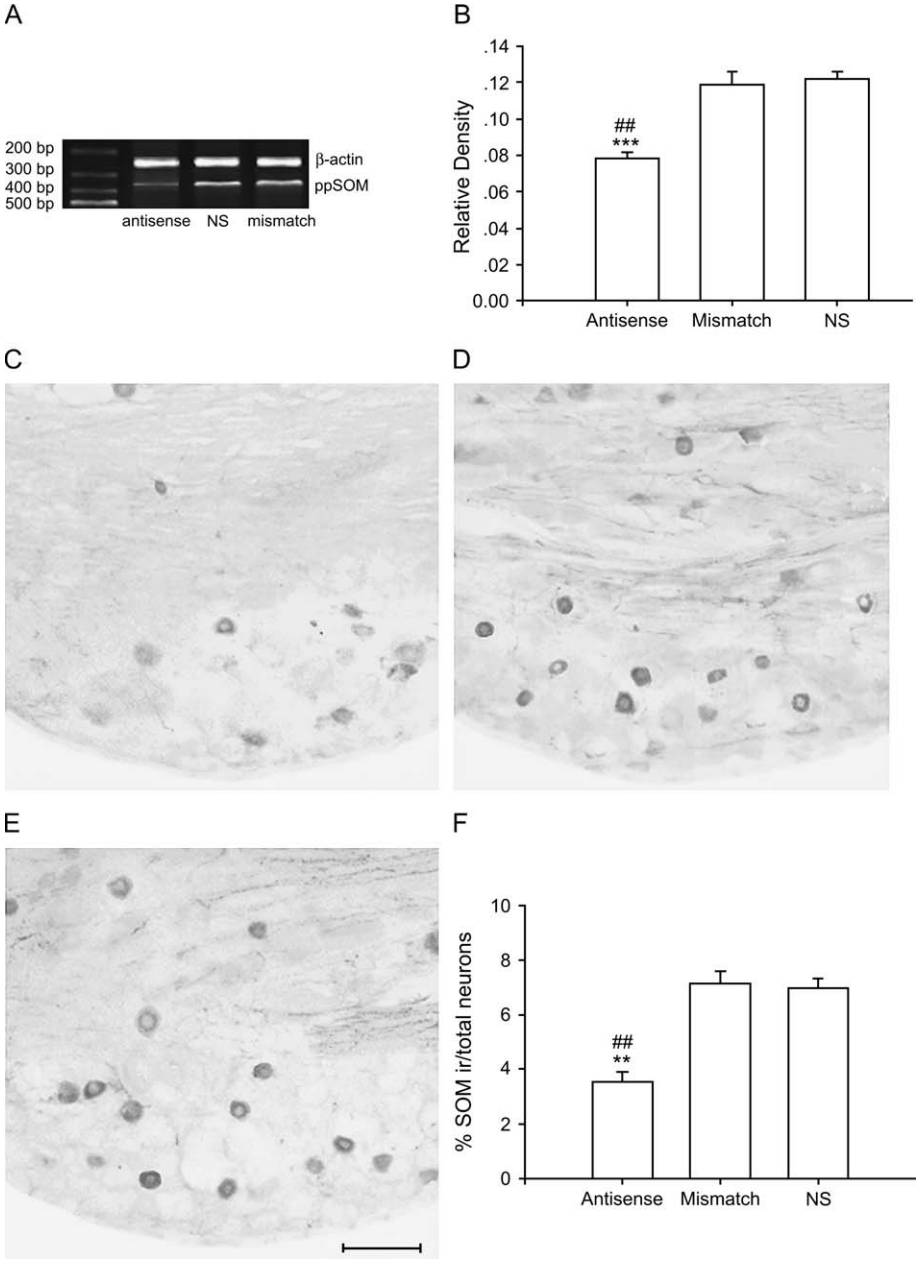

Fig. 5. Expression changes of SOM and ppSOM mRNA after GFRα-1 expression were knocked down by antisense ODN treatment. Expected size PCR products of ppSOM were acquired (A) and the mRNA level was expressed as a ratio to that of corresponding β-actin (B). Images were shown for SOM immunostaining in the ipsilateral L5 DRG of antisense ODN treatment group (C), mismatch ODN treatment group (D) and NS treatment group (E). The number of GFRα-1-ir neurons was expressed as a percentage of SOM-ir neurons to total neurons (F). (G–I) shows SOM immunoreaction in spinal dorsal horn of antisense, NS and mismatch group, respectively. SOM-ir signals were mainly limited to the superficial layers of spinal dorsal horn. The optical density was expressed as a percentage to that of the NS group sample (100%). 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.

1990). GDNF has been reported to increase activity-induced release of SOM in the dorsal horn, and prolonged intrathecal administration of GDNF to adult rats increased the number of small-diameter cells in the DRG that can express SOM (Charbel et al., 2001). The present study found a decrease in expression of SOM in DRG and spinal dorsal horn as well as a decrease in ppSOM mRNA level in DRG after GFRα-1 was knocked down by antisense ODN treatment. This indicated that endogenous GDNF might be a factor that contributes to the maintenance of the synthesis of SOM, which gave an explanation to the results of the present study that hyperalgesia of neuropathic pain rats was aggravated by antisense ODN treatment. The effect of GDNF on endogenous SOM may be a mechanism that underlines the role of GDNF system in neuropathic pain. However, since GDNF was reported to protect against multiple phenotypic changes induced by peripheral nerve injury, there may also be other mechanisms by which GDNF could be involved in the modulation of neuropathic pain. It has been shown that GDNF could prevent A-fibre sprouting into lamina II and the latter has been suggested as

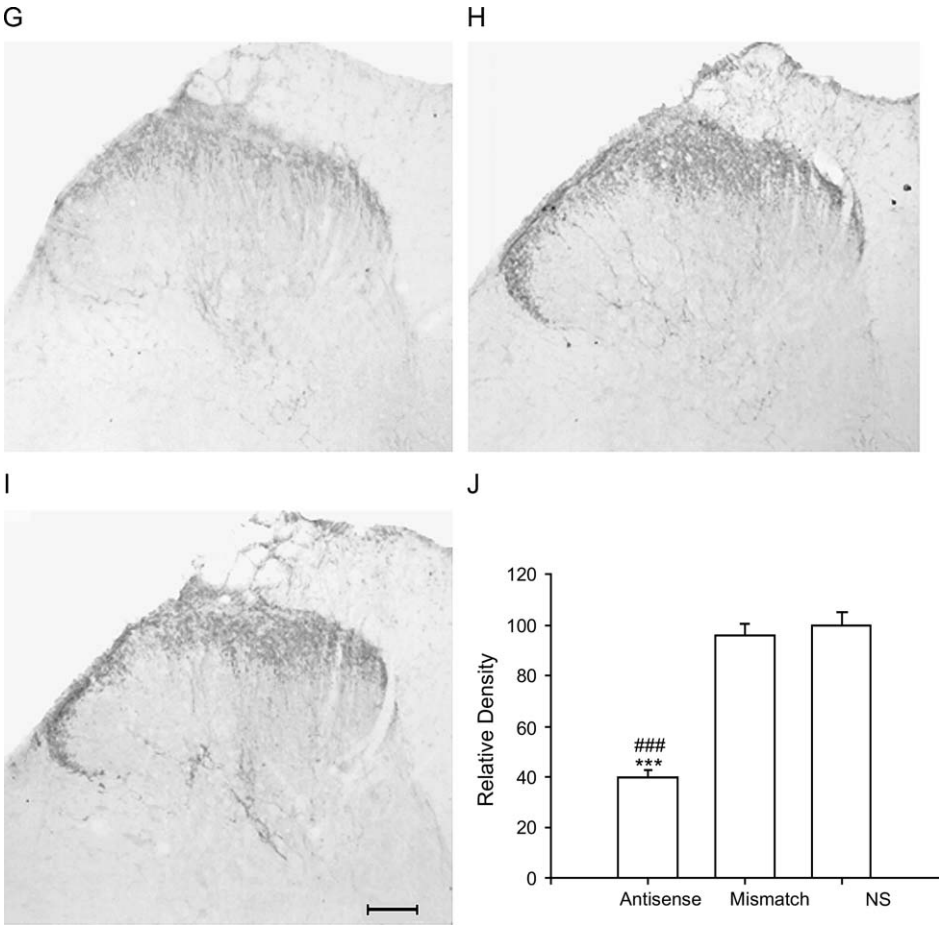

Fig. 5 (*continued*)

an underlying mechanism of neuropathic pain. GDNF has also been demonstrated to partially reverse axotomy-induced increases in a sodium channel subunit, Nav1.3, resulting in partial reversal of axotomy-induced changes in repriming kinetics of the sodium current. The ability of GDNF to block the expression of NaV1.3 in the injured nerve has been proposed to be a critical mechanism of GDNF's antiallodynic and anti-hyperalgesic effects (Boucher et al., 2000; Leffler et al., 2002). GDNF treatment also blocked spontaneous ectopic discharges in large-diameter myelinated afferent fibres induced by nerve injury. A GDNF-induced suppression of NPY production that is increased during neuropathic pain may also be causal to GDNF's ability to block tactile hypersensitivity (Wang et al., 2003).

The present study also observed a down-regulation of GFRα-1 expression in the spinal cord following antisense ODN treatment (data not shown). Since the expression of GDNF could also be detected in the superficial layer of spinal dorsal horn, it may be hard to determine the definite acting site of GDNF. Most possibly, GDNF may play a role both peripherally and centrally.

The present study addressed the role of endogenous GDNF and GFRα-1 system in neuropathic pain. The results provide a rational basis for a new therapeutic strategy for neuropathic pain by potentiating the function of GDNF and GFRα-1 system.

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