Nerve growth factor governs the enhanced ability of opioids to suppress inflammatory pain

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Nerve growth factor (NGF) regulates sensory neuron phenotype by elevated expression of ion channels and receptors contributing to pain. Peripheral opioid antinociception is dependent on sensory neuron μ-opioid receptor (MOR) expression, coupling and efficacy. This study investigates the role of NGF in the upregulation of the number and efficacy of sensory MORs rendering sites of painful inflammation more susceptible to opioids.

We identified co-localization of MOR with calcitonin gene-related peptides (CGRP) and with NGF receptors tyrosine receptor kinase (TrkA) and p75NTR within rat dorsal root ganglia (DRG). We showed that unilateral hind paw inflammation induced with Freund’s complete adjuvant (FCA) or intraplantar (i.pl.) NGF increased NGF’s retrograde transport and MOR expression in TrkA positive DRG which was prevented by the disruption of this NGF transport. MOR upregulation in DRG was followed by enhanced axonal MOR transport towards peripheral nerve terminals and subsequent increase of MOR-ir nerve fibres within skin. Furthermore, peripheral antinociception elicited by i.pl. fentanyl was naloxone reversible and potentiated exclusively in inflamed and NGF-treated paws. Both FCA- and NGF-induced effects occurring through DRG to peripheral nerve fibres and the potentiation of antinociception were abrogated by NGF neutralization. Therefore, our results suggest that NGF not only contributes to inflammatory pain but also governs the upregulation in the number and efficacy of sensory neuron MOR, resulting in enhanced opioid susceptibility towards better pain control. This suggests the potential to overcome the unresponsiveness to opioids of certain neuropathic pain states.

Keywords: opioid receptors; sensory neuron; nerve growth factor; pain, antinociception

Abbreviations: CGRP = calcitonin gene-related peptides; DRG = dorsal root ganglia; FCA = Freund’s complete adjuvant; i.pl. = intraplantar; MOR = μ-opioid receptor; NGF = nerve growth factor; TrkA = tyrosine receptor kinase


Introduction

Opioid receptors comprise a subfamily of structurally homologous G-protein-coupled receptors that mediate analgesic effects not only in the central but also peripheral nervous system (Stein et al., 2003). Previous studies have identified opioid receptors in dorsal root ganglia (DRG) of sensory neurons which undergo axonal transport to reach peripheral nerve terminals (Ninkovic et al., 1982; Laduron, 1984). Recent studies have shown that inflammation-induced increases in MOR binding and immunoreactivity within DRG result in enhanced MOR G-protein coupling and MOR agonist efficacy (Mousa et al., 2001; Zöllner et al., 2003; Shaqura et al., 2004). Since both exogenous (Schäfer et al., 1994; Zhou et al., 1998) and endogenous (Brack et al., 2004) peripheral opioid antinociception depend on the number of opioid receptors expressed in primary afferent neurons, the upregulation process of opioid receptors during inflammatory pain plays an important role in peripheral opioid pain control.

It has been shown that altered retrograde supply of neurotrophic factors contributes to the neuronal response to injury and inflammation (Woolf and Costigan, 1999). One such factor is the neurotrophin nerve growth factor...
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(NGF), which acts on nociceptive neurons via interactions with the tyrosine kinase receptors TrkA and p75NTR (Chao, 1992; Barbacid, 1995). Acute intradermal injection of NGF induces hyperalgesia (Andreev et al., 1995; Dyck et al., 1997), while sequestration of NGF prevents hyperalgesia, which normally accompanies inflammation (Lewin et al., 1994; McMahon et al., 1995). As principal regulator of altered neuronal phenotype following inflammation (McMahon et al., 1995; Woolf and Costigan, 1999), NGF is implicated in elevated neuropeptide expression (Donnerer et al., 1992; Cho et al., 1997), and upregulation of ion channels (Ji et al., 2002; Mamet et al., 2002) as well as G-protein-coupled receptors (Lee et al., 2002) in nociceptive neurons. All this may contribute to an enhanced sensitivity to painful stimuli.

Moreover, NGF is known to increase enkephalin-binding sites in cell culture (Inoue and Hatanaka, 1982), and to raise diprenorphine binding sites in isolated DRG (Chen et al., 1997). Since MOR upregulation on nociceptive neurons is essential for peripheral opioid-mediated pain control under inflammatory conditions, this study was undertaken to test in the in vivo model of Freund’s complete adjuvant (FCA) hind paw inflammation whether NGF contributes to the upregulation in the number and efficacy of sensory neuron MOR, rendering sites of painful inflammation more susceptible to analgesic effects of opioids. To this end, we studied (i) the subpopulation of MOR-ir sensory neurons and the co-localization of these neurons with the NGF receptors TrkA and p75NTR; (ii) the alterations in NGF content retrogradely transported from peripheral tissue towards DRG following intraplantar (i.pl.) FCA and NGF treatment; (iii) the changes in MOR binding and/or immunoreactivity in DRG and in peripherally projecting sciatic nerves of i.pl. FCA- and NGF-treated rats; (iv) the differences in the number of MOR-ir sensory nerve fibres within the deep layers of the epidermis of FCA- and NGF-treated rats; (v) the naloxone (NLX) reversibility and changes in potency of the i.pl. administered MOR agonist fentanyl in FCA and NGF-treated rats. Finally, we assessed whether FCA- and NGF-dependent changes were prevented by neutralization of local NGF with an i.pl. NGF-specific antibody, sterile isotonic saline (fentanyl).

Material and methods

Animals, induction of inflammation

Experiments were conducted in male Wistar rats (140–170 gm) (Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany). Rats were housed individually in cages and maintained on a 12 h light/dark schedule with food pellets and water ad libitum. Room temperature was maintained at 22 ± 0.5°C and at a relative humidity between 60 and 65%. Experiments and animal care were performed according to the Policy on Ethics approved by the Society for Neuroscience, and were approved by the local animal care committee of the Senate of Berlin, Germany. All efforts were made to minimize the number of animals used and their suffering. Rats sedated by brief halothane (Willy Rusch GmbH, Böblingen, Germany) anaesthesia received an i.pl. injection of 0.15 ml FCA into the right hind paw. This treatment consistently produces a localized inflammation of the inoculated paw characterized by an increase in paw volume, paw temperature and infiltration with various types of immune cells (Rittner et al., 2001). Saline-treated animals served as controls.

Drugs and antibodies for in vivo experiments

General laboratory chemicals of analytical grade were purchased from Merck (Darmstadt, Germany). Speciality reagents were obtained from Sigma Chemie GmbH (Deisenhofen, Germany), if not stated otherwise. Anti-NGF monoclonal antibodies (clone 27/21), partly conjugated with β-D-galactosidase, were purchased from Boehringer (Mannheim, Germany). Moreover, the following substances were used: [3H]DAMGO (50 Ci/nmol) (Amersham Biosciences, Little Chalfont, UK); naloxone (Sigma, St Louis, MO); fentanyl (Sigma); β-NGF (Sigma); scintillation fluid (Perkin Elmer Wallac, Turku, Finland); NGF antibody (affinity purified 2.5s anti-NGF antisera) (Cedarlane, Ontario, Canada); IgG control serum (Sigma); FCA (Calbiochem, San Diego, CA); NGF (β-NGF, Sigma, St Louis, USA). Drugs were dissolved in the following vehicles: phosphate buffered saline (PBS, pH. 7.4) (β-NGF and NGF antibody), sterile isotonic saline (fentanyl).

Immunohistochemistry

Experimental protocols and tissue preparation

For visualization of anterograde axonally transported MOR along the sciatic nerve, MOR immunohistochemistry was done at 48 h after the onset of treatment and 24 h after nerve ligation. Rats were divided into five groups (six rats each): the first group of rats received i.pl. FCA and 24 h later a tight ligation of the sciatic nerve at the mid-high level (as described in detail elsewhere; Mousa et al., 2001) under sodium pentobarbital (40–60 mg/kg, i.p.) anaesthesia. The second group of rats received i.pl. FCA at time 0 and 12 h after FCA treatment, then the nerve was ligated at 24 h after FCA. The third group received i.pl. NGF (4 µg/100 µl) at 0 and 12 h after FCA treatment, then the nerve was ligated at 24 h after the first NGF treatment. The fourth group received NGF (4 µg/100 µl NGF) and anti-NGF (8 µg/100 µl) simultaneously at 0 and 12 h, then the nerve was ligated at 24 h after the first treatment. The fifth group received saline only and served as a control and the nerve was ligated 24 h later. Taking into account the time delay of opioid axonal transport from DRG towards subcutaneous tissue which usually takes 2–3 days (Hassan et al., 1993; Mousa et al., 2001), we determined MOR immunoreactivity within the deep layers of the epidermis after 96 h of the following treatments: FCA, FCA/anti-NGF, NGF and NGF/anti-NGF. Rats were divided into five groups (six rats each): the first group of rats received i.pl. FCA at time 0 only; the second group of rats received i.pl. FCA at time 0 and then i.pl. anti-NGF (8 µg/100 µl) or control IgG (100 µl) at 0, 12, 24, 36, 48, 60, 72 and 84 h after FCA treatment; the third group received i.pl. NGF (4 µg/100 µl) at 0, 12, 24, 48 and 72 h. The fourth group received NGF at 0, 12, 24, 48 and 72 h together with anti-NGF or control IgG at 0, 12, 24, 36, 48, 60, 72 and 84 h. The fifth group received saline only and served as a control. Rats were deeply anaesthetized...
mounted onto gelatin-coated slides. Thick (0.5 cm) were prepared on cryostat and collected in PBS (floating sections), but DRG or sciatic nerve sections (10 μm thick) were mounted onto gelatin-coated slides.

**Immunofluorescence**

For single or double immunofluorescence, DRG or sciatic nerve mounted tissue sections were incubated with the following rabbit primary antibodies: MOR (Drs S. Schulz and V. Höllt, Magdeburg, Germany), TrkA (Abcam Inc., Cambridge, MA, USA) or p75NTR (Covance, University Avenue, Berkeley, CA) antibody (1:1000) alone (sciatic nerve sections) or in combination (DRG sections) with guinea pig polyclonal antibody against calcitonin gene-related peptides (CGRP) (Peninsula Laboratories, Belmont, CA, USA) (1:1000). The tissue sections were washed with PBS and then incubated with the appropriate secondary antibodies; Texas red conjugated goat anti-rabbit antibody alone or in combination with FITC conjugated donkey anti-guinea pig antibody. Thereafter, sections were washed with PBS, mounted in vectashield (Vector Laboratories) and viewed under a Zeiss 510 laser scanning microscope for DRG or under a fluorescence microscope (Zeiss Axioskop 2) with appropriate filters, then a MC 200 CHIP microscope camera was used for photography (sciatic nerve).

**Peroxidase immunohistochemistry**

For single and double immunohistochemistry, subcutaneous or DRG tissue sections were processed for immunohistochemistry with a Vectastain–avidin–biotin peroxidase complex kit (Vector Laboratories) as described previously (Mousa et al., 2004). Unless otherwise stated, all incubations were done at room temperature and PBS was used for washing (three times for 10 min) after each step. Sections were incubated for 45 min in PBS with 0.6% H2O2, and 30% methanol to block endogenous peroxidase. To prevent non-specific binding, sections were incubated for 60 min in PBS containing 0.3% Triton X-100, 1% BSA, 5% goat serum and 5% horse serum (blocking solution). Sections were then incubated overnight with the following rabbit primary antibodies: MOR, TrkA or p75NTR antibody (1:1000). Thereafter, sections were incubated for 90 min with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories). Sections were then incubated with avidin–biotin-conjugated peroxidase for 90 min. Finally, sections were washed and stained with 3',3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma) containing 0.01% H2O2 in 0.05 M Tris-buffered saline (pH 7.6) for 3 min. After the enzyme reaction, sections were washed in tap water, mounted onto gelatin-coated slides, dehydrated in alcohol, cleared in xylene and mounted in DPX (Merck, Darmstadt, Germany).

To study co-localization of MOR with TrkA or p75NTR in DRG using double peroxidase immunohistochemistry, sections immunostained with antibody against either MOR, TrkA or p75NTR (as described above) were treated with 0.6% H2O2 for 30 min to inactivate peroxidase in ABC, washed in several changes of PBS. Sections stained for MOR were then incubated with a second primary antibody against TrkA or p75NTR. For light microscopy, all incubations were done overnight at 4°C, slides were then washed in PBS, exposed to the biotinylated secondary antibody (Vector Laboratories) for 1 h and to avidin–biotin-conjugated peroxidase for 45 min. Finally, sections were washed and stained using a Histogreen Peroxidase Substrate Kit (Linaris, Wertheim–Bettingen, Germany). The chromogen DAB used for the first primary antiserum appears brown, whereas the Histogreen used for the second primary antiserum appears green (Mousa et al., 2004). After the enzyme reaction, sections were washed in distilled water, dehydrated in alcohol, cleared in xylene and mounted in DPX. The double immunostained DRG sections were viewed under a Zeiss Axioskop 2, then a 3 CCD colour video camera (Sony) was used for photography.

**Specificity controls**

To demonstrate specificity of staining, the following controls were included as mentioned in detail elsewhere (Brack et al., 2004): (i) pre-absorption of antibody against MOR with a synthetic peptide for MOR (Grampsch Laboratories) for 24 h at 4°C; (ii) omission of either the primary antiserum, the secondary antibodies or avidin–biotin complex; (iii) omission of either the first or second primary antibody and either the first or second secondary antibody.

**Quantification of immunostaining**

The method of quantification for DRG staining has been described previously (Ji et al., 1995; Walsh et al., 1999). Briefly, we stained every fourth section of DRG that was serially cut at 10 μm for each animal (n = 5). For neuron counting, only those immunostained neurons containing a distinct nucleus were counted for a total of minimum 400 neurons using the microscope (40x objective). Following MOR immunostaining, the total number of MOR-ir 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 MOR, trkA- and p75-ir neurons which were also immunoreactive for CGRP were counted and the proportion calculated as percentage of the total number of MOR-, trkA- or p75-ir neurons. In a similar way, the number of MOR/trkA- or MOR/p75-ir neurons was counted and this number was divided by the total number of trkA- or p75-ir neurons in each DRG section and represented as percentages. Also, the number of trkA/MOR-ir neurons was counted and this number was divided by the total number of MOR-ir neurons in each DRG section and represented as percentages for control and FCA or NGF-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 an epifluorescence microscope (Zeiss Axioskop 2) with a 20x objective and appropriate filters. Images were corrected and the Image-Pro Analysis package (Media Cybernetics) was applied to quantify changes in immunodensities as described in detail elsewhere (Mousa et al., 2002; Frank and Tilby, 2003). The upper and lower threshold density ranges were adjusted to encompass and match the immunoreactivity (red fluorescence) to provide an image with immunoreactive material appearing in colour (red) pixels, and non-immunoreactive material as black pixels. A standardized box was positioned over the proximal part of the ligated sciatic nerve of all groups and the product of the
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area and density of pixels within the threshold value were calculated. Data represent the following differences: FCA, control; NGF, control; FCA/anti-NGF, control. In subcutaneous paw tissue, MOR-ir nerve fibres were counted within the deep layers of the epidermis by a blinded experimenter in three tissue sections per animal. Five squares (38.4 mm² each) per section were analysed using a Zeiss microscope as described previously (Mousa et al., 2002; Brack et al., 2004). Six rats per group were used for analysis.

NGF fluorometric enzyme-linked immunoassay

The following experiments aimed at quantification of the alterations in NGF concentration within the distal and proximal parts of the ligated sciatic nerve at 24 h after nerve ligation and 48 h after i.pl. FCA/anti-NGF, NGF, NGF/anti-NGF or saline treatments. Rats were divided into five groups (10 rats each) receiving the same treatments that were used for immunofluorescence staining of sciatic nerve preparations (see above). All rats were killed 24 h after nerve ligation and 48 h after i.pl. treatment by deep anaesthesia, the distal and proximal parts (about 0.5 cm each) of the sciatic nerve were quickly dissected from the surrounding soft tissue, slightly stretched onto aluminium foil, and rapidly frozen in liquid nitrogen. The nerve segments (about 5 mm each) were cut while still frozen with a steel razor blade as described elsewhere (Raivich et al., 1991), and stored at −80°C for further analysis. After thawing and weighing of the wet tissue, the samples were homogenized on ice in 700 μl of homogenization buffer, and again stored at −80°C until NGF measurement by a highly sensitive and specific fluorometric enzyme-linked immunoassay as described in detail previously (Hellweg et al., 1989, 2001). The endogenous NGF content was calculated per pg NGF/mg wet weight. In order to quantify the amount of retrogradely transported NGF, the net concentration of NGF in the distal part of the ligated sciatic nerve was calculated by subtracting the proximal content (where NGF synthesis occurs due to the ligation) always from the distal content (where the amount of NGF is the result of both retrograde transport and local ligation-related synthesis) as detailed elsewhere (Raivich et al., 1991).

Opioid receptor binding experiments

The following experiments aimed at measuring MOR binding in DRG at 24 h of treatment, the time of peak increase in MOR binding compared with control according to previous studies (Zöllner et al., 2003, Shaqura et al., 2004). Rats were divided into five groups: the first group of rats received i.pl. FCA at time 0; the second group of rats received i.pl. FCA at time 0 and i.pl. anti-NGF (8 μg/100 μl) or control IgG at 0 and 12 h; the third group received i.pl. NGF (4 μg/100 μl) at 0 and 12 h. The fourth group received NGF (4 μg/100 μl) and simultaneous anti-NGF (8 μg/100 μl) or control IgG at 0 and 12 h; the fifth group received saline only and served as a control. At 24 h of the first treatment, rats were killed by halothane anaesthesia and lumbar (L3–L5) DRGs were removed separately from the ipsilateral and contralateral sides. In total, 30 DRG from 10 rats were pooled for one experiment; equal numbers of DRG and animals were used for each treatment group. The tissue was placed immediately on ice in cold assay buffer (50 mM Tris–HCl, 1 mM EGTA, 5 mM MgCl₂, pH 7.4). Membranes were aliquoted and stored at −80°C (for details see Zöllner et al., 2003).

Rats received the same treatments similar to that used for immunofluorescence staining of sciatic nerve preparations (see above). At 48 h after the first treatment and 24 h after nerve ligation, rats were killed by halothane anaesthesia, then the proximal part of sciatic nerve (about 5 mm) was removed quickly and placed immediately on ice in cold assay buffer (50 mM Tris–HCl, 1 mM EGTA, 5 mM MgCl₂, pH 7.4). Membranes were prepared as described above. DRG membranes were diluted in assay buffer. Specific binding of [³H]DAMGO was performed by incubating 150 μg of membrane protein with 2 nM [³H]DAMGO in the presence or absence of 10 μM unlabelled naloxone to determine non-specific binding. The accumulation of [³H]DAMGO binding sites in the proximal part of the ligated sciatic nerve was determined by incubating 150 μg of membrane protein with 2 nM [³H]DAMGO in the presence or absence of 10 μM unlabelled naloxone. Membranes were incubated for 1 h at 30°C in assay buffer. The reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass fibre filters, followed by four washes with cold buffer (50 mM Tris–HCl, pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry at 60% counter efficiency (Zöllner et al., 2003) after overnight extraction of the filters in 3 ml of scintillation fluid. Four to five independent experiments were performed for each condition.

Assessment of nociceptive thresholds

Nociceptive thresholds were assessed by paw pressure algesiometer (modified Randall-Selitto test; Ugo Basile, Comerio, Italy). Rats were handled before testing and then gently restrained under paper wadding and incremental pressure (maximum 250 g) applied onto the dorsal surface of the hind paw. The pressure required to elicit paw withdrawal, the paw pressure threshold (PPT), was determined by averaging three consecutive trials separated by 10 s (Stein et al., 1988). The sequence of left and right paws was alternated between animals to avoid bias. Drugs or saline were administered intraplantarly (in 100 μl). The experimenter was blind to the treatments. Values were given as raw data in g or as % maximum possible effect (% MPE) according to the formula: PPTpost-injection/PPTbasal/(250cut-off/PPTbasal). ED₅₀ values were calculated by non-linear regression analysis (GraphPad Prism, San Diego, USA).

Behavioural experiments

PPT measurements were performed at 96 h following treatments. Rats were divided into five groups (six rats each) receiving the same treatments as described for immunohistochemical staining of subcutaneous paw tissue (see above). After baseline PPT measurement, rats i.pl. treated with FCA, FCA/anti-NGF, NGF, NGF/anti-NGF or saline were acutely challenged with different peripherally but not systemically effective doses of fentanyl (i.pl.) (Antonijevic et al., 1995), and nociceptive thresholds were measured 5 min later by paw pressure test (PPT). The highest effective dose of i.pl. fentanyl following each treatment was also tested i.v. to exclude systemic effects. In addition, the reversibility of fentanyl-induced PPT elevations was tested following increasing...
doses of i.pl. naloxone (1–10 μg) co-administered with 0.7 μg fentanyl. The maximum effective dose of naloxone was also tested following systemic (s.c.) administration.

**Statistical analysis**

Data were analysed using one-way ANOVA followed by Student–Newman–Keuls or Dunnett’s post hoc test. For data not normally distributed, Kruskal–Wallis one-way ANOVA on ranks was performed, followed by Dunn’s or Tukey post hoc test. Dose–response curves were analysed by one-way ANOVA followed by linear regression. Comparisons of two dose–response curves were analysed by two-way ANOVA. Differences were considered significant if $P < 0.05$. All tests were performed using Sigma Stat 2.03 (SPSS Science, Chicago, IL) software. Data are expressed as means ± SEM.

**Results**

**Co-localization of sensory neuron MOR with CGRP and with the NGF receptors TrkA and p75NTR**

Single immunohistochemical staining in control rats showed that 18% of DRG neurons contained MOR ir. Following FCA and NGF treatment, 27 and 25% of ipsilateral DRG neurons were MOR-positive representing a 37 and 34% increase, respectively ($P < 0.05$). Confocal analysis of MOR and CGRP double-immunofluorescence staining of DRG showed coexistence of MOR and CGRP in DRG neurons (Fig. 1iA–C). All MOR-ir DRG neurons co-expressed CGRP; however, some DRG neurons expressed CGRP only. Moreover, double-immunofluorescence staining demonstrated that a high population of TrkA (87.5 ± 3.3%)- or p75NTR (73.7 ± 3.2%)-ir neurons co-expressed CGRP, whereas some neurons expressed TrkA (12.5 ± 1.3%) or p75NTR (26.3 ± 3.2%) alone (Fig. 1iD–F and iG–I).

**Fluorometric enzyme-linked immunosorbert assay of neuronal NGF concentration distal to a sciatic nerve ligation**

Netto (i.e. distal–proximal NGF fraction) NGF content (pg/mg) in the distal part of the ligated sciatic nerve innervating non-inflamed paw tissue was not significantly altered by saline treatment ($P > 0.05$, ANOVA and Student–Newman–Keuls test). However, i.pl. FCA and NGF treatment significantly increased NGF content in the distal part of the ligated sciatic nerve compared with saline treatment ($P < 0.05$, ANOVA and Student–Newman–Keuls test) (Fig. 2). Co-treatment of rats with i.pl. NGF antibody but not control serum prevented this increase in NGF in the distal part of ligated sciatic nerve after FCA treatment ($P < 0.05$) (Fig. 2).

**NGF-dependent alterations of MOR binding sites in DRG**

[$^3$H]DAMGO binding sites were evaluated in DRG of rats at 24 h of i.pl. FCA and FCA/anti-NGF (Fig. 4A) as well as i.pl. NGF and NGF/anti-NGF (Fig. 3B) compared with i.pl. saline treatment. Saturation binding of [$^3$H]DAMGO showed similar binding affinities (Kd) for DRG and brain.
tissue as well as for animals with or without i.pl. treatments, as shown previously (Zöllner et al., 2003; Shaqura et al., 2004). Intraplantar FCA (Fig. 3A) or NGF (Fig. 3B) treatment significantly increased the number of [3H]DAMGO binding sites in innervating DRG (P < 0.05; ANOVA, Student–Newman–Keuls test). However, no significant change in [3H]DAMGO binding sites was detectable in DRG membranes of the contralateral side (Fig. 3B) or of saline-treated rats (Fig. 3A and B). This increase in [3H]DAMGO binding sites induced by either FCA or NGF administration was significantly reduced following i.pl. NGF antibody but not control serum (P < 0.05) (Fig. 3A and B). In addition, interruption of the sciatic nerve retrograde axonal transport by tight nerve ligation (according to Bussmann and Sofroniew, 1999) starting at the onset of FCA-induced inflammation completely prevented the FCA-induced up-regulation in [3H]DAMGO binding sites (P < 0.05) (Fig. 3C).

NGF-dependent alterations of MOR binding sites and immunoreactivity in the sciatic nerve

Following 24 h i.pl. FCA or NGF treatment and sciatic nerve ligation for another 24 h, measurement of [3H]DAMGO binding sites proximal to the ligation revealed that the number of anterogradely transported MOR binding sites was significantly increased in rats with i.pl. FCA (Fig. 4A) or NGF (Fig. 4B) compared with rats with i.pl. saline treatment (P < 0.05; ANOVA and Student–Newman–Keuls test). Immunoneutralization of NGF by i.pl. NGF antibody but
NGF-dependent changes in the number of MOR-ir nerve endings within plantar skin

Following 96 h of FCA, FCA/anti-NGF, NGF, NGF/anti-NGF or saline treatment, we investigated the changes of MOR-ir nerve fibres within the deep layer of the epidermis using a specific antibody against MOR (Fig. 6A–D). We found a significantly increased number of MOR-ir nerve fibres within the deep layer of the epidermis in FCA (9.4 ± 0.5/38.4 mm²) and NGF (8.9 ± 0.6/38.4 mm²) compared with saline (4.4 ± 0.4/38.4 mm²) (P < 0.05, Kruskal–Wallis test, post hoc Tukey test) (Fig. 6). Intraplantar anti-NGF treatment but not control serum significantly reduced this increase in the number of MOR-ir nerve fibres induced either with FCA (5.4 ± 0.4/38.4 mm²) or NGF (5.0 ± 0.4/38.4 mm²) (P < 0.05) (Fig. 6).

NGF-dependent alterations in peripheral fentanyl-induced analgesia

In control animals, i.pl. injection of fentanyl (0.7 μg) resulted in a moderate but significant increase in PPT (Fig. 7A). In comparison, FCA pre-treatment led to a decrease in baseline PPT, but a much more pronounced fentanyl-induced PPT elevation (Fig. 7A). This difference in PPT elevation (between control and FCA-treated animals) is significantly different and is attenuated following anti-NGF treatment (Fig. 7A). Similarly, NGF pre-treatment leads to a decrease in baseline, but a more pronounced fentanyl-induced elevation in PPT (Fig. 7B). This difference in PPT elevation is also significantly different (Fig. 7B). Fentanyl (0.7 μg)-induced PPT elevations in FCA-treated animals were dose-dependently reversed following increasing i.pl. doses of the opioid receptor antagonist naloxone (Fig. 7C). In contrast to the i.pl. injection, systemic injection of the maximally effective dose of naloxone was ineffective (data not shown). In addition, fentanyl (0.5–1.0 μg)-induced PPT elevations were dose-dependent (P < 0.05, linear regression ANOVA) (Fig. 8A). The highest dose (1.0 μg) given intravenously showed an effect on PPT neither in inflamed nor in non-inflamed paws (data not shown). In FCA-treated animals, significantly lower doses of fentanyl produced a dose-dependent elevation of PPT indicating a leftward shift in potency (P < 0.05, two-way ANOVA) (Fig. 8A). In i.pl.-treated animals a similar leftward shift of dose-dependent antinociceptive effects of fentanyl was observed (Fig. 8B). Immunoneutralization of NGF with i.pl. anti-NGF antiserum but not with control serum significantly reversed this leftward shift in fentanyl-induced antinociception (P < 0.05, two-way ANOVA) (Fig. 8C). ED₅₀ values for dose-dependent i.pl. fentanyl analgesia were significantly different following FCA and NGF compared with saline treatment (P < 0.05, ANOVA and Dunnett’s test) (Table 1). In addition, the ED₅₀ values for dose-dependent i.pl. fentanyl analgesia were significantly different following FCA plus anti-NGF compared with FCA treatment alone (P < 0.05, ANOVA and Dunnett’s test) (Table 1).
Discussion

The major finding of this study is that during localized inflammatory pain endogenous NGF enhances the susceptibility to locally applied opioids by an upregulation in the number and efficacy of MOR in primary afferent neurons. This is established in several ways: (i) co-localization of MOR-ir neurons in DRG with the sensory neuron marker CGRP and the majority of these co-expressing the NGF receptors TrkA and p75NTR; (ii) increased NGF concentrations retrogradely transported within the sciatic nerve innervating FCA or NGF-treated tissue; (iii) upregulation of MOR predominantly in trkA positive DRG ipsi- but not contralateral to i.pl. FCA or NGF and its prevention by disruption of the retrograde axonal transport; (iv) enhanced peripheral transport of MOR along the sciatic nerve following FCA or NGF; (v) increased number of MOR-ir sensory nerve endings within FCA or NGF-treated skin; (vi) potentiation of dose-dependent antinociceptive effects of i.pl. fentanyl following local FCA or NGF, which are naloxone reversible. All these changes are prevented by neutralization of local NGF with an i.pl. NGF-specific antiserum.

The neurotrophin NGF has been suggested to be an important mediator of inflammatory pain: neutralization of endogenous NGF attenuates hyperalgesia during inflammation (McMahon et al., 1995; Woolf, 1996), whereas enhanced NGF production is induced at the site of inflammation (Safieh-Garabedian et al., 1995). NGF is taken up by sensory nerve terminals within subcutaneous tissue and is retrogradely transported to their cell bodies within DRG (Greene and Kaplan, 1995; Delcroix et al., 2003), a process which starts as early as 3 h (Johnson et al., 1978). NGF exerts its effects on nociceptive neurons via two types of receptors, a high-affinity receptor (TrkA) and a low-affinity receptor (p75NTR) (Chao, 1992; Barbacid, 1995). In this study, double-immunofluorescent labelling and confocal analysis demonstrated that all MOR-ir neurons and a high population of TrkA (87%) and p75NTR (74%) positive neurons co-localize with the sensory neuron specific marker CGRP in DRG of rats. This is in agreement with previous studies (Li et al., 1998) demonstrating that the majority of TrkA- or p75NTR-ir neurons express CGRP in DRG (Averill et al., 1995; Chao and Hempstead, 1995; Pezet et al., 2001). Extending these findings, we showed the majority of MOR-ir neurons co-express TrkA and p75NTR in DRG, which is consistent with electrophysiological evidence of acute NGF and opioid effects within the same DRG neuron (McDowell, 2004). Moreover, following FCA or NGF treatment, this study showed that more neurons

Fig. 5 NGF-dependent alterations in sciatic nerve MOR immunoreactivity. Following 48 h i.pl. FCA and 24 h of sciatic nerve ligation, MOR immunoreactivity was assessed at the sciatic nerve ligature ipsilateral to i.pl. FCA (upper left panel), saline (upper right), NGF (lower left) or FCA/anti-NGF (lower right) treatment of rat hind paws. Note the elevated accumulation of MOR immunoreactivity in the sciatic nerve ipsilateral to the FCA or NGF injected hind paw compared to that of saline (upper right) or FCA/anti-NGF (lower right) treated hind paw. This accumulation is more pronounced proximal than distal to the ligature. Bar = 20 μm.

Fig. 6 Immuno-histochemical localization of MOR immunoreactivity on nerve endings within the deep layers of the epidermis (epi): Localization of MOR immunoreactivity at 96 h in saline (control) (A + B), FCA (C + D), NGF (E + F) and FCA/anti-NGF (G + H) within the plantar skin of injected hind paws. Note MOR-ir nerve fibres were abundant within the basal layer of the epidermis of FCA (C + D) or NGF (E + F) injected hind paw compared to that of saline (A + B) or FCA/anti-NGF (G + H) treated hind paw. Bar = 40 μm for A, C, E and G and 20 μm for B, D, F and H.
expressed MOR and that this increase in MOR expression occurs predominantly in the trkA positive population. This suggests a role for NGF as a regulator of MOR expression in sensory neurons via activation of its receptors (TrkA or p75NTR). Support for this notion comes from recent findings that exogenous as well as increased endogenous NGF following FCA-induced inflammation enhance the expression of the neuropeptide pituitary adenylate cyclase activating polypeptide in sensory neurons (Jongsma Wallin et al., 2001).

It is well described that endogenous NGF concentrations are highly increased within inflamed subcutaneous tissue (Safieh-Garabedian et al., 1995). In line with a retrograde axonal transport of NGF from the target tissue to the
innervating DRG (Richardson and Riopelle, 1984), we showed that i.pl. FCA and NGF highly increased NGF concentrations retrogradely transported within the sciatic nerve towards ipsilateral DRG and that this was prevented by immunoneutralization of endogenous NGF within inflamed tissue. In a previous report, sensory neuron NGF content peaked at 24 h of FCA inflammation (Donnerer et al., 1992), which coincides with a peak upregulation in MOR binding as well MOR-ir neurons within DRG (Shaqura et al., 2004). Therefore, we investigated the role of NGF in MOR upregulation in DRG following FCA inflammation.

Extending our recent findings of a significant increase in MOR binding, coupling and immunoreactivity in DRG (Shaqura et al., 2004), we have now identified NGF as an important regulator of this upregulation. Sequestration of endogenous NGF by i.pl. NGF antibody significantly prevented the MOR increase in DRG following i.pl. FCA inoculation. Furthermore, local treatment with NGF caused a similar marked increase in MOR of ipsilateral DRG compared with that of control rats and this increase was prevented by i.pl. NGF antibody treatment. No significant changes in MOR binding were observed in contralateral DRG of FCA or NGF-treated rats suggesting that MOR upregulation occurs exclusively within ipsilateral DRG due to local and not systemic effects. Since enhanced retrogradely transported NGF seems to contribute to this upregulation, we investigated whether disruption of the retrograde transport during the entire period of FCA inflammation interferes with MOR upregulation in DRG. Previous studies have shown that tight sciatic nerve ligation effectively blocks retrograde axonal transport (Frigell et al., 1975; Bussmann and Sofroniew, 1999). In our hands, tight sciatic nerve ligation simultaneously with FCA hind paw inoculation prevented MOR upregulation in DRG. Taken together, our results show that FCA inflammation increases MOR in DRG which is dependent on the retrograde transport of NGF from the inflamed paw tissue towards the DRG. This is further supported by our demonstration of MOR upregulation following i.pl. NGF. Our findings are consistent with reports that demonstrated MOR mRNA increase following FCA (Püehler et al., 2004; Molliver et al., 2005), and a 3.2-fold MOR mRNA upregulation in NGF-overexpressing compared with wild-type mice (Zwick et al., 2003). These findings strongly support our hypothesis that NGF contributes to MOR upregulation in sensory neurons.

It is generally accepted that opioid receptors synthesized in DRG neurons undergo axonal transport towards peripheral nerve terminals which leads to enhanced density of opioid receptors on cutaneous nerve fibres (Ninkovic et al., 1982; Hassan et al., 1993; Jeanjean et al., 1995; Mousa et al., 2001). In our binding and immunohistochemical experiments, we demonstrated a significant increase of MOR transport along the sciatic nerve at 24 h nerve ligation and following 48 h of either i.pl. FCA or i.pl. NGF treatment. Moreover, we showed that the enhanced anterograde transport of MOR was attenuated by i.pl. NGF neutralization. In line with a delay of 2–3 days required for the axonal transport of MOR from the DRG towards peripheral nerve terminals (Hassan et al., 1993; Mousa et al., 2001), we could show a significant increase in MOR-ir nerve fibres within the deep layers of the epidermis following 96 h of i.pl. FCA or NGF and this increase was abolished by local anti-NGF treatment. Taken together, these findings suggest that NGF contributes to MOR upregulation in DRG and consecutive enhanced MOR axonal transport towards the periphery resulting in an increased number of MOR-ir nerve fibres within inflamed subcutaneous tissue.

Finally, we examined whether local exogenous or increased endogenous NGF following FCA also leads to enhanced analgesic efficacy of local opioids. Intraplantar, but not systemic injection of the μ-agonist fentanyl elicited dose-dependent antinociceptive effects in normal rats. In rats treated with i.pl. NGF or FCA, dose-dependent antinociceptive effects of fentanyl shifted to the left suggesting a potentiation. This was confirmed by comparing the respective dose–response curves as well as by analysing the calculated ED50 values. Furthermore, we demonstrated that fentanyl-induced analgesia was naloxone reversible and thus opioid receptor specific and that the observed fentanyl potentiation was abolished by local anti-NGF. Together, our findings suggest enhanced peripheral fentanyl-induced antinociception via MOR upregulation in sensory neurons following i.pl. NGF or elevated endogenous NGF following i.pl. FCA. Our present findings do not rule out the involvement of other mechanisms in MOR upregulation, since preliminary results have shown at very early stages of FCA a possible contribution of neuronal electrical activity to enhanced MOR mRNA transcription (Püehler et al., 2004), and others have suggested that interleukin-1β is able to enhance the axonal transport of MOR (Jeanjean et al., 1995). However, this study identifies NGF as an important regulator of MOR expression in sensory neurons which is consistent with the reports by Molliver et al. (2005) and Zwick et al. (2003) that NGF overexpression causes a robust upregulation of MOR in transgenic mice. Interestingly, these mice recovered more rapidly and became more hypo- and not hyperalgesic despite extensive paw swelling during FCA inflammation which can possibly be explained by an enhanced active tone of immune cell-derived

### Table 1 ED50 values (mean ± SEM) of dose-dependent i.pl. fentanyl analgesia in control, FCA-, NGF-, and FCA + anti-NGF treated animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ED50 (μg)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td>NGF</td>
<td>0.54 ± 0.03*</td>
</tr>
<tr>
<td>FCA</td>
<td>0.56 ± 0.01*</td>
</tr>
<tr>
<td>FCA + anti-NGF</td>
<td>0.85 ± 0.03†</td>
</tr>
</tbody>
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*P < 0.05, versus control (Student t-test).
†P < 0.05, versus FCA (Student t-test).
opioid peptides on increased sensory neuron MOR, a mechanism which has been described previously (Stein et al., 2003). Consistent with NGF as a regulator of MOR expression, i.t. application of NGF can rescue the reduced opioid responsiveness in neuropathic pain (Cahill et al., 2003).

In summary, we have shown that exogenous as well as elevated endogenous NGF not only contribute to inflammatory pain but also to an upregulation in the number and efficacy of sensory neuron MOR resulting in an enhanced opioid susceptibility towards better pain control. Consequently, dose-dependent antinociceptive effects of locally applied opioids such as fentanyl are potentiated following i.pl. FCA and NGF treatment and this is reversed by sequestration of NGF. Thus, in addition to the growing evidence that NGF up-regulates ion channels and receptors in primary afferent neurons contributing to enhanced sensitivity to pain, it may—as a compensatory response—also up-regulate opioid receptors, leading to enhanced pain control upon activation. This suggests the therapeutic potential for pathological conditions with a reduced susceptibility to opioids such as certain neuropathic pain states.

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