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# REGULATION OF PROINFLAMMATORY CYTOKINES GENE EXPRESSION BY NOCICEPTIN/ORPHANIN FQ IN THE SPINAL CORD AND THE CULTURED ASTROCYTES

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**Abstract—Peripheral inflammation induces central sensitization characterized by the development of allodynia and hyperalgesia to thermal stimuli. Recent evidence suggests that activation of glial cells and a subsequent increase in proinflammatory cytokines contribute to the development of behavioral hypersensitivity after nerve injury or peripheral inflammation. The neuropeptide nociceptin/orphanin FQ (N/OFQ), the endogenous agonist of the N/OFQ peptide receptor (ORL1 receptor), has been demonstrated to play an important role in modulation of nociceptive signals. In the present study, we investigated: (1) astrocyte activation and proinflammatory cytokine expression at the lumbar spinal cord following intraplantar administration of complete Freund's adjuvant (CFA) in rats; (2) the mechanism of N/OFQ on nociception modulation, the relationship between N/OFQ and cytokines in the rat CNS *in vivo* and *in vitro*. The results showed: (1) CFA-induced peripheral inflammation evoked robust astrocyte activation and proinflammatory cytokines spinally; (2) down-regulation of cytokine mRNA transcripts by intrathecal administration of N/OFQ, the effects produced by N/OFQ were abolished by combination with ORL1 receptor-specific antagonist [Nphe$^1$]N/OFQ(1–13)NH$_2$; (3) ORL1 receptor was expressed on astrocytes of rat spinal cord; (4) cytokine gene expression was inhibited in astrocyte cultures exposed to N/OFQ, the inhibiting effects of N/OFQ were significantly blocked by [Nphe$^1$]N/OFQ(1–13)NH$_2$. The present data demonstrated that astrocyte activation and enhanced cytokine expression at the CNS had a role in eliciting behavioral hypersensitivity; the anti-nociception function of N/OFQ might be dependent on cytokines derived from astrocytes, the effects were attributable to the ORL1 receptor pathway.** 

**Key words: astrocytes, proinflammatory cytokines, N/OFQ, ORL1 receptor, antinociception, peripheral inflammation.**

From sensation to perception, pain and pain modulation are classically viewed as being mediated solely by neurons. Glia in the CNS have been presumed to have no role because they lack axons and so have not been thought of in terms of cell-to-cell signaling. For pain, this view is dramatically changing. New research implicates spinal cord glia (both microglia and astrocytes) as key players in the initiation and maintenance of persistent pain states (DeLeo and Yezierski, 2001; Watkins et al., 2001a,b; Raghavendra and DeLeo, 2003). Potential activation of microglia and astrocytes was observed at the lumbar spinal cord; the activation is involved in hypersensitivity and persistent pain induced by inflammation and damage to peripheral tissues, peripheral nerves, spinal nerves and spinal cord (Watkins et al., 2001b; Sweitzer et al., 1999; Raghavendra et al., 2003a,b). Upon activation, a variety of algesic substances is released by glia that may potentiate pain transmission by neurons (Raghavendra and DeLeo, 2003; Sommer, 2003; Watkins and Maier, 2003). Of these glial products, proinflammatory cytokines, such as interleukin-1$\beta$ (IL-1$\beta$), interleukin-6 (IL-6) and tumor necrosis factor-$\alpha$ (TNF-$\alpha$), have been demonstrated to be common mediators of allodynia and hyperalgesia (Sommer and Kress, 2004; Raghavendra and DeLeo, 2003; Sommer, 2003; Watkins and Maier, 2003; Boddeke, 2001).

Although the role of astrocytes and cytokines in the persistent pain states has been reported and reviewed (DeLeo and Yezierski, 2001; Watkins et al., 2001b; Watkins and Maier, 2003), few investigations have well shown the time course of its activation in the CNS in different animal models of persistent pain.

Discovery of the opioid-receptor-like (ORL1) receptor and its endogenous ligand nociceptin/orphanin FQ (N/OFQ) has led to a new understanding of the endogenous pain modulation system (Meunier et al., 1995; Reinscheid et al., 1995). N/OFQ transcripts and the precursor protein for nociceptin are highly expressed in the dorsal horn of the spinal cord and medulla, particularly in the superficial laminae (Neal et al., 1999b). In addition, ORL1 receptor and its mRNA are densely distributed in the spinal dorsal horn (Bunzow et al., 1994; Mollereau et al., 1994; Neal et al., 1999a). The dorsal horn of the spinal cord is an important site for nociceptive transmission. Therefore, the anatomical distribution of N/OFQ and ORL1 receptor has been related to the modulation of nociceptive signals. The idea was further highlighted by our work concentrated on directly evaluating the nociception modulation of N/OFQ by demonstrating its reversal of a hyperalgesic response elicited by peripheral inflammation following intrathecal (i.t.) administration (Fu et al., 2006). Furthermore, it has recently been demonstrated that ORL1 receptor was ex-

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*Abbreviations:* ANOVA, analysis of variance; CFA, complete Freund's adjuvant; DAB, 3,3-diaminobenzidine; GFAP, glial fibrillary acid protein; IgG, immunoglobulin G; IL-1$\beta$, interleukin-1$\beta$; IL-6, interleukin-6; i.t., intrathecal; LPS, lipopolysaccharide; NGST, normal goat serum in 0.01 M PBS with 0.3% Triton-X 100; N/OFQ, nociceptin/orphanin FQ; ORL1, opioid-receptor-like; PB, phosphate buffer; $PGE_2$, prostaglandin E2; PWL, paw withdrawal latency; RT-PCR, reverse transcription–polymerase chain reaction; TNF-$\alpha$, tumor necrosis factor-$\alpha$.

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pressed on astrocytes of rat brain (Zhao et al., 2002), indicating that N/OFQ might influence cytokines function via the ORL1 receptor pathway to exert its effects. However, it has not been established whether nociception modulation of N/OFQ is associated with cytokines in the CNS.

The biological activities of N/OFQ, including pain modulation, can be better elucidated using a selective ORL1 receptor antagonist. Several potential ORL1 antagonists have been identified using *in vitro* studies but only a peptidil compound, [Nphe$^1$]N/OFQ(1–13)$NH_2$, has demonstrated a competitive antagonist profile in a variety of preparations, essentially devoid of any agonist activity, and it is highly selective for ORL1 receptor over the mu-, delta- and kappa-opioid peptide, receptors (Calo et al., 2000a). Therefore, as a member of the opioid family, it would be important using [Nphe$^1$]N/OFQ(1–13)$NH_2$ as selective antagonist to study the detailed role of N/OFQ in pathophysiology.

Thus, the present study was designed to characterize the time course of activation of astrocytes and cytokines in the lumbar spinal cord of rats following intraplantar administration of complete Freund's adjuvant (CFA) and investigate the effect of N/OFQ on expression of proinflammatory cytokines (IL-1$\beta$, IL-6 and TNF-$\alpha$) gene expression *in vitro* and *in vivo* in order to further elucidate the central mechanism by which N/OFQ modulates nociception. We aimed to establish: (1) that the expression of protein for astrocytic marker (glial fibrillary acid protein, GFAP) and mRNA for cytokines was characterized with respect to the development of thermal sensitivity during the acute (4 h), subacute (day 4) and chronic (10, 14 days) phases of CFA-induced peripheral inflammation; (2) whether ORL1 receptor is expressed on astrocytes of rat spinal cord at mRNA and protein levels; (3) the effect of N/OFQ on the gene expression of IL-1$\beta$, IL-6 and TNF-$\alpha$ in spinal cord and in spinal astrocyte culture.

## EXPERIMENTAL PROCEDURES

### Animals

Adult male Sprague–Dawley rats (weighing 200–220 g, from Experimental Animal Center, Shanghai Medical College, Fudan University, Shanghai, China) were allowed to acclimate for 1 week and maintained at a room temperature of 22±2 °C and a 12-h light/dark cycle with free access to food and water. The animal protocols were conducted according to the Animals Care and Use Committee of Shanghai Medical College, Fudan University, and conformed to the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983). Every effort was made to minimize the number of animals used and their suffering.

### Induction of chronic inflammation

As previously described, chronic inflammation was induced by an s.c. injection of 100 $\mu$l CFA (Sigma, St. Louis, MO, USA), which was suspended in a 1:1 oil/saline emulsion and contained 40 $\mu$g *Mycobacterium tuberculosis*, in the plantar surface of the left hind paws using a 25-gauge hypodermic needle (Stein et al., 1988) under brief isoflurane anesthesia. The inflammation, manifesting as redness, edema and hyper-responsiveness to noxious stimuli, was limited to the injected paw, appeared shortly after the injection, and lasted about 2 weeks. Hyperalgesia was determined by a decrease in paw withdrawal latency (PWL) to a noxious thermal stimulus. The rats serving as the control group were injected with 100 $\mu$l saline to the left hind paw.

### Behavioral test

Rats were tested for hind paw thermal hyperalgesia by a method developed previously (Hargreaves et al., 1988). Briefly, the rats were placed under a clear plastic chamber on the glass surface of the Model 390 paw stimulator analgesia meter (IITC/Life Science Instruments, Woodland Hills, CA, USA) and allowed to acclimatize for 30 min. The radiant light focused onto the plantar surface of each hind paw. The intensity of the thermal stimulus was adjusted to derive an average baseline PWL of approximately 10.0 s in naive animals. A cutoff time of 20 s for the stimulation was used to prevent tissue damage.

Hyperalgesia score was established prior to CFA injection and 24 h following injection of CFA for each rat by averaging the latency of four tests with a 10-min interval between each test according to the formula:

$$\text{Hyperalgesia score (s)} = \text{PWL of inflammatory side} - \text{PWL of normal side}$$

The time points of analysis were selected as day 0 (i.e. prior to intraplantar injection), 4 h, 4, 10 and 14 days after CFA or saline injection, which were chosen to represent various phases of inflammation. At each time point, eight animals of each group were used for every analysis.

### I.t. injection technique

Under pentobarbital sodium anesthesia (40 mg/kg i.p.), the rats were prepared for i.t. injection and allowed to recover for 7 days prior to induction of hyperalgesia. A chronic catheter was implanted into the spinal cord of rats in order to administer compounds locally (Storkson et al., 1996). Before the introduction of chronic inflammation, a 7.5-cm length of PE-10 tube flushed with sterilized saline was inserted through the gap between vertebrae L3 and L4 and extended to the subarachnoid space of the lumbar enlargement. The external end of the tube was passed subdermally and secured to the back of the neck where an incision had been made to allow exit. After completion of the surgical operation, 10 $\mu$l of lidocaine (50 mg/ml) was administered in order to confirm that the cannula was in the suitable position. Lidocaine usually induced paralysis of hind paws when injected into the lumbar enlargement; if paralysis did not occur within 5 min, the animal was excluded from the study. Rats were used at day 4 following the injection of CFA or saline. N/OFQ (Tocris, Balwin, MO, USA) and [Nphe$^1$]N/OFQ(1–13)$NH_2$ (a selective ORL1 receptor antagonist, Tocris) were delivered via the catheter in a volume of 10 $\mu$l followed by a flush with 10 $\mu$l of saline. Rats from the control group were s.c. injection of 100 $\mu$l saline with i.t. injection of 10 $\mu$l saline.

### Astrocyte cultures and cell treatment

Primary spinal cord astrocyte cultures were prepared from 1- to 2-day-old rat pups according to the method described previously (Saneto and de Vellis, 1987). Briefly, spinal cords were dissociated in 0.25% trypsin (Gibco BRL, Carlsbad, CA, USA) for 30 min at 37 °C. Cells were collected by centrifugation, filtered through an 80 $\mu$m mesh cell dissociation sieve, and plated at a density of $1.5\times10^6$ cells per 25 cm$^2$ in a flask (Nunc, Naperville, IL, USA) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, Hepes (3.6 g/l), penicillin (100 IU/ml), and streptomycin (100 $\mu$g/ml). When confluent, cultures were shaken for 48 h at 250 r.p.m. at 37 °C, incubated for another 48 h with 10 $\mu$M cytosine arabinoside, and then amplified to $2\times10^4$ cells/cm$^2$ in a 35 mm Petri dish or 24-well plates (Nunc). The astrocyte

monolayers were >98% pure as determined by GFAP immunoreactivity, and were devoid of OX42-positive microglial cells.

Prior to experimental treatments, cultures of astrocytes were passaged twice and the cells were allowed to reach 90% confluence, when lipopolysaccharide (LPS, *Escherichia coli*, serotype O111:B4, Sigma), or N/OFQ or [Nphe$^1$]N/OFQ(1–13)$NH_2$, a selective ORL1 receptor antagonist, was added.

## Immunohistochemistry

For the study of GFAP immunoreactivity, the time points of analysis were selected as 4 h, and 4, 10 and 14 days after CFA injection. At each time point, six animals of each group were used for every analysis. Rats were given an overdose of urethane (1.5 g/kg, i.p.) and surgery proceeded with sternotomy, transcardiac aortic needle cannulation, and perfusion with 200 ml of normal saline followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Spinal cords were then removed and postfixed in fixative solution for 4 h at 4 °C. After immersion in PB containing 30% sucrose for 24–48 h at 4 °C for cryoprotection, the L4/5/6 segments of spinal cord were sectioned at 30 $\mu$m thickness on a cryostat and collected in cryoprotectant solution (0.05 M PB, 30% sucrose, 30% ethylene glycol) and then stored at −20 °C until use. The L4/5/6 segments of spinal cord were identified by identification of the lumbar enlargement and nerve roots. Free-floating tissue sections were processed for GFAP immunoreactivity by the avidin–biotin method. Sections were removed from storage at −20 °C and placed directly into 0.1 M PB for 10 min. Following 3×15 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). The sections were then incubated in rabbit anti-GFAP polyclonal antibody (1:10,000, Dako, Carpinteria, CA, 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, USA) for 1 h at room temperature, washed three times in 1% NGST and incubated for 1 h in avidin–biotin–peroxidase complex (1:100, Vector Laboratories) at room temperature. Finally, the sections were washed three times in 0.01 M PBS, and the GFAP-like immunoreactive product was 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, air dried, dehydrated in alcohol in a graded manner, cleared in xylenes and coverslipped. 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. The GFAP-immunoreactive astroglial cells were observed and analyzed using a Leica Q500IW image analysis system (Leica, Wetzlar, Germany). Five sections from the L4, L5 or L6 segments of the spinal cord of each rat were randomly selected. The superficial laminae (laminae I and II) on the side of the spinal cord ipsilateral to the site of inflammation were taken and the average of the number of positive cells in five slices was defined as the number of GFAP-immunoreactive astroglial cells. The investigator responsible for image analysis was blind to the experimental condition of each rat. At least three sections were used to determine scoring for each animal. Scoring was done according to (Colburn et al., 1997): +, basal staining; ++, moderate activation; and +++, intense activation.

## Tissue collection and preparation of whole-cell extracts for Western blot analysis

For determination of GFAP protein level, protein extracts were obtained from L4/5/6 segments of spinal cord according to the following protocol. The time points of analysis were selected as 4 h, and 4, 10 and 14 days after CFA injection. At each time point, six animals of each group were used for every analysis. Rats were killed with an overdose of urethane (1.5 g/kg, i.p.) and spinal cord tissues were collected in dry ice and stored at −70 °C until assayed. 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. Furthermore, to determine the expression of ORL1 receptor on primary cultures of astrocytes of rat spinal cord at protein level, whole-cell extracts were prepared by scraping the cells directly into 1× Laemmli buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, and 0.1% Bromphenol Blue). Cells were disrupted by brief sonication. Supernatant after 12,000 r.p.m. centrifugation for 10 min was used for Western blotting.

Samples (50 $\mu$g 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 rabbit anti-GFAP polyclonal antibody (1:5000, Dako) or anti-ORL1 receptor (KOR-3 (H-85) rabbit polyclonal antibody, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and $\beta$-actin (Actin H-196 rabbit polyclonal antibody, 1:200; Santa Cruz Biotechnology) to control for equal protein loading, diluted in TBST containing 5% BSA. Blots were washed extensively in TBST and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Biosciences, Little Chalfont, UK) in TBST/1.25% BSA for 1 h at room temperature. Immunoblots were developed by enhanced chemiluminescence ECL (Amersham Biosciences) and visualized on 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, Cambridge, UK).

## Extraction of tissue and cells for assay of reverse transcription–polymerase chain reaction (RT-PCR)

RT-PCR was performed to investigate the time course of proinflammatory cytokines expression in CFA-injected rats, the effect of N/OFQ on mRNA for proinflammatory cytokines (IL-1$\beta$, IL-6 and TNF-a) *in vitro* and *in vivo*, the expression of ORL1 receptor on astrocytes of rat spinal cord, and characterization of LPS-induced cytokines release from astrocytes. Total RNA was extracted from the lumbar enlargement of the spinal cord at the time points of 4 h, 4, 10 and 14 days post-CFA injection and day 4 when i.t. N/OFQ, also that was from untreated or treated astrocytes with LPS or N/OFQ or [Nphe$^1$]N/OFQ(1–13)$NH_2$ for various periods using trizol reagent as recommended by the manufacturer. RNA was further purified using the RNeasy kit according to the RNA clean-up protocol, and eluted in 50 $\mu$l of RNase-free distilled $H_2O$. The amount of RNA was measured spectrophotometrically; 1 $\mu$g of total RNA was used for the synthesis of the first strand of cDNA using the SuperScript reverse transcriptase. Briefly, RNA, oligo (dT)18 primers (0.5 $\mu$g /$\mu$l) 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 $\mu$l 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. Oligonucleotide primers used for RT-PCR amplification were designed according to the published sequences. Primer sequences that synthesized and purified by Shanghai Institute of Biochemistry, Chinese Academy of Science used for PCR were: IL-1$\beta$ (sense, 5′-CAGGATGAGGACATGAGCACC-3′; antisense, 5′-CTCTGCAGACTCAAACTCCAC-3′, 447 bp); IL-6 (sense, 5′-GACAAAGCCAGAGTCCTTCAGAGAG-3′; antisense, 5′-CTAGGTTTGCCGAGTAGATCCTC-3′, 227 bp); TNF-$\alpha$ (sense, 5′-ATGAGCACAGAAAGCATGATC-3′; antisense, 5′-TACAGGCTTGTCACTCGAATT-3′, 275 bp); $\beta$-actin (sense, 5′-GACATGGAGAAGATCTGGCACCACA-3′; antisense, 5′-ATCTCCTGCTCGAAGTCTAGAGCAA-3′, 440 bp); ORL1 receptor (sense, 5′-GTTCAAGGACTGGGTGTTCAGCCAGGTAGT-3′; antisense, 5′-TGCTGGCCGTGGTACTGTCTCAGAAGTCTT-3′,

258 bp). PCR reaction was performed as follows: denatured at 94 °C for 30 s, annealed at 60 °C for 1 min, with extension at 72 °C for 1 min. Twenty-nine cycles, 28 cycles and 40 cycles were used to amplify ORL1 receptor, IL-1$\beta$ and TNF-$\alpha$, IL-6, respectively. Ten microliters of each PCR production was electrophoresed in 1% agarose gel, visualized by ethidium bromide staining and scanned with ultraviolet transilluminator (GDS 8000, Gene Tools from Syngene software). The results were expressed as ratios of the intensity of the cytokines cDNA bands to that of $\beta$-actin band.

### Immunocytochemistry

Astrocyte cultures were grown on poly-lysine-coated glass coverslips. When the cells were adherent (growing overnight), they were fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 min at 4 °C. The cell walls were then perforated by treating with 0.1% polyoxyethylene sorbitane monolaurate (Sigma) for 5 min. To prevent unspecific binding, the preparation was incubated in blocking solution (10% bovine serum albumin in PBS) for 30 min. Primary incubation was done overnight at 4 °C with anti-ORL1 receptor (KOR-3 (H-85) rabbit polyclonal antibody, Santa Cruz Biotechnology) at a dilution of 1:200. Staining for ORL1 receptor was performed by use of DAKO EnVision+System Peroxidase (DAB) Rabbit Kit (Dako). Negative controls were conducted by excluding primary antibody in the procedure. Immunoreactivity was visualized with Leica Q500IW image analysis system.

### Statistical analysis

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

## RESULTS

### Time course of behavioral hypersensitivity

Following a peripheral administration of CFA into the left hind paw, animals showed evident signs of inflammation, which were assessed by localized erythema and an increase in the swelling of the paw, and caused a decrease in hyperalgesia score to noxious thermal stimuli. The hyperalgesia score for thermal stimuli was significantly different from the saline-treated rats. The hyperalgesia score to thermal stimuli decreased markedly at 4 h, and 4, 10 and 14 days in the CFA-treated rats. The hyperalgesia score to thermal stimuli in the saline-treated rats remained stable over the observation period (Fig. 1).

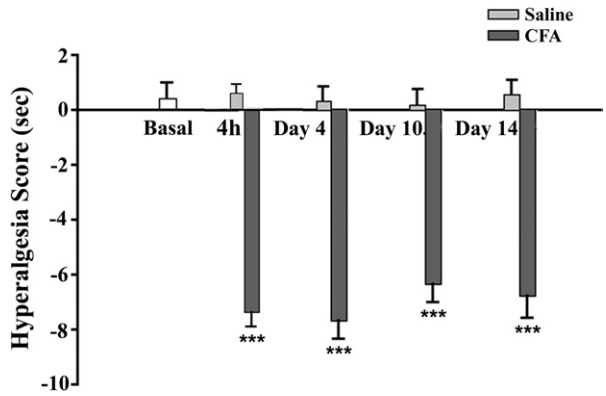



**Fig. 1.** Time courses of CFA-induced thermal hyperalgesia. The hyperalgesia score was expressed as the means±S.E.M. The nociceptive thresholds (s) were measured on day 0 (i.e. prior to intraplantar injection), 4 h, and 4, 10 and 14 days after intraplantar injection of CFA or saline. Statistical analysis by ANOVA indicated that during the observation period, the hyperalgesia score to thermal stimuli remained stable in the saline-treated rats ($n=8$); following a 100 $\mu$l injection of CFA into the left hind paw ($n=8$), its hyperalgesia score markedly decreased compared with control (saline) group. *** $P<0.001$ vs. control (saline) group.

**Table 1.** Immunohistochemistry results for astrocytic activation in the CNS during various phases of CFA-induced peripheral inflammation

| Treatment | L4/5/6 lumbar spinal cord | | | |
|---|---|---|---|---|
| | 4 h | d4 | d10 | d14 |
| Saline | + | + | + | + |
| CFA | + | ++ | +++ | +++ |

Scoring of GFAP positive cells is as follows [see Colburn et al. (1997) for representative scoring photomicrograph]: +, baseline staining; ++, mild to moderate staining; +++, intense staining.

### Time course of glial activation

Immunohistochemistry revealed that intraplantar administration of CFA produced a significant increase in the expression for the astroglial marker, GFAP, in the lumbar spinal cord after the subacute phase (day 4) of inflammation, compared with the control group (Table 1). As shown in Fig. 2 (in the dorsal horn of the lumbar spinal cord), GFAP-immunoreactive astroglial cells showing fine branches were sparsely distributed in the saline-treated control rats. At day 4 (subacute phase) after intraplantar CFA administration, a large number of GFAP-positive astroglial cells exhibited intense immunoreactivity and appeared hypertrophied with thick processes.

### Time course of GFAP protein level in CFA-injected rats

Quantification of protein by Western blot showed that a single protein band of the expected size (~50 kDa) for GFAP was detected with the GFAP-specific primary antibody (Fig. 3). In addition, no band was detected when the primary antibody was omitted (data not shown). The results showed that increased expression of GFAP level at the lumbar spinal cord in CFA-injected rats was significant after the subacute phase (day 4) of CFA-induced inflammation.

### Time course of proinflammatory cytokines expression and effect of N/OFQ on mRNA for proinflammatory cytokines in CFA-injected rats

RT-PCR showed the increased expression of IL-1$\beta$, IL-6 and TNF-$\alpha$ at the lumbar spinal cord was significant throughout the various phases of inflammation (Table 2).

In order to investigate the effect of N/OFQ on mRNA for proinflammatory cytokines, we chose i.t. administration with 15 nmol N/OFQ and 20 nmol [Nphe$^1$]nociceptin(1–13)$NH_2$. The samples were divided into five groups

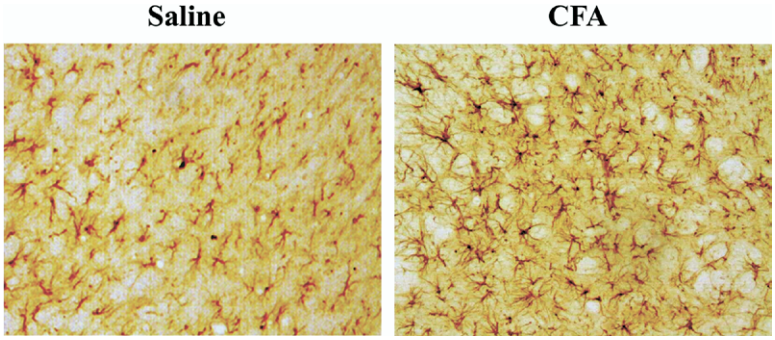



**Fig. 2.** Changes of expression of GFAP immunoreactivity in the dorsal horn of the spinal cord detected by immunohistochemistry in saline- and CFA-injected rats. Representative images were shown for GFAP immunostaining in the spinal dorsal horn of control (saline) group and CFA group at the time point of 4 days after the injection. As compared with control (saline) group, after intraplantar CFA administration, a large number of GFAP-positive astroglial cells exhibited intense immunoreactivity and appeared hypertrophia.

($n$=5/group): (1) control (2) CFA (3) CFA+N/OFQ (4) CFA+[Nphe$^1$]N/OFQ(1–13)$NH_2$ (5) CFA+N/OFQ+[Nphe$^1$]N/OFQ(1–13)$NH_2$. Table 3 showed that cytokines mRNA transcripts were expressed at low levels in the saline control group. However, these levels were significantly increased following CFA, and antagonized by treatment with N/OFQ. This effect was antagonized by co-treatment with an ORL1 receptor antagonist.

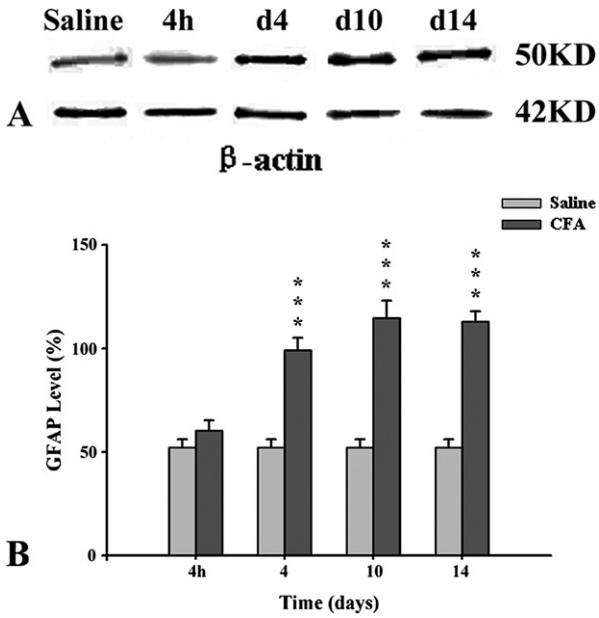



**Fig. 3.** Changes of GFAP protein level detected by Western blot in the dorsal horn of the spinal cord after saline or CFA injection. Images were shown for protein bands corresponding to GFAP in the spinal dorsal horn of control (saline) group and CFA group at the time point of 4 h, and 4, 10, and 14 days after the injection (A). (B) The immunoblot results of above time points were quantified and demonstrated. The protein level was expressed as a ratio to that of corresponding $\beta$-actin. Data were represented as mean±S.E.M. (n=6/group at each time point). GFAP protein level was significantly increased during various phases of inflammation. *** $P<0.001$ vs. control (saline) group.

### Immunocytochemistry

Micrographs of cultivated astrocytes showed that cells were stained well in the presence of primary antibody, compared with no staining of negative control (Fig. 4). This indicated that ORL1 receptor was present on cultured astrocytes of rat spinal cord.

### Detection of ORL1 receptor mRNA on astrocytes by RT-PCR

RT-PCR was performed to determine whether ORL1 receptor was expressed on astrocytes of rat spinal cord at mRNA level. As shown in Fig. 5A, the cDNA detected by gel electrophoresis indicated that mRNA corresponding to 258 bp was present. Blank control (no templates) contained no ORL1 receptor mRNA expression. These results demonstrated that cultured astrocytes expressed ORL1 receptor mRNA.

### Western blot for ORL1 receptor protein on astrocytes

To investigate whether the expression of ORL1 receptor mRNA correlated with protein expression, total ORL1 receptor proteins were measured by Western blot in membranes prepared from the astrocytes of rat spinal cord. A single protein band of the expected size (~40 kDa) for ORL1 receptor was detected in Western blot with the ORL1 receptor-specific primary antibody (Fig. 5B). Blank control (incubation medium) contained no ORL1 receptor immunoreactivity. The results indicated that cultured astrocytes possessed ORL1 receptor.

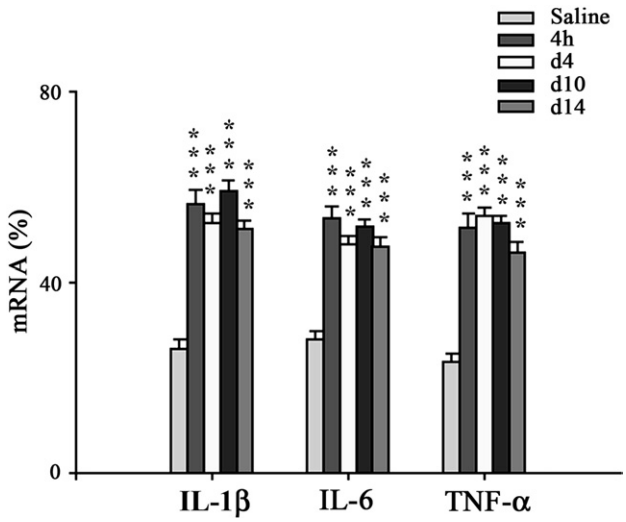
### Characterization of LPS-induced proinflammatory cytokine release and effect of N/OFQ on proinflammatory cytokine gene expression in cultured astrocytes

Primary cultures of rat astrocytes obtained from spinal cord of neonatal rats appeared as a monolayer of flat polygonal-shaped cells. They were identified by their immunoreactivity with anti-GFAP antiserum (data not shown). Then we performed RT-PCR analysis in order to identify the time course of proinflammatory cytokines

**Table 2.** Time course of upregulation of mRNA for proinflammatory cytokines in the spinal cord following CFA-induced peripheral inflammation

| Cytokine | L4/5/6 lumbar spinal cord | | | | |
|---|---|---|---|---|---|
| | Saline | 4 h | d4 | d10 | d14 |
| IL-1$\beta$ | 26.2±1.934 | 56.4±3.023*** | 52.6±1.806*** | 59.2±2.223*** | 51.2±1.855*** |
| IL-6 | 28.2±1.715 | 53.5±2.453*** | 48.0±1.789*** | 51.7±1.549*** | 47.6±1.965*** |
| TNF-$\alpha$ | 23.4±1.720 | 51.6±2.853*** | 54.0±1.732*** | 52.4±1.720*** | 46.4±1.990*** |

The mRNA was quantified by RT-PCR and was expressed as a ratio to that of corresponding $\beta$-actin (%). Values were fold increase over saline-injected control group ($n$=5/group). *** $P<0.001$ vs. control (saline) group.




(IL-1$\beta$, IL-6 and TNF-$\alpha$) gene expression after exposure to LPS. LPS was reported to be an effective stimulant of IL-1$\beta$ mRNA expression at a concentration of 30 mg/l (Zhao et al., 2002), IL-6 mRNA expression and TNF-$\alpha$ mRNA expression at a concentration of 100 ng/ml (Sawada et al., 1992; Kucher and Neary, 2005). Fig. 6 shows characterization of LPS-induced proinflammatory cytokines release. After exposure to 30 mg/l LPS, IL-1$\beta$ gene expression could be detected as early as 30 min, reached a maximum level at 2 h, and then gradually decreased. IL-6 mRNA expression was observed at 3 h after 100 ng/ml LPS stimulation in astrocytes, reached a maximum level at 5 h, and then slowly declined. Astrocyte cultures were treated with 100 ng/ml LPS for various times, significant TNF-$\alpha$ release was observed at 3 h and the maximum response occurred following 24 h treatment.

We next examined the effect of N/OFQ on IL-1$\beta$, IL-6 or TNF-$\alpha$ gene expression in cultured astrocytes, responses occurring 2 h, 5 h or 24 h after treatment with LPS, 100 nM N/OFQ or 100 nM ORL1 receptor antagonist were focused on, respectively. Cultured cells were divided into four groups: (1) LPS, (2) LPS +N/OFQ, (3) LPS +[Nphe$^1$]N/OFQ(1–13)NH$_2$, (4) LPS +N/OFQ +[Nphe$^1$]N/OFQ(1–13)NH$_2$ ($n$=6/group). As shown in Fig. 7, LPS-induced IL-1$\beta$, IL-6 or TNF-$\alpha$ release was attenuated by N/OFQ. The reduced proinflammatory cytokines gene expression was significantly reduced by [Nphe$^1$]N/OFQ(1–13)NH$_2$. These results indicated that the increased IL-1$\beta$, IL-6 and TNF-$\alpha$ gene expression induced by LPS could be inhibited by N/OFQ, the effect was attributable to the ORL1 receptor pathway.

## DISCUSSION

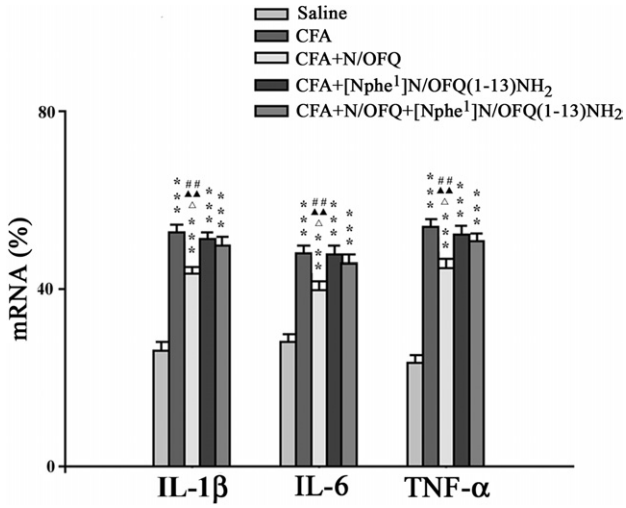
We report herein the temporal expression profile of specific proinflammatory cytokines and glial activation in a rodent model of chronic inflammatory pain. Astrocytes in normal CNS are basally active and carry out various functions, including regulation of extracellular ion and neurotransmitter concentrations, availability of neurotransmitter/neuromodulator precursors to nearby neurons and maintenance of extracellular pH. After a lesion, astrocytes undergo extensive hypertrophy of their cell bodies and cytoplasmic processes. Upregulation of intermediate filament proteins (GFAP, vimentin and/or nestin) and increased production of a variety of proinflammatory substances are the biochemical hallmarks of astrogliosis (Raghavendra and DeLeo, 2003; Watkins and Maier, 2003). In line with these findings, we also observed the immunoreactivity and protein expression of GFAP and the expression of proinflammatory cytokines mRNA transcripts were significantly upregulated.

Central neuroimmune activation, characterized by glial activation and proinflammatory cytokine expression, may be integral to the generation and maintenance of hyperalgesia following both peripheral and central insults (DeLeo and Yezierski, 2001; Watkins and Maier, 2003). Our results showed that a significant increase in GFAP immunoreactivity was observed only after the subacute phase, suggesting a delayed astroglial activation in response to

**Table 3.** Effect of N/OFQ on mRNA for proinflammatory cytokines in the spinal cord at day 4 following CFA-induced peripheral inflammation

| Cytokine | Day 4 | | | | |
|---|---|---|---|---|---|
| | Saline | CFA | CFA+N/OFQ | CFA+[Nphe$^1$]N/OFQ(1–13)$NH_2$ | CFA+N/OFQ+[Nphe$^1$]N/OFQ(1–13)$NH_2$ |
| IL-1$\beta$ | 26.2±1.934 | 52.6±1.806*** | 43.4±1.720***,##,▲▲,△ | 51.1±1.524*** | 49.6±1.990*** |
| IL-6 | 28.2±1.715 | 48.0±1.789*** | 39.8±1.985***,##,▲▲,△ | 47.0±2.016*** | 45.8±1.934*** |
| TNF-$\alpha$ | 23.4±1.720 | 54.0±1.732*** | 44.8±1.934***,##,▲▲,△ | 52.3±1.781*** | 50.6±1.778*** |

The mRNA was quantified by RT-PCR and was expressed as a ratio to that of corresponding $\beta$-actin (%). Values following CFA were fold increase over saline-injected control group, N/OFQ had an inhibitory effect on CFA. This effect was antagonized by co-treatment with an ORL1 receptor antagonist ($n$=5/group). *** $P<0.001$ vs. control (saline) group; ## $P<0.01$ vs. CFA; ▲▲ $P<0.01$ vs. CFA+[Nphe$^1$]N/OFQ(1–13)$NH_2$; △ $P<0.05$ vs. CFA+N/OFQ+[Nphe$^1$]N/OFQ(1–13)$NH_2$.




peripheral administration of CFA and might have a role in maintaining behavioral hyperalgesia during later phases (subacute and chronic phase) of CFA-induced peripheral inflammation. In addition, increased expression of the proinflammatory cytokines was observed throughout the phases of inflammation, demonstrating the time course of the up-regulation of proinflammatory cytokines paralleled the initiation of hyperalgesia during the acute of phase of inflammation.

Previous studies demonstrated that acute peripheral inflammation induced by intraplantar formalin or zymogen administration produced only mild astrocytic activation in the lumbar spinal cord in contrast to robust activation following L5 spinal nerve transection (Sweitzer et

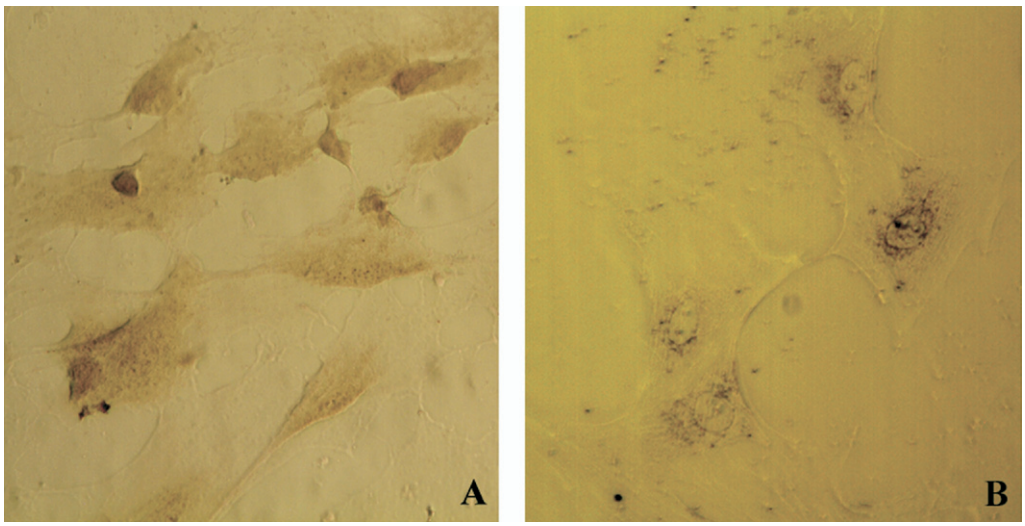



**Fig. 4.** Micrographs of cultured astrocytes positively stained with polyclonal rabbit anti ORL1 receptor antibody (A), and without primary antibody (B) (negative control). Magnifications are 40×.

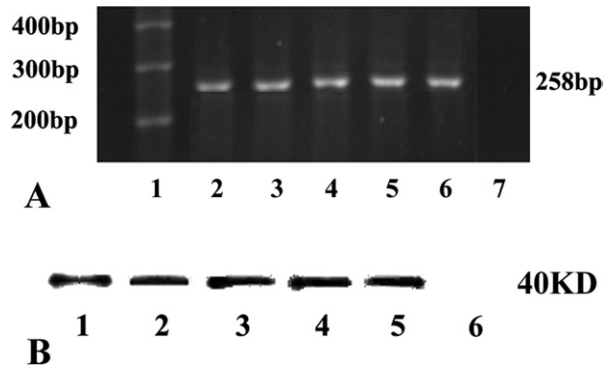



**Fig. 5.** ORL1 receptor was expressed on astrocytes of rat spinal cord. RT-PCR analysis revealed ORL1 receptor on mRNA level in astrocytes of rat spinal cord. Lanes 2–6: an expected 258 bp product for ORL1 receptor mRNA. Lane 7: blank control which contains no templates. One hundred base pair ladders were seen in lane 1 (A). The membranes from chemiluminescent Western blot with antibody detecting ORL1 receptor. There were bands (~40 kDa) for the ORL1 receptor protein in lanes 1–5. Lane 6: medium incubated without cells (blank control) (B).

al., 1999, 2002). In our present studies, compared with peripheral nerve transection (Tanga et al., 2004), CFA-induced peripheral inflammation produced a similar characteristic pattern of astrocytic activation and proinflammatory cytokines expression. In agreement with our findings, another study also observed increased expression of proinflammatory cytokines with intense glial activation following the peripheral administration of CFA (Raghavendra et al., 2004). This might be discrepant between intraplantar formalin and zymogen produced transient behavioral hypersensitivity and CFA treatment and nerve injury-induced persistent pain.

N/OFQ, an endogenous opioid peptide, has been believed to play a role in the modulation of nociception (Calo et al., 2000b). Our previous behavioral work demonstrated that i.t. administration with 15 nmol of N/OFQ could significantly attenuate hyperalgesia which was induced by CFA. The antinociceptive effects of N/OFQ were significantly blocked by i.t. injection 20 nmol of [Nphe$^1$]N/OFQ(1–13)$NH_2$ (Fu et al., 2006). Therefore in present study, we chose the same dose ratio of N/OFQ and [Nphe$^1$]N/OFQ(1–13)$NH_2$ to investigate the effect of N/OFQ on mRNA for proinflammatory cytokines, which was shown an inhibitory effect and was attributable to the ORL1 pathway. Previous research demonstrated that blocking proinflammatory cytokine activity blocks hyperalgesia arising from various causes, including tissue inflammation, peripheral nerve inflammation, peripheral and spinal nerve trauma, spinal cord inflammation, spinal cord trauma, and spinal dynorphin (Watkins et al., 1994, 1997; Laughlin et al., 2000; Milligan et al., 2001, 2003; Plunkett et al., 2001; Sweitzer et al., 2001; Raghavendra et al., 2002). Therefore, findings concerning modulation of nociception by N/OFQ might be associated with proinflammatory cytokines in the CNS. The main cellular source of cytokines is astrocytes, but their role in modulation of nociception by N/OFQ was not investigated. Thus, we need to further observe the effect of N/OFQ on astroglial activation and numbers.

Previous observations in animals have suggested the presence of ORL1 receptor in astrocytes of the cerebral cortex, hippocampus and hypothalamus (Zhao et al., 2002). The results from the present study indicated that ORL1 receptor existed in cultured astrocytes of rat spinal cord both at the mRNA and at the protein levels. RNA was isolated from cultured astrocytes of rat spinal cord, and reverse transcriptase PCR, amplification of DNA, and sequencing revealed a 258 bp DNA fragment identical to the ORL1 receptor from rat spinal cord (Xie et al., 1999). Immunocytochemistry using an antibody against ORL1 receptor resulted in strong staining of astrocytes and Western blot analysis of cultured astrocytes revealed the presence of the receptor protein. To our knowledge, this is the first demonstration that rat spinal dorsal horn astrocytes express ORL1 receptor, indicating that N/OFQ might influence glial function via ORL1 receptor pathway to exert its effects. In addition, proinflammatory cytokines are created and released by various type of glia within the CNS. Accordingly, it was necessary to examine the cellular actions of N/OFQ on the proinflammatory cytokines.

Previous studies have demonstrated that proinflammatory cytokines modulated spinal pain processing in several ways. Activation of their receptors on spinal neurons can lead to rapid changes in neuronal excitability (Obreja et al., 2002). They might also act indirectly through the release of nitric oxide (Holguin et al., 2004; Wu et al., 2001) and prostaglandin E2 ($PGE_2$) (Durrenberger et al., 2004). However, spinal cord cytokines alone are probably not sufficient to explain pathological pain. A recent report showed that regulation of N/OFQ by inflammatory mediators in astrocytes suggested that proinflammatory cytokines worked in concert with N/OFQ (Buzas et al., 2002). In our study, an-

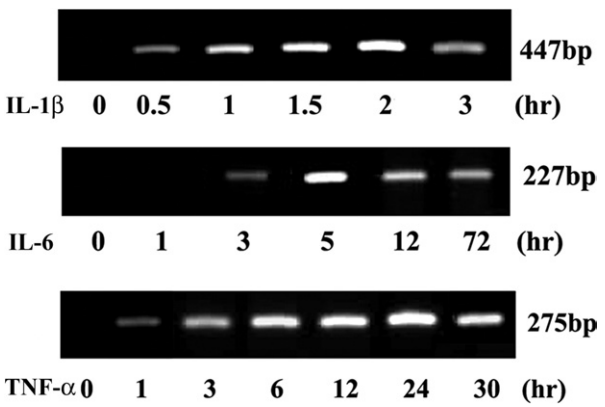



**Fig. 6.** RT-PCR detection of the time course of proinflammatory cytokine gene expression after exposure to LPS. Astrocyte cultures were treated with 30 mg/l LPS, IL-1$\beta$ gene expression could be detected as early as 30 min, reached a maximum level at 2 h, and then gradually decreased. IL-6 mRNA expression was observed at 3 h after 100 ng/ml LPS stimulation in astrocytes, the maximum level was reached at 5 h, and then slowly declined. After exposure to 100 ng/ml LPS, significant TNF-$\alpha$ release was observed at 3 h and the maximum response occurred following 24 h treatment.

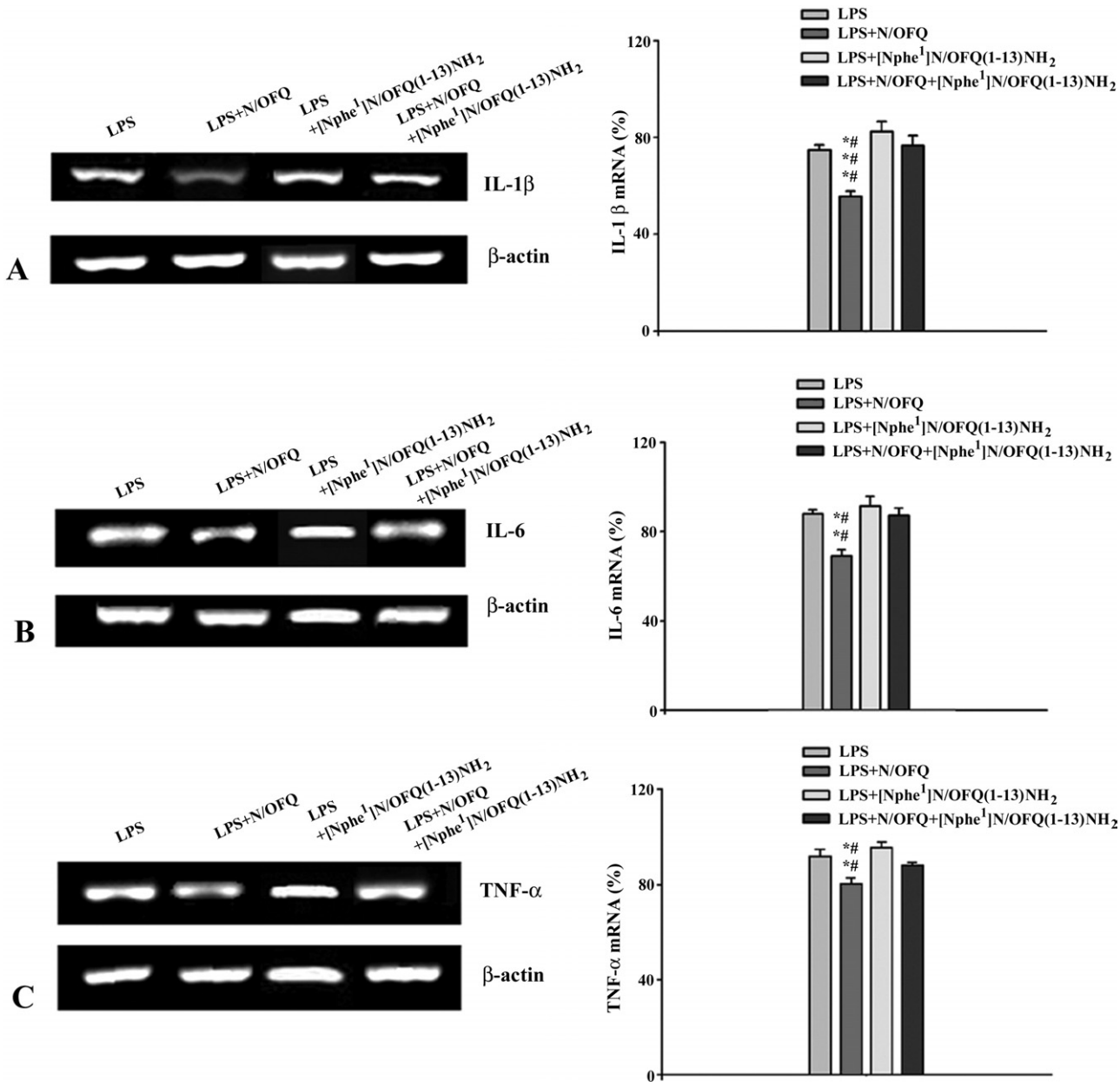



**Fig. 7.** Changes of proinflammatory cytokine gene expression in cultured astrocytes of rat spinal cord detected by RT-PCR after treatment with LPS, N/OFQ or [Nphe$^1$]N/OFQ(1–13)$NH_2$. Expected size PCR products were acquired corresponding to IL-1$\beta$ (A), IL-6 (B) or TNF-$\alpha$ (C). The time point was chosen at 2 h (A), 5 h (B) or 24 h (C) after the treatment, respectively. The results of all time points were quantified and demonstrated. The mRNA level was expressed as a ratio to that of corresponding $\beta$-actin. Data were represented as mean±S.E.M. ($n$=6/group at each time point). As compared with LPS group, N/OFQ could attenuate LPS-induced proinflammatory cytokines release, which was significantly abolished by [Nphe$^1$]N/OFQ (1–13)$NH_2$. ** $P<0.01$, *** $P<0.001$ vs. LPS group and $^{\#\#}$ $P<0.01$, $^{\#\#\#}$ $P<0.001$ vs. ORL1 receptor antagonist group.

other significant finding was that 100 nM N/OFQ, a concentration which has been described to inhibit cell activity through membrane hyperpolarization in current clamp (Lai et al., 1997), could reduce proinflammatory cytokine gene expression induced by LPS in cultured astrocytes and the effect of N/OFQ was significantly abolished by [Nphe$^1$]N/OFQ(1–13)$NH_2$. The evidence presented in this study showed that IL-1$\beta$, IL-6 or TNF-$\alpha$ was regulated by N/OFQ, which, considering the nociception modulatory role of N/OFQ, suggested that cytokines stemming from astrocytes might function as a mediator of inflammation. This might shed some light on the mechanism of the antinociceptive effect of N/OFQ. N/OFQ might act on the ORL1 receptor expressed by astrocytes to prevent cytokines from activating their receptors on spinal neurons and accordingly blocked rapid changes in neuronal excitability; or N/OFQ might act on ORL1 receptor to inhibit the indirect effect of cytokines and prevent nitric oxide and $PGE_2$ from being released. Although the detailed relationships of the present N/OFQ-induced decreased expression of proin-

flammatory cytokines gene to the anti-nociception effect of N/OFQ remain to be determined, IL-1$\beta$, IL-6 or TNF-$\alpha$ derived from astrocytes could play an important role of nociception modulation of N/OFQ.

## CONCLUSION

In summary, the present study showed that astrocyte activation and enhanced cytokine expression in the spinal cord might have a role in eliciting behavioral hypersensitivity. N/OFQ shared a close relationship with cytokines in the CNS. ORL1 receptor was expressed in cultured astrocytes of rat spinal cord. Further it was shown that N/OFQ had a regulatory effect on cytokines mRNA transcripts, which suggested that modulation of nociception by N/OFQ might be dependent on proinflammatory cytokines in the CNS.

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