p38 Mitogen–activated Protein Kinase Activation by Nerve Growth Factor in Primary Sensory Neurons Upregulates μ-Opioid Receptors to Enhance Opioid Responsiveness Toward Better Pain Control

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ABSTRACT

Background: Sensory neuron opioid receptors are targets for spinal, epidural, and peripheral opioid application. Although local nerve growth factor (NGF) has been identified as a mediator of sensory neuron μ-opioid receptor (MOR) up-regulation, the signaling pathways involved have not been yet identified.

Methods: Wistar rats were treated with intraplantar vehicle, Freund's complete adjuvant, NGF, NGF plus intrathecal p38 mitogen-activated protein kinase (MAPK) inhibitors, or NGF plus extracellular signal–regulated kinase–1/2 MAPK inhibitors. After 4 days of treatment, paw pressure thresholds of an intraplantar full (fentanyl) or partial (buprenorphine) opioid agonist were determined by algesiometry. Tissue samples from rat dorsal root ganglia were subjected to radiolabeled ligand binding, Western blot analysis, and confocal immunofluorescence.

Results: Exogenous and endogenous NGF resulting from Freund's complete adjuvant inflammation produced significant potentiation and enhanced efficacy in fentanyl- and buprenorphine-induced dose-dependent antinociception, respectively. Furthermore, in the ipsilateral dorsal root ganglia, NGF produced a significant increase in MOR binding sites, proteins, and immunoreactive neurons. In parallel, phosphorylated p38-MAPK protein, the number of phosphorylated p38–MAPK immunoreactive neurons expressing MOR in dorsal root ganglia, and the peripherally directed axonal transport of MOR significantly increased. Finally, NGF-induced effects occurring in dorsal root ganglia, on axonal transport, and on the potentiation or enhanced efficacy of opioid antinociception were abrogated by inhibition of p38, but not extracellular signal–regulated kinase–1/2, MAPK.

Conclusions: Local NGF through activation of the p38-MAPK pathway leads to adaptive changes in sensory neuron MOR toward enhanced susceptibility to local opioids. This effect may act as a counter-regulatory response to p38-MAPK–induced pain (*e.g.*, inflammatory pain) to facilitate opioid-mediated antinociception.

What We Already Know about This Topic

- Inflammation sensitizes nociceptive peripheral nerves, in part, by increasing nerve growth factor release— but nerve growth factor also increases their expression of μ-opioid receptors.

What This Article Tells Us That Is New

- In rats, peripheral inflammation increased μ-opioid receptor expression in nociceptive peripheral nerves by nerve growth factor activation of p38-mitogen–activated protein kinase.

DAILY clinical practice shows that opioids are the most effective and widely used drugs in the treatment of severe pain (*e.g.*, acute postoperative pain, chronic cancer-related pain).[1,2] Most opioids are administered systemically, passing the blood-brain barrier to activate corresponding opioid receptors in pain-related brain areas.[3] However, opioids are also effective on the central terminals of peripheral sensory neurons *via* intrathecal or epidural delivery.[4,5] Likewise, they are effective at the peripheral terminals of sensory neurons *via* local or topical application.[6]

It has been shown that the μ-opioid receptor (MOR) is expressed mainly in cell bodies of nociceptive C and Aδ neurons within dorsal root ganglia (DRG).[7–9] In addition, there is growing evidence that inflammatory pain increases MOR expression in these neurons,[10–12] resulting in en-

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hanced axonal transport of receptors from the DRG toward nerve terminals of sensory neurons.[13,14] This up-regulation in MOR expression occurs exclusively in ipsilateral DRG, suggesting that local (but not systemic) inflammatory mediators regulate opioid receptor expression of peripheral sensory neurons.[13]

It is well established that nerve growth factor (NGF), interleukin-1, and other inflammatory mediators can affect the phenotype of nociceptive neurons by increased expression of neuropeptides, such as substance P and calcitonin gene–related peptide (CGRP),[15–18] as well as receptors and ion channels, such as transient receptor potential channel, vanilloid subfamily member 1, acid-sensing ion channel receptors, and purinoreceptors.[19–21] This inflammatory-associated neuroplasticity is the consequence of a combination of activity-dependent changes and specific signal-transduction pathways, which phosphorylate cellular proteins, activate transcription factors, and finally alter gene expression.[22] Several studies have investigated the signal-transduction pathways that are mainly involved in this enhanced expression.[19,21,23] However, little is known about the signaling pathways responsible for the regulation of sensory neuron opioid receptor expression during painful conditions, such as inflammatory pain.

Mitogen-activated protein kinases (MAPK) transduce extracellular stimuli into intracellular responses and, thereby, induce changes in transcription and posttranslational modifications of target proteins.[24] p38 MAPK and extracellular signal–regulated kinase (ERK) 1/2 are activated in primary sensory neurons after peripheral inflammation.[25,26] A recent study demonstrated that the transient receptor potential ion channel, vanilloid subfamily member 1, is regulated by NGF-induced activation of the ERK pathway in DRG neurons *in vitro*.[27] Moreover, Ji *et al.*[19] showed that p38-MAPK activation in the DRG is responsible for the inflammation-induced transient receptor potential ion channel, vanilloid subfamily member 1, up-regulation, and hyperalgesia. In addition, p38 MAPK is involved in substance P and *N*-methyl-D-aspartate receptor–induced pain sensitivity as well as the regulation of cyclooxygenase-2 up-regulation and prostaglandin E2 release in the spinal cord.[28]

Because NGF has been implicated in the up-regulation of the number and efficacy of sensory neuron MOR, resulting in enhanced analgesic effects of opioids,[13] we set out to identify the NGF-dependent signaling pathways. Therefore, we examined whether: (1) exogenous as well as endogenous NGF, raised by Freund's complete adjuvant (FCA), contributes to enhanced antinociception of a full (fentanyl) or partial (buprenorphine) opioid agonist; (2) NGF-dependent increases in MOR binding, protein, and immunoreactive cells in peripheral sensory neurons are abolished by p38 MAPK and/or ERK-1/2 inhibition; (3) the number of immunoreactive MOR neuronal cells colocalizing with activated p-p38 MAPK within DRG neurons is increased after intraplantar NGF but return to baseline levels after concomitant intrathecal inhibition of p38 and/or ERK-1/2 MAPK; (4) local NGF enhances the anterograde axonal transport of MOR along the sciatic nerve *via* activation of p38 MAPK and/or ERK 1/2; (5) NGF-induced enhanced antinociceptive effects of local opioids are abolished by intrathecal inhibition of p38 and/or ERK-1/2 MAPK.

Materials and Methods

Animals

Experiments were conducted in male Wistar rats (200–250 g) housed in cages lined with ground corncob bedding. Rats were kept in climate- and light-controlled rooms (22 ± 0.5°C; relative humidity, 60–65%; 12-h light/dark cycle) with standard rodent food pellets and water *ad libitum*. Experiments were performed in accordance with *The Development of Science-based Guidelines for Laboratory Animal Care*‖ and were approved by the local animal care committee (Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit, LAGetSi, Berlin, Germany). All efforts were made to minimize the number of animals used and their suffering.

Intrathecal Catheter Implantation

Intrathecal catheters were used according to previously published protocols.[29] In brief, animals were anesthetized with isoflurane in oxygen *via* nose cone. A longitudinal skin incision was made in the lumbar region directly above the spinous processes of the L4–L6 vertebrae. The needle, through which the catheter was set up, was inserted at a 30° angle between the L5 and L6 vertebra. The catheter (PE10, 15 cm, 0.61-mm OD; Portex Ltd, Hythe, Kent, United Kingdom) was carefully advanced while rotating it between the thumb and forefinger. This rotation facilitated the penetration through the intervertebral space and dura. Dura penetration was observed by movement of the tail and hind limbs. The catheter was then carefully pushed upward 1–2 cm into the intrathecal space. The needle was then carefully removed before the catheter was fixed with glue to the tissue and secured with two additional sutures. Another skin incision was made at the dorsal neck, where the catheter was tunneled under the skin and pulled out at the neck before suture. Intrathecal catheter location was confirmed after surgery by administration of 10 μl lidocaine, 2%. Lidocaine caused 10–15 min of bilateral hind limb paresis whereas no deficits occurred in animals injected with sterile phosphate buffered saline (PBS). Animals were allowed to recover for at least 2 days before experiments. Only animals exhibiting no motor deficits were used for further testing.

‖ National Research Council Institute for Laboratory Animal Research. The Development of Science-based Guidelines for Laboratory Animal Care: Proceedings of the November 2003 International Workshop. Washington, National Academies Press, 2004. Available at: http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=nap11138. Accessed October 29, 2010.

Drugs

FCA, a water-in-oil emulsion with killed mycobacteria (Calbiochem, San Diego, CA) was injected (150 μl) intraplantarly. Affinity-purified polyclonal rabbit anti-NGF antiserum (CEDARLANE, Burlington, Ontario, Canada) was dissolved in PBS at 8 μg/100 μl. The first and intraplantar injection was administered 30 min before FCA.[13] According to a previous protocol,[30] the β subunit of NGF (β-NGF; R&D Systems, Inc., Minneapolis, MN) was reconstituted in sterile PBS at 4 μg/100 μl and injected intraplantarly once daily for 4 consecutive days. According to manufacturer instructions, the p38-MAPK inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580) and the MEK/ERK-1/2 inhibitor 2′-amino-3′-methoxyflavone (PD98059; Calbiochem) were dissolved in dimethyl sulfoxide diluted with sterile distilled water to a final concentration of 2%. Twice before removal of DRG, intrathecal boluses (1 μg/10 μl) were injected for each drug.[17,31] The first injection was delivered 30 min before intraplantar NGF. For 96-h experiments, mini-pumps (1 μg/1 μl; delivery rate 0.5 μl/h; ALZET Osmotic Pumps, Cupertino, CA) were filled with drugs or vehicle, implanted subcutaneously, and attached to anchored intrathecal catheters.[29,32] Fentanyl (*N*-(1-phenethyl-4-piperidyl) propionanilide citrate (1:1) or buprenorphine hydrochloride (21-(cyclopropyl-7α-[(S)-1-hydroxy-1,2,2-trimethylpropyl]-6,14-endo-ethano-6,7,8,14-tetrahydrooripavine hydrochloride) (Sigma-Aldrich, Taufkirchen, Germany) were dissolved in isotonic saline at different concentrations before application.

Experimental Groups

Rats were divided into nine groups with six to eight animals per group. The first group was treated with intraplantar FCA for 96 h. The second group of rats received intraplantar FCA plus intraplantar affinity-purified anti-NGF antiserum 30 min before FCA and then every 12 h. The third group, which served as a control, was injected intraplantar with isotonic saline. The fourth group received intraplantar NGF once daily for 1 or 4 days (24 or 96 h). The fifth group received intraplantar NGF and intrathecal SB203580. The sixth group received intraplantar NGF and intrathecal PD98059.

The dose of each MAPK inhibitor was selected based on previously published experimental protocols.[19,31] For visualization of anterograde axonally transported MOR, rats from the last three groups also received a tight ligation of the sciatic nerve at the midthigh level 24 h after the onset of treatment under isoflurane anesthesia and were sacrificed 24 h later.[13,14] Taken together, all experimental groups were tested at 24 h for changes at the level of the DRG, at 48 h for changes at the level of the sciatic nerve, and at 96 h (4 days) for behavioral changes at the level of the hind paw after local NGF treatment to examine consecutive changes along the peripheral sensory neuron.

Algesiometric Testing

At 96 h after treatment initiation, paw pressure threshold (PPT) was assessed using an algesiometer (modified Randall-Selitto test; Ugo Basile, Comerio, Italy) before (baseline) and after intraplantar injection of two different MOR opioid agonists: the full opioid agonist fentanyl (0–1.2 μg/100 μl) and the partial opioid agonist buprenorphine (1–5 μg/100 μl). Animals were gently restrained under paper wadding. Using a wedge-shaped, blunt piston, incremental pressure was applied on the dorsal surface of the hind paw by means of an automated gauge. The pressure required to elicit paw withdrawal, the PPT (250-g cutoff), was determined by averaging three consecutive trials separated by 10 s. The sequence of left and right paws was alternated among animals to preclude order effects. The experimenter was blind to group assignment.

Western Blot Analysis

At 24 h after treatment initiation, animals from the different groups were sacrificed. Ipsilateral L3–L5 DRGs were immediately removed, frozen in liquid nitrogen, and stored at −80°C. Western blot analysis was performed as previously described by Ji and Rupp.[33] In brief, samples were homogenized in boiling sodium dodecyl sulfate polyacrylamide sample buffer (100 mM Tris-hydrochloric acid, 2% sodium dodecyl sulfate, 20% glycerol). Protein concentration was measured using bicinchoninic acid assay (Pierce BCA Protein Assay; Pierce Biotechnology, Inc., Rockford, IL). 2-Mercaptoethanol and bromphenol blue were added before loading. Extracts were separated using 10% (MOR) or 12% (p38, p-p38, ERK 1/2) sodium dodecyl sulfate polyacrylamide gel electrophoresis 60-μg protein per lane and transferred onto nitrocellulose filters. Filters were blocked in 5% milk for 1 h and incubated with the following antibodies at 4°C overnight: rabbit polyclonal MOR antiserum (1:1,000 in 5% bicinchoninic acid; Gramsch Laboratories, Schwabhausen, Germany), rabbit polyclonal antiphospho-p44/p42 MAPK (p-ERK 1/2, 1:500), mouse monoclonal anti-p38 MAPK (1:1,000), and antiphospho-p38 MAPK (p-p38 MAPK, 1:500; Cell Signaling Technology, Inc., Beverly, MA). After incubation at room temperature for 2 h with secondary antibodies, specifically peroxidase-conjugated goat anti-rabbit (1:5,000; Amersham Pharmacia Biotech Europe, Freiburg, Germany) and rabbit anti-mouse (1:5,000; Abcam, Cambridge, MA), reactive bands were visualized in enhanced chemiluminescence solutions (Amersham Pharmacia Biotech Europe) for 1 min and immediately exposed to autoradiograph film for 5–20 min. Finally, blots were incubated in stripping buffer (62.6 mM Tris-hydrochloric acid [pH 6.7], 2% sodium dodecyl sulfate, 100 mM mercaptoethanol) at 56°C for 30 min and reprobed with monoclonal mouse anti–β-actin antibody (1:10,000; Sigma-Aldrich) as a loading control. These experiments were performed in duplicate and carried out three times.

Western blot bands of MOR, p38, and p-p38 MAPK were then quantified using an open-source image software program

(Java Image; ImageJ#).[34] The area and density of pixels within the threshold values representing immunoreactivity were measured. The integrated density (*i.e.*, the product of the area and mean of gray value) was calculated. Integrated immunodensities of controls and treated groups were compared and analyzed.

Opioid Receptor Binding

At 24 h after treatment initiation, animals from the different groups were killed by an overdose of isoflurane. The lumbar (L3–L5) DRG ipsilateral to the NGF-injected hind paw were removed. Membranes were obtained from DRGs as previously described.[35] In brief, the tissue was placed immediately on ice in cold assay buffer (50 mM Tris-hydrochloric acid, 1 mM EGTA, 5 mM $MgCl_2$ [pH 7.4]). Tissue was homogenized (POLYTRON; Kinematica, Littau, Switzerland) and centrifuged at 48,000*g* at 4°C for 20 min. The pellet was resuspended in assay buffer after 10-min incubation at 37°C to remove endogenous ligands. The homogenate was centrifuged at 42,000*g* and resuspended in assay buffer. Membranes were aliquoted and stored at −80°C. DRG membranes were diluted in assay buffer. Specific binding of [^{3}H]DAMGO (DAMGO = [D-Ala2, N-MePhe4, Gly-ol]-enkephalin) was performed by incubating 100 μg membrane protein with 2 nM [^{3}H]DAMGO in the presence or absence of 10 μM unlabeled naloxone to determine nonspecific binding. Membranes were incubated at 22°C for 1 h in assay buffer. Reactions were terminated by rapid filtration under vacuum through Whatman grade GF/B glass fiber filters followed by four washes with cold buffer (50 mM Tris-hydrochloric acid [pH 7.4]). Bound radioactivity was determined by liquid scintillation spectrophotometry (PerkinElmer, Inc., Rodgau, Germany) at 60% counter efficiency after overnight extraction of the filters in 3 ml scintillation fluid.[35] All experiments were performed in duplicate and carried out three times.

Immunohistochemistry

For visualization of anterograde axonally transported MOR with CGRP along the sciatic nerve, immunohistochemistry was done 48 h after treatment initiation and 24 h after nerve ligation. However, for visualization of MOR with p-p38 MAPK in DRG, immunohistochemistry was done at 24 h after treatment initiation. Rats were deeply anesthetized with isoflurane and transcardially perfused with 100 ml PBS (pH 7.4) and 500 ml paraformaldehyde, 4% w/v, in phosphate buffer (pH 7.4). After perfusion, the DRG and the ligated sciatic nerve (0.5 cm) were removed from treated and control animals, postfixed in the same fixatives for 90 min, and then cryoprotected at 4°C overnight in PBS with 10% sucrose. The DRG or sciatic nerve was embedded in Tissue-Tek OCT compound (Bayer Corporation, Pittsburgh, PA), frozen, and cut into 8-μm sections.

Double Immunofluorescence

DRG or sciatic nerve–mounted tissue sections were incubated with the following rabbit primary antibodies: rabbit polyclonal MOR antibody (1:1,000; Gramsch Laboratories) alone or in combination with mouse monoclonal p38-MAPK antibody (1:1,000), mouse monoclonal p-p38-MAPK antibody (Cell Signaling Technology, Inc.), or guinea pig polyclonal antibody against CGRP (1:1,000; Peninsula Laboratories, Belmont, CA). Tissue sections were washed with PBS and incubated with the appropriate secondary antibodies: Texas red conjugated goat anti-rabbit antibody alone or in combination with fluorescein isothiocyanate conjugated donkey anti-mouse or anti–guinea pig antibody. Finally, tissues were washed in PBS, mounted on VECTASHIELD (Vector Laboratories, Inc., Burlingame, CA), and viewed under a laser-scanning microscope (LSM 510; Carl Zeiss MicroImaging, Göttingen, Germany).

Specificity Controls

To demonstrate staining specificity, the following controls were included as detailed elsewhere: (1) preabsorption of antibody against MOR with a synthetic peptide for MOR (Gramsch Laboratories) at 4°C for 24 h; (2) omission of either the primary antisera, the secondary antibodies, or the avidin-biotin complex; (3) omission of either the first or second primary antibody and either the first or second secondary antibody.[13]

Quantification of Immunostaining

The method of quantification for DRG staining has been detailed elsewhere.[9,13,36] In brief, every fourth section of DRG that was serially cut at 10 μm for each animal (n = 5) was stained. For neuron counting, only those immunostained neurons containing a distinct nucleus were counted for a total of a minimum of 400 neurons using the microscope (×40). After MOR immunostaining, the total number of immunoreactive MOR neurons was counted by an observer blinded to the experimental protocol. This number was divided by the total number of neurons in each DRG section, and the percentage was calculated. The immunoreactive MOR neurons, which were also immunoreactive for p-p38 MAPK, were counted. The proportion was calculated as a percentage of the total number of immunoreactive MOR neurons in each DRG section and represented as percentages for vehicle (NGF *vs.* NGF/p38-MAPK inhibitor–treated animals). Data were obtained from four sections of each DRG and five rats per group.

For quantification of MOR immunoreactivity at the ligated area of the sciatic nerve, images of red (Texas red) immunofluorescence were obtained using a laser scanning microscope laser-scanning microscope (Carl Zeiss MicroImaging). To quantify changes in immunodensities, Modular Image-Analysis software was applied (version 2.5 [service pack 2]; Carl Zeiss MicroImaging).[13,37] The settings of the confocal microscope were established using a control section and kept unchanged for all subsequent image acquisitions. Six to eight images were sampled per animal. Images were thresholded to exclude background fluorescence. They were gated to include intensity measurements only from positively stained cells. For images analysis, a standardized box was positioned over the proximal part of the li-

National Institutes of Health. ImageJ: Image Processing and Analysis in Java. Available at: http://rsb.info.nih.gov/ij/. Accessed June 28, 2010.

gated sciatic nerve of all groups to determine the mean product of the area (μm^2) and the mean intensity of pixels within the threshold value as well as to calculate the integrated optical intensity (product of area and mean intensity). Five rats per group were used for analysis.

Statistics

The results of behavioral experiments are given as mean ± SD or SEM of the percentage of maximum possible effect (% MPE) according to the following formula: $(PPT_{postinjection} - PPT_{basal})/(250_{cut\text{-}off} - PPT_{basal})$. All statistics (two-tailed testing) were performed using STAT software (version 2.03; SPSS Science Software, Chicago, IL). Data were compared by a one-way or two-way analysis of variance (ANOVA) if the normality test passed (Kolmogorov-Smirnov test). Otherwise, the Kruskal-Wallis test was used. *Post hoc* multiple pair-wise comparisons were performed by the Newman-Keuls test. Data of two groups were compared using the unpaired Student *t* test. For the analysis of behavioral dose-dependent effects, a linear regression ANOVA was applied. A *P* value of less than 0.05 was considered statistically significant.

Results

Sensory Neuron NGF-dependent Enhancement of Antinociceptive Effects

In PBS-treated Wistar rats, intraplantar injection of the full MOR agonist fentanyl (0.5–1.0 μg) elicited a significant and dose-dependent increase in PPT consistent with the development of dose-dependent antinociception (fig. 1A; $P < 0.05$, linear regression one-way ANOVA, n = 6–8). In rats with FCA hind paw inflammation, the dose-response curve elicited by 0.5–1.0 μg fentanyl significantly shifted to the left, toward enhanced potency. Immunoneutralization of endogenous NGF in FCA-inflamed hindpaws reversed this leftward shift in potency ($P < 0.05$, two-way ANOVA, Newman-Keuls, n = 6–8). In contrast, pretreatment of naive rats with exogenous NGF resulted in a similar leftward shift toward enhanced potency of intraplantar fentanyl (fig. 1B). In PBS- treated Wistar rats, intraplantar injection of the partial opioid agonist buprenorphine (1–5 μg) did not increase PPT consistent with a lack of peripheral efficacy (fig. 1C), although higher doses revealed systemic antinociceptive effects (data not shown). In rats with FCA hind paw inflammation, administration of buprenorphine (1–5 μg) significantly and dose-dependently increased PPT ($P < 0.05$, linear regression one-way ANOVA, n = 6–8). However, the maximum peripheral antinociceptive effects of buprenorphine were lower than those elicited by fentanyl. Immunoneutralization of NGF in FCA-treated rat hindpaws significantly abolished the antinociceptive efficacy elicited by buprenorphine ($P < 0.05$, two-way ANOVA, Newman-Keuls, n = 6–8). In contrast, pretreatment of naive rats with exogenous NGF resulted in a similar degree of enhanced efficacy of intraplantar fentanyl (fig. 1D).

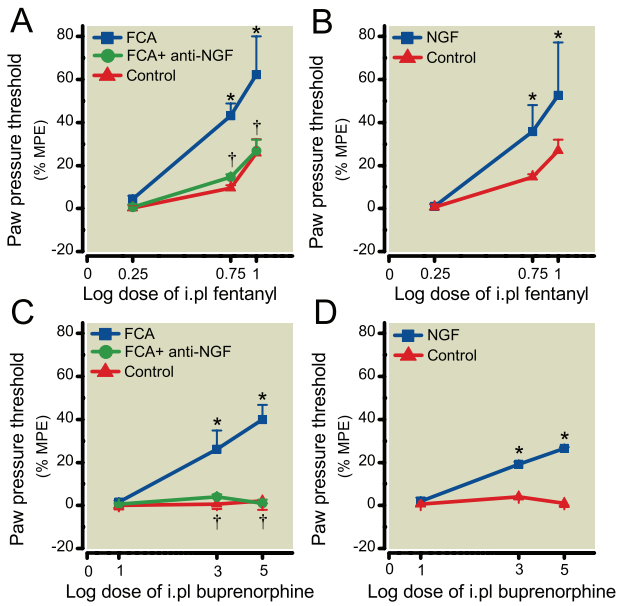

Fig. 1. Effects of intraplantar administration of the full opioid agonist fentanyl (*A, B*) or the partial opioid agonist buprenorphine (*C, D*) on paw pressure thresholds (PPTs) after Freund's complete adjuvant (FCA), FCA plus anti–nerve growth factor (NGF), or vehicle treatment. PPT values are given as mean ± SD of the percentage of maximum possible effect (% MPE) according to the following formula: $(PPT_{postinjection} - PPT_{basal})/(250_{cut\text{-}off} - PPT_{basal})$. (*A*) Intraplantar injection of fentanyl produced a significant and dose-dependent increase in PPT of vehicle-treated rats. In FCA-treated animals, significantly lower doses of fentanyl produced a dose-dependent elevation of PPT, indicating a leftward shift in potency. (*B*) Similar to intraplantar FCA, intraplantar NGF treatment resulted in a leftward shift in the dose-dependent antinociceptive effects of intraplantar fentanyl compared with vehicle treatment. (*C, D*) Intraplantar buprenorphine produced a significant and dose-dependent increase in PPT of FCA- and NGF-treated, but not vehicle-treated, rats. Immunoneutralization of endogenous NGF in FCA-treated animals with intraplantar anti-NGF antiserum reversed the leftward shift in fentanyl-induced antinociception (*A*) or abolished the buprenorphine evoked antinociceptive efficacy (*B*). * $P < 0.05$ *versus* vehicle (two-way ANOVA, Newman-Keuls test). † $P < 0.05$ *versus* FCA (two-way ANOVA, Newman-Keuls test).

Activation of p38 MAPK Mediates NGF-dependent Increases of MOR Binding Sites, Immunoreactive Cells, and Protein

MOR-specific [^{3}H]DAMGO binding sites were evaluated in the DRGs of rats treated with intraplantar NGF with or without intrathecal p38-MAPK inhibitor SB203580 or MEK/ERK-1/2 inhibitor PD98059 compared with vehicle. Twenty-four hours after NGF treatment, the number of [^{3}H]DAMGO binding sites in DRG was significantly increased compared with that of vehicle-treated rats (fig. 2A; $P < 0.05$; one-way ANOVA, Newman-Keuls, n = 6). This increase in MOR-specific [^{3}H]DAMGO binding sites induced by NGF was significantly reversed after intrathecal p38-MAPK inhibitor SB203580 ($P < 0.05$; one-way

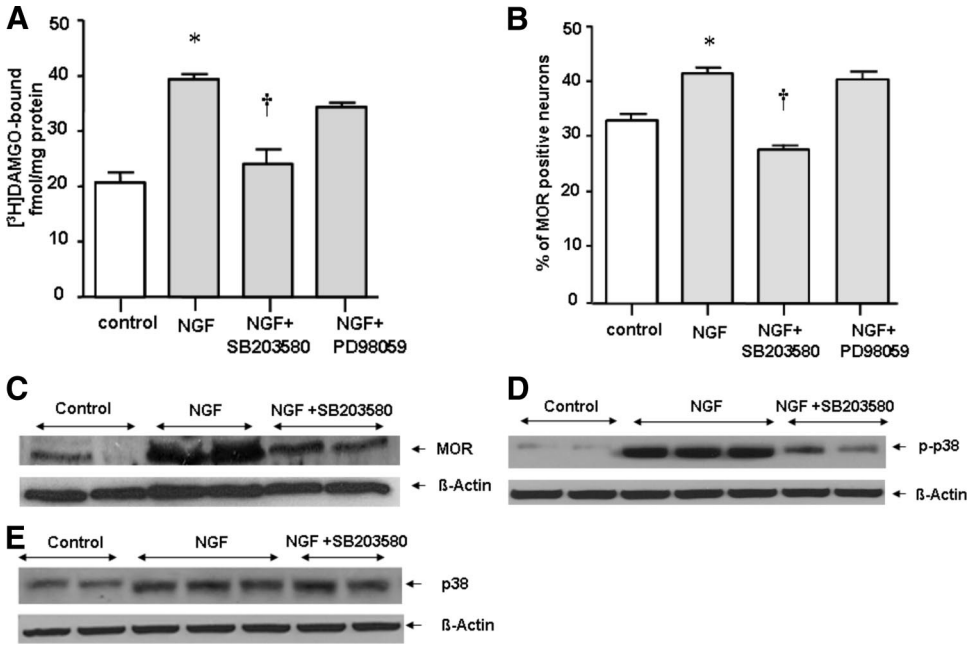

Fig. 2. Activated p38 mitogen-activated protein kinase (MAPK), but not extracellular signal–regulated kinase (ERK) 1/2, mediate nerve growth factor (NGF)–dependent increases in μ-opioid receptor (MOR) binding sites, immunoreactive cells, and protein concentration in dorsal root ganglia. (*A*) MOR-specific [^{3}H]-labeled [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO)–binding experiments with dorsal root ganglion membrane preparations from animals that received intraplantar treatment with vehicle, NGF, NGF plus intrathecal p38-MAPK inhibitor SB203580, or NGF plus intrathecal ERK-1/2 inhibitor PD98059 were performed in the presence or absence of 10 μM unlabeled naloxone to exclude nonspecific binding. After NGF, [^{3}H]DAMGO binding sites significantly increased compared with vehicle. This increase was significantly reversed by intrathecal p38-MAPK inhibitor SB20358, but not by ERK-1/2 inhibitor PD98059. Data are expressed as mean ± SEM. Statistically significant differences *versus* control (*) and inhibitor (†) are noted ($P < 0.05$; one-way ANOVA, Newman-Keuls test). (*B*) Quantitative analysis of MOR-specific immunofluorescent cell staining of dorsal root ganglia from animals with intraplantar vehicle (control), intraplantar NGF, intraplantar NGF plus intrathecal p38-MAPK inhibitor SB20358, or NGF plus intrathecal ERK-1/2 inhibitor PD98059 treatment. Data are expressed as mean ± SEM. Statistically significant differences compared with vehicle or p38-MAPK inhibitor treatment are noted (*† $P < 0.05$; one-way ANOVA, Newman-Keuls test). (*C–E*) Western blot analysis of dorsal root ganglia (DRG) immunoblotted with anti-MOR, antiphospho-p38 MAPK, or anti-p38 MAPK showing bands of MOR (50 kDa) (*C*), phospho-p38 MAPK (43 kDa) (*D*), and p38 MAPK (38 kDa) (*E*) in animals with vehicle, NGF, NGF/p38-MAPK inhibitor, or NGF/ERK-1/2 inhibitor treatment. ß-Actin was used as a loading control. The optical density of the protein bands of MOR and p-p38 MAPK, but not p38 MAPK, were significantly increased in NGF-treated groups. This increase was reversed only after treatment with intrathecal p38-MAPK inhibitor SB203580.

ANOVA, Newman-Keuls, n = 6), but not after intrathecal MEK/ERK-1/2 inhibitor PD98059.

Analysis of MOR immunofluorescence staining showed a significant up-regulation in the percentage of immunoreactive MOR cells in DRG (40.2 ± 7.3%,) 24 h after local administration of NGF in comparison with vehicle-treated animals (32.1 ± 6.2%). Intrathecal application of the p38-MAPK inhibitor SB203580 (27.7 ± 4.6%) significantly reduced the percentage of immunoreactive MOR cells in comparison with NGF-treated animals (fig. 2B; $P < 0.05$; one-way ANOVA, Newman-Keuls, n = 20).

Western blot analysis of DRG tissue extracts of control animals revealed immunoreactive MOR protein bands at the expected molecular weight of 50 kDa (fig. 2C). Twenty-four hours after intraplantar NGF treatment, the integrated optical density of immunoreactive MOR protein bands was increased by 67% compared with that of vehicle-treated animals ($P < 0.05$; one-way ANOVA, Newman-Keuls, n = 6). This increase was again prevented after intrathecal administration of p38-MAPK inhibitor SB203580 ($P < 0.05$; one-way ANOVA, Newman-Keuls, n = 6).

In parallel, at 24 h after NGF treatment, the integrated optical density of immunoreactive phosphorylated p-p38–MAPK protein bands at the expected molecular weight of 43 kDa increased eightfold compared with that of vehicle-treated animals (fig. 2D; $P < 0.05$; one-way ANOVA, Newman-Keuls, n = 6). This increase was reduced 5.6-fold by intrathecal administration of p38-MAPK inhibitor SB203580 ($P < 0.05$; one-way ANOVA, Newman-Keuls, n = 6).

In contrast, the integrated optical density of total immunoreactive p38-MAPK protein bands at the expected molecular weight of 38 kDa increased slightly by 0.4-fold in NGF-treated animals compared with vehicle-treated animals and did not change significantly after intrathecal treatment with p38-MAPK inhibitor SB203580 (fig. 2E; $P > 0.05$, one-way ANOVA, Newman-Keuls, n = 6).

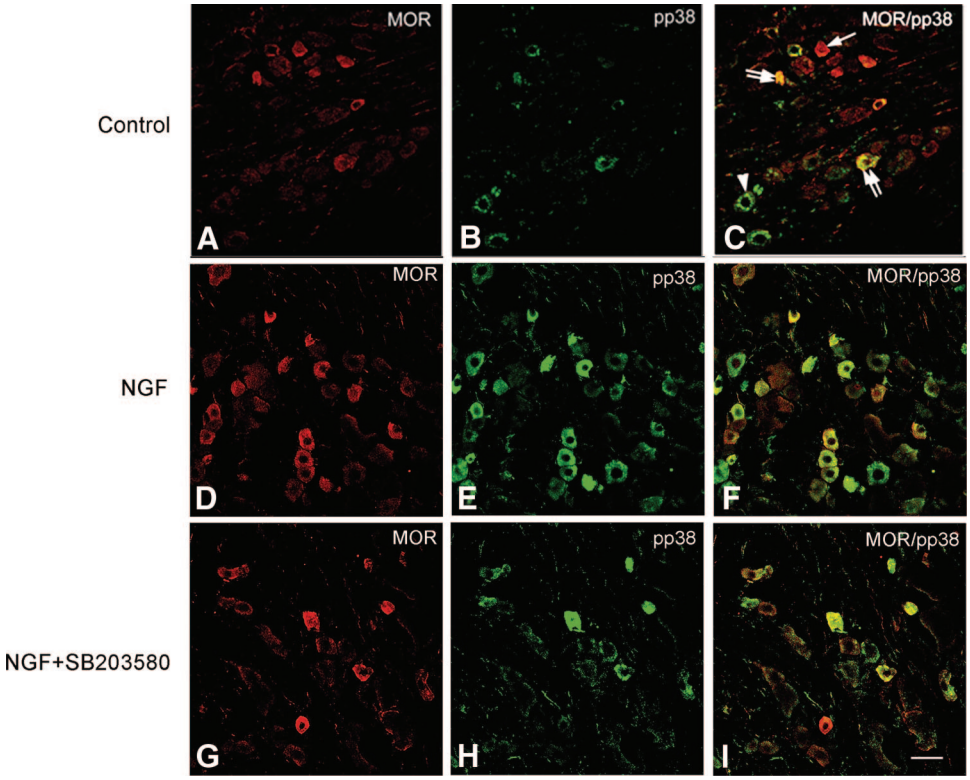

Fig. 3. (*A, D, G*) Double confocal immunofluorescence microscopy of μ-opioid receptor (MOR; *red fluorescence*) and (*B, E, H*) phosphorylated p38 mitogen-activated protein kinase (MAPK; *green fluorescence*) immunoreactivity in dorsal root ganglia (DRG) neurons of intraplantar vehicle (*A–C*), intraplantar nerve growth factor (NGF) (*D–F*), and intraplantar NGF plus intrathecal p38-MAPK inhibitor SB20358-treated animals (*G–I*). (*C, F, I*) Combined images showing colocalization of MOR with phosphorylated p38 MAPK (p-p38 MAPK). Note the coexistence of MOR (red) with p-p38 MAPK (green) (*double arrows*) with few cells containing only MOR (*single arrow*) or p-p38 MAPK (▽). After intraplantar NGF, the percentage of immunoreactive MOR neurons colocalizing with p-p38 MAPK was increased compared with intraplantar vehicle, which was attenuated after treatment with intrathecal p38-MAPK inhibitor SB203580. Bar = 20 μm.

NGF-dependent Increase in Phosphorylated p-p38 MAPK of MOR-expressing Neurons is Prevented

Double confocal immunofluorescence microscopy of MOR and phosphorylated p-p38 MAPK in DRG of control animals showed immunoreactive MOR neurons (51.1 ± 2.2%) colocalizing with phosphorylated p-p38 MAPK, although some neurons expressed MOR (48.9 ± 2.2%) alone (figs. 3A, B, and C). Twenty-four hours after intraplantar NGF treatment, the percentage of immunoreactive MOR neurons colocalizing with phosphorylated p-p38 MAPK increased significantly (83.2 ± 3.5%) compared with that of vehicle-treated animals (figs. 3A, B, C, D, E, and F; $P < 0.05$, one-way ANOVA, Newman-Keuls, n = 20). This NGF-induced elevation in MOR colocalization with p-p38 MAPK in DRG neurons (55 ± 2.9%) was significantly attenuated after intrathecal p38-MAPK inhibitor SB203580 (figs. 3G, H, and I; $P < 0.05$, one-way ANOVA, Newman-Keuls, n = 20).

Activation of p38 MAPK Mediates NGF-dependent Increases in the Axonal Transport of Sciatic Nerve MOR

Double confocal immunofluorescence microscopy examined the axonal MOR immunoreactivity proximal to a sciatic nerve ligature in rats treated with intraplantar vehicle (figs. 4A, B, and C), NGF (figs. 4D, E, and F), or NGF with intrathecal p38-MAPK inhibitor SB20358 (figs. 4G, H, and I). In vehicle-treated rats, MOR immunoreactivity expressed in CGRP nerve fibers accumulated mainly proximal to the ligature of the sciatic nerve. Compared with vehicle-treated animals, intraplantar NGF treatment induced a 44% increase in axonally transported MOR immunoreactivity on CGRP nerve fibers proximal to the ligature ($P < 0.05$, one-way ANOVA, Newman-Keuls, n = 30). Intrathecal pretreatment with the p38-MAPK inhibitor SB20358 prevented this increase in axonally transported MOR of immunoreactive CGRP nerve fibers ($P < 0.05$, one-way ANOVA, Newman-Keuls, n = 30).

Activation of p38 MAPK Mediates NGF-dependent Increases in Enhanced Antinociceptive Effects

Intraplantar NGF-dependent potentiation of intraplantar fentanyl antinociception was reversed after intrathecal administration of the p38-MAPK inhibitor SB203580 ($P < 0.05$; two-way ANOVA, n = 6–8), but not after the MEK/ERK-1/2 inhibitor PD98059 (figs. 5A and B). NGF-dependent enhanced efficacy of intraplantar buprenorphine antinociception was inhibited after intrathecal administration

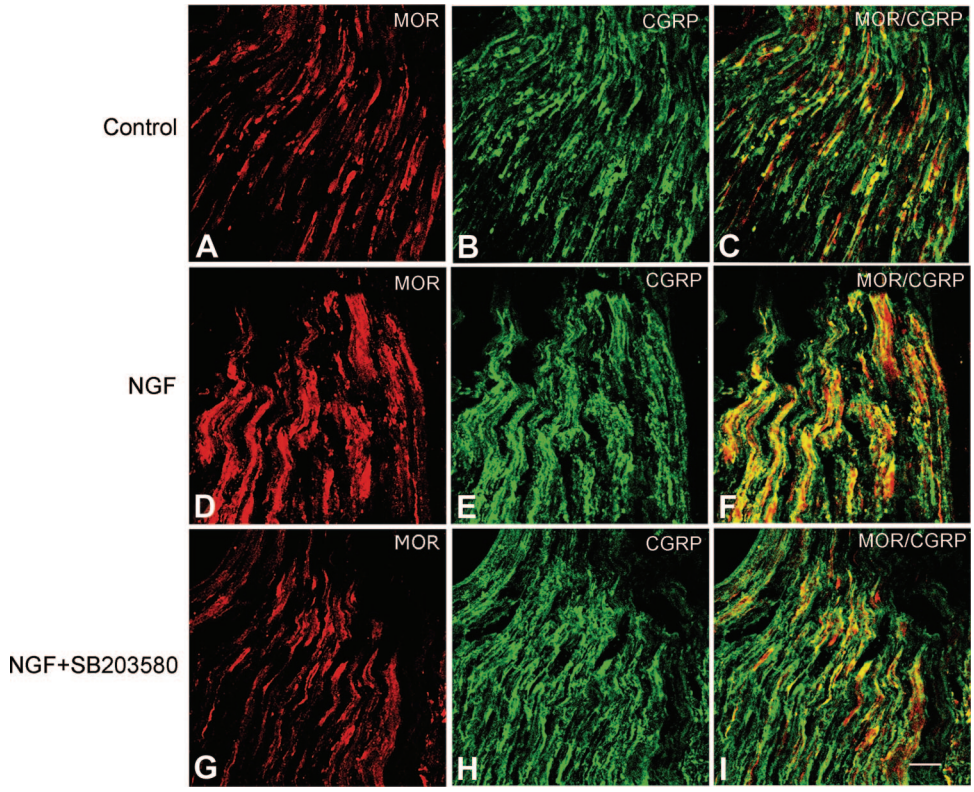

Fig. 4. Activated p38 mitogen-activated protein kinase (MAPK) mediates nerve growth factor (NGF)–dependent increases in immunoreactive μ-opioid receptor (MOR) (*red fluorescence*) of calcitonin gene–related peptide (CGRP) positive (*green fluorescence*) sciatic nerve neurons. After intraplantar Freund's complete adjuvant (FCA), anterogradely transported MOR immunoreactivity (*A, D, G*) in immunoreactive CGRP neurons (*B, E, H*) was assessed at the site of a sciatic nerve ligature after intraplantar vehicle (*A–C*), intraplantar NGF (*D–F*), and intraplantar NGF plus intrathecal p38-MAPK inhibitor SB203580 (*G–I*). (*C, F, I*) Combined confocal immunofluorescent images show the increased accumulation of MOR immunoreactivity proximal to the ligature of the sciatic nerve ipsilateral to the NGF-injected hind paw (*D–F*) compared with that of vehicle (*A–C*). This increase was abolished after treatment with intraplantar p38-MAPK inhibitor SB203580 (*G–I*). Bar = 20 μm.

of the p38-MAPK inhibitor SB203580 ($P < 0.05$; two-way ANOVA, n = 6–8), but not after the MEK/ERK-1/2 inhibitor PD98059 (figs. 5C and D). It is noteworthy that intraplantar NGF immediately decreased PPT (*i.e.*, hyperalgesia) when compared with vehicle-treated animals (table 1). However, this hyperalgesia was reversed by intrathecal administration of p38-MAPK inhibitor SB203580, but not MEK/ERK-1/2 inhibitor PD98059.

Discussion

The major finding of this study is that exogenous and endogenously up-regulated NGF, through activation of the p38-MAPK pathway, contributes to adaptive changes of the sensory neuron opioid system toward enhanced susceptibility to local opioids. This effect is established in five ways: (1) a potentiation of dose-dependent local antinociceptive effects of the full opioid agonist fentanyl as well as an enhanced antinociceptive efficacy of the partial opioid agonist buprenorphine 96 h after increased local NGF; (2) at 24 h, NGF treatment up-regulation of MOR in DRG ipsilateral to local NGF and its attenuation by intrathecal application of a p38-MAPK, but not a MEK/ERK-1/2, inhibitor; (3) increased phosphorylation and, thus, activation of p38 MAPK and its enhanced colocalization in immunoreactive MOR neurons of DRG 24 h after local NGF treatment; (4) increased anterograde axonal transport of MOR along the sciatic nerve 48 h after local NGF and its inhibition by intrathecal p38-MAPK inhibitor; (5) reversal of the NGF-induced enhanced antinociceptive effects of local opioids by intrathecal application of a p38-MAPK, but not a MEK/ERK-1/2, inhibitor.

In the present study, we examined whether endogenously increased NGF in inflamed paws, or exogenously applied intraplantar NGF, leads to enhanced antinociceptive effects of local opioids. Intraplantar injection of low, systemically inactive doses of the full MOR agonist fentanyl elicits dose-dependent antinociceptive effects in normal rats. In rats treated with intraplantar NGF or FCA, the dose-dependent antinociceptive effects of fentanyl shift to the left, indicating a potentiation. Moreover, intraplantar anti-NGF reversed this potentiation in FCA-induced inflammatory pain, suggesting a role of endogenous NGF that is commonly known to be up-regulated during FCA inflammation.[38] Consistent with a required higher receptor occupancy for partial opioid

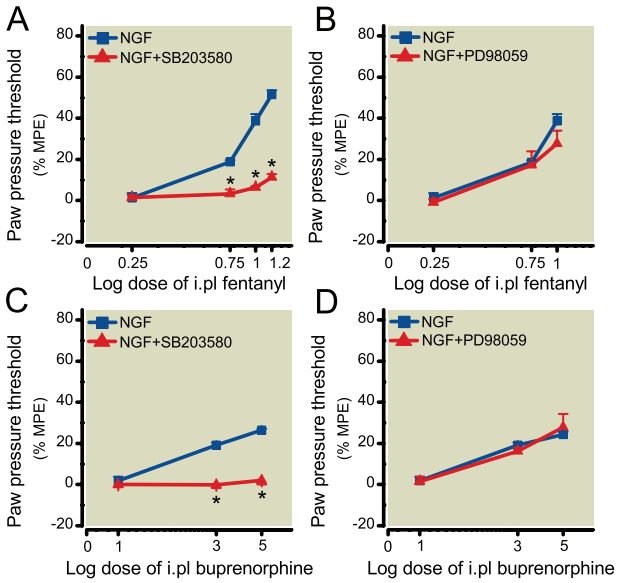

Fig. 5. Activated p38 mitogen-activated protein kinase (MAPK), but not extracellular signal–regulated kinase (ERK), mediates nerve growth factor (NGF)–dependent enhanced antinociceptive effects of the peripheral full opioid agonist fentanyl or partial opioid agonist buprenorphine. Paw pressure threshold (PPT) values are given as mean ± SD of the percentage of maximum possible effect (% MPE) according to the following formula: $(PPT_{postinjection} - PPT_{basal})/(250_{cut\text{-}off} - PPT_{basal})$. (*A, B*) Fentanyl-induced, dose-dependent increases in PPT after intraplantar NGF (*closed diamonds*) were shifted to the right after intrathecal p38-MAPK inhibitor SB203580 (*A*), but not after intrathecal ERK-1/2 inhibitor PD98059 (*B*). Buprenorphine-induced, dose-dependent increase in PPT after intraplantar NGF was abolished after intrathecal p38-MAPK inhibitor SB203580 (*C*), but not after ERK-1/2 inhibitor PD98059 (*D*). * $P < 0.05$ *versus* NGF plus p38-MAPK inhibitor SB203580 (two-way ANOVA, Newman-Keuls test).

agonists[39,40] than for full agonists (*e.g.*, fentanyl) and a relatively low maximal number of MOR binding sites in DRG of normal rats,[41] peripheral administration of buprenorphine did not show antinociceptive efficacy in untreated rats. However, after local FCA or NGF treatment, intraplantar buprenorphine elicited significant and dose-dependent antinociceptive effects. However, these effects remained small compared with those observed for intraplantar fentanyl. The potentiation in the antinociception of the full opioid agonist fentanyl and the rise in the antinociceptive efficacy of the partial opioid agonist buprenorphine strongly suggest an increase in the number of MOR. Indeed, Western blot analysis of MOR receptor protein, MOR-specific [^{3}H]DAMGO binding sites, and the number of immunoreactive MOR cells in DRG showed a significant up-regulation after local FCA or NGF treatment. These findings are consistent with those of Mousa *et al.*[13] and other reports,[42,43] showing an up-regulation of MOR in NGF-overexpressing transgenic mice. These findings are also consistent with a report by Cahill *et al.*,[44] which showed that intrathecal application of NGF can rescue the reduced opioid responsiveness seen in neuropathic pain.

Table 1. Mean ± SD (g) Paw Pressure Threshold After Intrathecal Administration of Vehicle *versus* p38-MAPK Inhibitor SB203580

—	Vehicle	SB203580
Control	73.33 ± 1.36	74.75 ± 1.78
NGF	50.94 ± 2.86†	79.51 ± 2.83*

* $P < 0.05$, nerve growth factor (NGF) *vs.* NGF + SB203580 (Student *t* test). † $P < 0.05$, NGF *vs.* control (Student *t* test).
MAPK = mitogen-activated protein kinase.

We extended these investigations to identify the NGF-dependent signaling pathways responsible for the up-regulation of sensory neuron opioid receptor expression and efficacy. Increased concentrations of endogenous NGF during inflammatory pain and exogenously applied NGF are known to bind to its receptors TrkA and p75.[45] They are then retrogradely transported as early endosomes to the cell somata of sensory neurons within the DRG.[45] These NGF TrkA-carrying early endosomes also colocalize the signaling proteins p38 MAPK and ERK 1/2,[45] which are involved in different pain conditions.[46] In Western blot analysis, the NGF-induced up-regulation of MOR receptor proteins was concomitant with a 5.6-fold increase in phosphorylated (*i.e.*, activated) p-p38 MAPK in DRG. However, total p38 MAPK increased only slightly and not at a statistically significant level. Moreover, intrathecal administration of the p38-MAPK inhibitor SB203580—but not the MEK/ERK-1/2 inhibitor PD98059—reversed NGF-induced increases in MOR receptor proteins, MOR-specific [^{3}H]DAMGO binding sites, and the number of immunoreactive MOR cells in DRG. Therefore, our findings suggest that the p38-MAPK–signaling pathway mainly contributes to the NGF-dependent up-regulation of sensory neuron MOR. These results are in line with previous studies[19] that showed increased activation of p38 MAPK after local NGF. However, the increased activation of p38 MAPK may also lead to enhanced nociception—most likely *via* an increased number of ion channels, such as capsaicin receptor TRPV1—in painful conditions.[19,46] In line with this theory, intraplantar NGF produced hyperalgesia in our study, as reflected by diminished PPT when compared with vehicle-treated animals (table 1). Hyperalgesia was attenuated after intrathecal application of the p38-MAPK inhibitor SB203580. However, our results support the notion that, in parallel to enhanced hyperalgesia through p38-MAPK activation, adaptive changes occur within sensory neurons toward an up-regulation in MOR number and efficacy—resulting in enhanced opioid susceptibility and pain control.[13] This conclusion was confirmed in double confocal immunofluorescence microscopy, which demonstrated an increase in the number of immunoreactive MOR neurons colocalizing with phosphorylated (*i.e.*, activated) p38 MAPK, an effect that was suppressed by the intrathecal p38-MAPK inhibitor SB203580. In line with

other studies,[19] this NGF-induced increase in p38-MAPK activation was restricted to small and medium size DRG neurons, most of which were nociceptors. This result indicates that p38-MAPK activation in DRG neurons is not a universal response of neurons to stress. Instead, it is restricted to nociceptive NGF-responsive and TrkA-expressing neurons.[13] Together, these results suggest that NGF, through the activation of the p38-MAPK pathway, contributes to enhanced expression of MOR within primary afferent sensory neurons. It is noteworthy that intrathecal administration of the MEK/ERK-1/2 inhibitor PD98059 had no effect on NGF-induced up-regulation of sensory neuron MOR, although the literature[26,47] has shown that, under these conditions, sensory neuron ERK-1/2 is activated.

How might up-regulation in the number of sensory neuron MOR be mechanistically explained? One possible mechanism is that p38 MAPK increases transcription of MOR in DRG neurons. In previous work,[11] we observed a moderate increase in MOR messenger RNA (mRNA) 1–2 h after intraplantar FCA. However, a lack of changes in MOR mRNA does not rule out possible changes in MOR receptor proteins.[10] Although some studies[48–50] have investigated possible transcription factors that regulate MOR expression, many were performed using cell lines that endogenously express MOR. Up-regulation of MOR mRNA in human neuroblastoma SH-SY5Y cells after interleukin-6 stimulation was dependent on the transcription factors STAT1 and STAT 3.[48] Recent studies showed that p38 MAPK is involved in the activation of STATs[49] and CREB.[50] It is noteworthy that phosphorylation of STAT3 and CREB are increased in mouse DRGs after peripheral inflammation.[51] Alternatively, p38 MAPK has been shown to regulate protein expression under inflammatory conditions by mRNA stabilization. Unstable mRNAs often contain AU-rich elements in their 3′-untranslated region. (Au refers to nucleic acid bases adenin, uracil.) The characteristic motif is AUUUA. The AU-rich element–regulated mRNA stability is mediated by RNA-binding proteins, such as human antigen R. In response to proinflammatory stimuli, HuR has been shown to increase the mRNA stability of tumor necrosis factor-α, cyclooxygenase-2, and interleukin 8 in a p38-MAPK–dependent manner.[52–55] This p38-MAPK dependence was blocked by use of the p38-inhibitor SB203580. The 3′-untranslated region of human and mouse MOR contains several AU-rich elements[56] that regulate MOR expression.[57,58]

Opioid receptors synthesized in DRG neurons undergo anterograde axonal transport toward peripheral nerve terminals. This process leads to enhanced density of opioid receptors on cutaneous nerve fibers.[13,14,59,60] Therefore, we examined whether the peripherally directed axonal transport of MOR is affected by NGF with or without intrathecal p38-MAPK inhibition. Double confocal immunofluorescence microscopy of MOR and immunoreactive CGRP neurons in the ligated sciatic nerve showed a significant increase of MOR after intraplantar NGF accumulating proximal to the ligature. This increase in axonally transported MOR was attenuated by intrathecal injection of the p38-MAPK inhibitor SB203580. These findings suggest that NGF enhanced the axonal transport of sensory neuron MOR toward the peripheral subcutaneous tissue through activation of the p38-MAPK pathway.

Finally, we examined whether NGF-induced MOR up-regulation in peripheral sensory neurons and enhanced antinociceptive effects of local opioids are mediated through the activation of the p38 or ERK-1/2 MAPK pathway. Axonal transport of MOR from the DRG toward peripheral nerve terminals requires a delay of approximately 2 days after FCA or NGF treatment.[13,14] Indeed, our previous work[13] showed a significant increase in immunoreactive MOR nerve fibers within the layers of the epidermis 96 h after intraplantar NGF, coinciding with the potentiation of opioid-mediated antinociception. Therefore, algesiometric experiments were performed 96 h after NGF treatment. Our current investigation found that, whereas the potentiation of dose-dependent antinociceptive effects of the full opioid agonist fentanyl was reversed by a rightward shift in the dose-response curve, the enhanced antinociceptive efficacy of the partial opioid agonist buprenorphine was abolished by intrathecal injection of the p38-MAPK inhibitor SB203580—but not the MEK/ERK-1/2 inhibitor PD98059. Thus, consistent with NGF-induced sensory neuron MOR up-regulation through p38-MAPK activation, p38-MAPK inhibition prevented the enhanced antinociceptive effects of local opioids.

In summary, we have shown that exogenous and endogenously up-regulated NGF, through activation of the p38-MAPK pathway, lead to adaptive changes in sensory neuron opioid receptors toward enhanced susceptibility to local opioids. After intraplantar NGF treatment, this effect occurs in three consecutive steps: increased MOR in DRG at 24 h, increased axonal MOR transport at 48 h, and increased MOR density at 96 h. Consequently, dose-dependent peripheral antinociceptive effects of locally applied full opioid agonists such as fentanyl are potentiated and those of partial opioid agonists such as buprenorphine are enhanced in efficacy which is reversed by intrathecal p38-MAPK inhibitor SB203580. This mechanism may act as a counter-regulatory response to p38-MAPK–induced painful conditions, such as inflammatory pain, to facilitate exogenously or endogenously mediated opioid antinociception.

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