Transcriptional and Translational Regulation of Glial Activation by Morphine in a Rodent Model of Neuropathic Pain

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ABSTRACT

Glia cells function in maintenance of homeostasis as well as in pathophysiology. In this study, we determined the time course of spinal glial cell activation during the development of morphine analgesic tolerance in an L5 spinal nerve transection rodent model of neuropathic pain. We also sought to assess whether the method of morphine administration affected neuroimmune activation at the levels of transcription and translation. Rats received L5 spinal nerve transection or no surgery on day 0. On day 6 post-transection, osmotic minipumps were implanted to deliver saline or morphine s.c. (1 or 10 mg/kg) or i.t. (5 or 20 nmol/h). Mechanical allodynia developed immediately after spinal nerve transection; this hypersensitivity was reversed with both low- and high-dose morphine by either route. Tolerance to antiallodynia developed after 3 days of i.t. morphine and after 6 days of s.c. morphine, indicating hastened tolerance following i.t. delivery. Analysis of mRNA revealed that s.c. morphine treatment did not lead to increases in glial activation markers. In contrast, i.t. morphine caused a biphasic alteration in glial fibrillary acidic protein (GFAP) and integrin α M mRNA. Protein levels for GFAP were elevated after s.c. and i.t. administration of morphine; however, induction was further enhanced in the latter group. Here, we show for the first time that there is differential recruitment of transcriptional and translational mechanisms of glial activation by systemic and i.t. morphine. Furthermore, we suggest that enhanced neuroimmune activation after i.t. dosing contributes to the hastened development of analgesic tolerance seen in these animals.

Effective treatments for neuropathic pain (i.e., chronic pain resulting from nerve injury) remain limited. Although morphine is successfully used to treat acute pain, many physicians believe that neuropathic pain is less responsive to opioid analgesia (Cherny et al., 1994). The dose required for even partial relief of neuropathic pain is often difficult to attain due to side effects and rapid development of tolerance to analgesia. Elucidation of the cellular and molecular mechanisms underlying opioid tolerance in neuropathic pain may thus benefit a large patient population that now has few pharmacological options.

Neuropathic pain, which arises as a result of ectopic firing from damaged nerve axons and intact neighboring neurons, leads to sensitizing neuroplastic changes in the brain and spinal cord. Such sensitization has also been implicated in the decreased efficacy of opioids in treating neuropathic pain and in the accelerated development of tolerance to opioids. Nerve injury-induced hyperalgesia may therefore share cellular and molecular mechanisms of neuronal plasticity with opioid tolerance (Mayer et al., 1999).

Recent work has shown that glial cell activation and increased CNS expression of proinflammatory cytokines, together referred to as neuroimmune activation, contribute significantly to long-term spinal changes associated with neuropathic pain and morphine tolerance. In particular, astrocytic and microglial activation has been demonstrated in the lumbar spinal cord dorsal horn after nerve injury (Colburn et al., 1997; Tanga et al., 2004) or morphine administration (Song and Zhao, 2001).

Our laboratory has previously shown that chronic treatment with the glial modulator, propentofylline, attenuates nerve injury-induced allodynia and hyperalgesia and reinstates the effectiveness of acute morphine (Raghavendra et al., 2003). In another study, we have shown that propentofylline attenuates the development of morphine tolerance as measured by mechanical hyperalgesia. At the molecular level, increases in mRNA for glial fibrillary acidic protein

ABBREVIATIONS: CNS, central nervous system; GFAP, glial fibrillary acidic protein; ITGAM, integrin α M; IL, interleukin; TNF, tumor necrosis factor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcripction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G_{T}, threshold values; MK-801, dizocilpine maleate.
(GFAP) and integrin α M (ITGAM) as well as mRNA and protein for the cytokines Interleukins (ILs) 1β and 6 and tumor necrosis factor (TNF) observed 16 h after the final morphine dose are suppressed in rats receiving both morphine and propofol (Raghavendra et al., 2004). Subsequently, Johnston et al. (2004) demonstrated, at 2 h postinjection, increases in cerebrospinal fluid IL-1β and spinal cord IL-1β, IL-6, and TNF mRNA following chronic but not acute central morphine administration. Furthermore, Song and Zhao (2001) have determined that coadministration of i.t. morphine with the glial metabolic inhibitor fluorocitrate significantly attenuates astrocyte activation and morphine analgentic tolerance. Our laboratory has sought to determine the effect on neuroimmune activation of twice daily s.c. morphine treatment in L5 spinal nerve-transected rats, thus examining both hyperalgesia and tolerance simultaneously. We observed glial activation in the dorsal horn (as visualized by immunohistochemistry) and increased levels of IL-1β, IL-6, and TNF mRNA and protein in the lumbar spinal cord 16 h after systemic morphine administration (Raghavendra et al., 2002). In contrast, acute morphine did not enhance spinal glial activation or cytokine expression, indicating that neuroimmune activation contributes to alterations related to prolonged opioid treatment. It is noteworthy that discontinuous morphine administration protocols have been shown to lead to variations in plasma drug concentrations that may result in successive periods of drug withdrawal (Dunbar and Pulai, 1998). This must be considered in the interpretation of the above data in which a twice daily administration protocol was utilized.

These results underscore the conceptual theory that opioid tolerance and neuropathic pain share common mechanisms not limited to the neuron. In particular, glial cells are also crucial. However, the mechanism by which CNS neuroimmune activation contributes to the induction and maintenance of morphine tolerance in a model of neuropathic pain remains unknown. The purpose of the present study was 3-fold: to further elucidate the relationship between morphine tolerance and neuroimmune activation in a neuropathic pain model, to compare the result of continuous morphine administration via two different methods (s.c. and i.t.) on transcriptional and translational control of spinal glial markers of activation, and to elucidate the time course of glial regulation with respect to morphine antiallodynia and the subsequent development of morphine tolerance.

Materials and Methods

Animals. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 150 to 175 g at the start of surgery were housed individually and maintained in a 12-12-h light/dark cycle with ad libitum access to food and water. The animals were allowed to habituate to the housing facilities for 1 week before experiments began. Behavioral studies were performed in a quiet room between the hours of 7:00 and 10:00 AM. The Institutional Animal Care and Use Committee at Dartmouth College approved the procedures in this study. Efforts were made to limit animal distress and use the minimum number of animals necessary to achieve statistical significance, in accord with guidelines set forth by International Association for the Study of Pain (Covino et al., 1980).

L5 Spinal Nerve Transection Surgery. Unilateral mononeuropathy was produced according to the method described by Colburn et al. (1999). Briefly, rats were anesthetized by inhalational halothane in an O2 carrier (induction, 4%; maintenance, 2%). A small incision to the skin overlying L5 to S1 was made, followed by retraction of the paravertebral musculature from the superior articular and transverse processes. The L6 transverse process was partially removed, exposing the L4 and L5 spinal nerves. The L5 spinal nerve was identified, separated, lifted, and transected, followed by removal of a 3-mm distal segment of nerve to prevent reconnection. The wound was irrigated with saline and closed in two layers with 5-0 polyester suture (fascial plane) and surgical skin staples.

Lumbar Catheterization and Osmotic Minipump Implantation. Osmotic minipumps (ALZET Osmotic Pumps; Durect Corporation, Cupertino, CA) were filled with test agents and primed overnight in 0.9% saline at 37°C. For i.t. delivery, 9 cm of PE-10 tubing was linked via 5 mm of silastic tubing to 4 cm of PE-60 tubing, which was then secured to the pump’s flow moderator before priming. For low-dose s.c. delivery for 3 and 6 days, pump model 1007D (0.5 μl/h, 100-μl capacity, 7 days) was used, and for 14 days, pump model 2002 (0.5 μl/h, 200-μl capacity, 14 days) was implanted. For high-dose s.c. delivery, model 2ML2 (5.0 μl/h, 2000-μl capacity, 14 days) was used for 3, 6, and 14 days of administration. The i.t. groups receiving 3 and 6 days of morphine or saline (low- and high-dose groups) were implanted with pump model 1007D, whereas the 14-day groups (low- and high-dose groups) received model 2002.

Rats were anesthetized under halothane inhalational anesthesia. A 1- to 3-cm skin incision was made on the lumbar back and skin was separated from the underlying fascia. The pump was placed in the s.c. pocket. For i.t. delivery, a modified hollow 18-gauge needle was inserted into the i.t. space at the L4 to L5 vertebral level (entry into the subarachnoid space was verified by a characteristic tail flick). The PE-10 catheter (already attached to the pump) was then advanced 3 cm cranially to reach the L5 spinal cord level using the hollowed needle as a track. The needle was then withdrawn, and the catheter was secured to the fascia using 3-0 polyester suture. The skin wound was closed with surgical staples.

Mechanical Allodynia. Mechanical sensitivity to non-noxious stimuli was measured by applying 2- and 12-g von Frey filaments (Stoelting, Wood Dale, IL) to the plantar surface of the ipsilateral hind paw (n = 8–12/group). Each round of testing consisted of three sets of 10 stimulations, with sets separated by 10 min from the previous (to avoid sensitization), for a total of 30 stimulations with each filament. The number of paw withdrawals observed is expressed out of a maximum of 30 possible withdrawals.

Immunohistochemistry. For assessment of GFAP and OX-42 (recognizing CR3/CD11b on microglia) immunoreactivity, a separate group of animals (n = 4/treatment) were anesthetized and transcardially perfused with 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde in PBS. Lumbar spinal cord sections were identified, isolated, and processed as described previously (Colburn et al., 1997). Immunohistochemistry was performed on 20-μm free-floating L5 spinal cord sections. Antibody to GFAP (1:15,000 working dilution; DakoCytomation California Inc., Carpinteria, CA) was used to label astrocytes, and OX-42 (1:2 working dilution, William F. Hickey, Dartmouth-Hitchcock Medical Center) was used to label CR3/CD11b expression on activated microglia. Scoring of tissue sections was carried out as previously described (Colburn et al., 1997).

Isolation of mRNA and Protein from Lumbar Spinal Cord. Tissue was collected from four to five rats/group on postoperative days 9, 12, and 20. To obtain both mRNA and protein from the same tissue sample, the L5 region of the spinal cord was isolated and placed in PBS supplemented with protease inhibitor cocktail (1:1000; Sigma-Aldrich, St. Louis, MO) and RNase inhibitor (0.75 U/μl; Ambion, Austin, TX). Samples were sonicated in five 1-s bursts at half-maximal power then centrifuged at 6500 rpm for 15 min at 4°C. Protein-containing supernatants were collected and stored at −80°C until further processing by Western blot analysis, whereas the pellet was treated with TRIzol reagent (Invitrogen, Carlsbad, CA) for the
Neuroimmune Activation in Morphine Tolerance and Pain

Western Blot for GFAP. Protein obtained from the L5 lumbar spinal cord was quantified using the Lowry method (Lowry et al., 1951) (DC assay; Bio-Rad, Hercules, CA). Twenty-five micrograms of protein and standard protein markers was subjected to SDS-polyacrylamide gel electrophoresis (4–20% gradient gel, Bio-Rad) and transferred to polyvinylidene difluoride (Bio-Rad) filters. Non-specific binding was blocked by incubation with 5% milk/PBS/Tween 20 at room temperature, and then membranes were incubated overnight at 4°C with monoclonal anti-GFAP primary antibody (1:250; BD Biosciences, San Jose, CA). The next day, blots were incubated for 1 h at room temperature with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:3000; Pierce Chemical, Rockford, IL), visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) for 1 min and imaged using the Typhoon Imaging System (Amersham Biosciences Inc., Piscataway, NJ). Finally, blots were incubated for 15 min in stripping buffer (Pierce) and reprobed with a monoclonal mouse anti-β-actin antibody (1:10,000; Abcam, Cambridge, MA) as a loading control. It was not possible to use this methodology to detect ITGAM protein.

Real-Time Reverse Transcription PCR. Total RNA was isolated from 60 to 80 mg of lumbar spinal cord tissue on post-transection days 9, 12, or 20 as described above. RNA samples were subsequently treated with DNase I (DNA-Free Kit; Ambion) to remove any contaminating genomic DNA. Reverse transcription (RT) was carried out using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA) according to the vendor’s protocol. Real-time RT-PCR reactions were carried out in a total reaction volume of 25 μL containing a final concentration of 1.5 U of Platinum Taq DNA polymerase (Invitrogen); 20 mM Tris HCl, pH 8.4; 50 mM KCl; 3 mM MgCl2; 200 μM dGTP, dCTP, and dATP; 400 μM dUTP and 1 U of uracyl DNA glycosylase; 900 mM forward and reverse primers; 300 nM TaqMan probe; and 5 μl of a 10-fold dilution of cDNA (50 ng) from the RT step. Primer and probe sequences for the genes of interest (GFAP, ITGAM, and GAPDH) are shown in Table 1. The iCycler Multicolor Real-Time PCR detection system (Bio-Rad) was used to quantify PCR product. The fluorescence and threshold values (Ct) thus obtained were used to compare the relative amount of target mRNA in experimental groups with those of controls using the 2^−ΔΔCt method (Livak and Schmittgen, 2001). Each experiment was run twice and samples were run in duplicate. For each sample, the mean Ct value for the control gene (GAPDH) was then subtracted from the mean Ct value for the gene of interest (GFAP, ITGAM) to obtain a ΔCt value. The ΔCt values for all animals in the control group (normal s.c. saline) were then averaged and subtracted from the ΔCt for each animal in the experimental groups to obtain the ΔΔCt. The relative fold change from control was then expressed by calculation of 2^−ΔΔCt for each sample, and the results are expressed as the group mean fold change ± S.E.M.

It has been shown that i.t. catheterization alone causes spinal glial activation (DeLeo et al., 1997). We determined the effect of catheterization alone by comparing i.t. saline uninjured values with s.c. saline-uninjured values by the 2^−ΔΔCt method and subtracting the effect of catheterization from the i.t. morphine-treated groups. This allowed us to compare the effect of direct CNS delivery of morphine with that of systemic administration without being confounded by the effects of catheterization.

Data Analysis. Values are expressed as means ± S.E.M. For behavioral and real-time RT-PCR data, comparisons between groups were performed using two-way analysis of variance with treatment group and length of exposure (day) as factors. Overall main effects of treatment and/or day are reported by the two-way analysis of variance P ratios and corresponding P values. Finally, individual group differences were ascertained by Bonferroni post tests. In all cases, P < 0.05 was considered significant.

Experimental Design. Rats received either an L5 spinal nerve transection or no surgery on day 0. The development of mechanical allodynia was monitored as described above on days 1, 3, and 5. Osmotic minipumps containing saline or morphine (1 or 10 mg/kg s.c.; 5 or 20 nmol/h i.t.; National Institute of Drug Abuse, Bethesda, MD) were implanted on day 6 post-transection; day 7 post-transection, therefore, corresponds to day 1 of morphine administration. Behavior was subsequently tested on days 7, 9, 12, and 20 post-transsection (i.e., days 1, 3, 6, and 14 of morphine administration). Rats were euthanized on days 9, 12, or 20 post-transsection (i.e., after 3, 6, or 14 days of continuous morphine or saline), and tissue was further processed by real-time RT-PCR, Western blot, or immunohistochemistry.

Results

General Results

As shown previously, L5 spinal nerve transection leads to the development of robust mechanical allodynia to 2- and 12-g von Frey filaments. Alldynia is maintained at least through day 20 of testing (P < 0.001 compared with uninjured controls). Uninjured control rats did not develop any sensitivity to non-noxious mechanical stimuli.

The Development of Tolerance to Antiallodynia Is Dependent on Dose and Method of Morphine Administration

Subcutaneous morphine administered via osmotic minipump on days 6 through 20 provided significant antiallodynia to L5 spinal nerve-transected animals on days 6, 7, and 9 (P < 0.001 compared with L5 spinal nerve-transected, saline group) in a dose-dependent manner (Fig. 1). After 6 days of morphine (i.e., post-transection day 12), low- and high-dose L5 spinal nerve-transected groups receiving morphine displayed increased numbers of withdrawals. However, these values remained significantly lower than for L5 spinal nerve-transected, saline controls (P < 0.05), indicating that tolerance was not complete.

Development of opioid-induced allodynia was recorded in uninjured rats after 14 days of s.c. morphine administration (post-transection day 20). Normal, morphine-treated rats dis-

<table>
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<tr>
<td></td>
<td></td>
<td>TaqMan probe</td>
<td>5’ ATCGAGAAGTTCGCTTCCGAAAG 3’</td>
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* The TaqMan probe has a reporter fluorescent dye, 6-carboxyfluorescein, at the 5’ end and fluorescence dye quencher, 6-carboxytetramethyl-rhodamine, at the 3’ end.
played greater paw withdrawals than saline-treated controls (4.7 ± 2.19 in 1 mg/kg morphine group and 5.8 ± 1.11 in 10 mg/kg morphine group versus 0.75 ± 0.25 in normal, saline-treated group, P > 0.05); however, this did not reach statistical significance.

Intrathecal morphine provided more effective antiallodynia to L5 spinal nerve-transected rats than s.c. morphine, as would be expected from the larger dose attained at the lumbar dorsal horn. Hastened antiallodynic tolerance was observed in i.t.-treated animals, as shown by the V-shaped curve (Fig. 2). On post-transection day 9 (i.e., after 3 days of morphine administration), paw withdrawals began to increase again, most notably in the high-dose group. Complete tolerance developed by post-transection day 12, at which time the number of paw withdrawals no longer differed significantly from that for L5 spinal nerve-transected, saline controls (22.9 ± 2.2 for group 6 versus 25.6 ± 0.7 for group 4, P = N.S.). After 14 days of i.t. morphine, normal (uninjured) rats displayed a trend toward evidence of mechanical allodynia (3.8 ± 1.3 paw withdrawals for group 3 versus 0.67 ± 0.33 for group 1).

Transcriptional Regulation of GFAP and ITGAM by Continuous Morphine

Subcutaneous Administration. After s.c. morphine treatment on days 6 through 20, we observed a significant main effect of treatment (F_{3,38} = 5.47, P < 0.01) and day (F_{2,38} = 4.7, P < 0.05) on GFAP mRNA expression. Treatment (F_{3,32} = 7.77, P < 0.001) and day (F_{2,32} = 3.55, P < 0.05) also contributed to the variation observed in ITGAM levels. Individual effects were also significant in s.c.-treated animals compared with normals; i.e., ITGAM mRNA levels in L5 spinal nerve-transected rats receiving high and low morphine were significantly increased compared with those of uninjured controls (Fig. 3B, P < 0.05) on days 9 and 20 post-transection (i.e., after 3 and 14 days of drug administration). However, alterations in astrocytic or microglial activation in the L5 spinal nerve-transected s.c. morphine group were not significantly different from those in L5 spinal nerve-transected s.c. saline controls at any time suggesting that s.c. morphine does not induce transcriptional alterations in glial markers above transection alone.

Intrathecal Administration. As described under Materials and Methods, i.t. catheterization alone produces spinal neuroimmune activation. Our results confirm this finding because uninjured rats receiving i.t. saline demonstrated increases in GFAP (Fig. 4A, P < 0.05 on days 12 and 20) and ITGAM (Fig. 4B, P < 0.01 on day 12) mRNA compared with s.c. saline-treated rats.

Following i.t. administration of low and high doses of morphine, a reliable main effect of treatment was observed (F_{3,39} = 8.74, P < 0.001 for GFAP; F_{3,37} = 7.09, P < 0.001 for ITGAM) and day (F_{2,39} = 14.10, P < 0.001 for GFAP; F_{2,37} = 1.595, P > 0.05, N.S. for ITGAM), in addition to a significant interaction between treatment and day for GFAP (F_{6,39} = 3.008, P < 0.05) (Fig. 5).

After i.t. administration of morphine to L5 spinal nerve-transected rats, a biphasic alteration occurred in both GFAP and ITGAM. GFAP mRNA in rats receiving 5 nmol/h morphine was 2.210 ± 0.257-fold higher than in controls on day 9, 1.38 ± 0.497-fold higher on day 12 (Fig. 5A, P < 0.001 versus day 20), and 3.85 ± 0.648-fold higher on day 20 (Fig. 5A, P < 0.001 versus L5 saline). In rats receiving 20 nmol/h morphine i.t., GFAP mRNA was 2.21 ± 0.278-fold higher than in controls on day 9, 1.325 ± 0.059-fold higher on day 12 (Fig. 5A, P < 0.01 versus day 20), and 3.24 ± 0.210-fold higher on day 20 (Fig. 5A, P < 0.05 versus L5 saline). These
results indicate that direct delivery of an opioid to the spinal cord activates astrocytes in a biphasic manner but did not seem dose-dependent (Fig. 5A).

Microglial activation as evidenced by increases in ITGAM mRNA levels in rats receiving 5 nmol/h morphine was 3.14 ± 0.535-fold higher than controls on day 9, 1.82 ± 0.668-fold higher on day 12, and 2.99 ± 0.710-fold higher on day 20. In rats receiving 20 nmol/h morphine ITGAM mRNA was 3.0 ± 0.481-fold on day 9 compared with controls, 1.3 ± 0.338-fold higher on day 12, and 2.77 ± 0.306-fold higher on day 20 (Fig. 5B). The enhanced fold increase in microglial ITGAM mRNA compared with astrocytic GFAP mRNA on day 9 demonstrates that these cells contribute to the development phase of tolerance induced by i.t. morphine more so than astrocytes.

**Translational Regulation of Glial Activation by Continuous Morphine**

In previous studies, we determined that GFAP and ITGAM protein increased following L5 spinal nerve transection alone (Sweitzer et al., 2001b; Raghavendra et al., 2003). In this study, no significant changes were seen in GFAP and ITGAM mRNA after continuous s.c. morphine treatment in L5 spinal nerve-transected rats compared with rats receiving L5 spinal nerve transection alone. Therefore, we selected a representative group (L5 spinal nerve-transected rats that had re-
ceived 6 days of 10 mg/kg morphine treatment) on which to perform immunohistochemistry to determine whether there were translational alterations in astrocytic or microglial markers and, if so, where such changes were observed in the spinal cord. As shown in Fig. 6, L5 spinal nerve transection alone (L5-txd, saline group) caused increased GFAP and OX-42 immunoreactivity in the dorsal horn of the lumbar spinal cord. Using this qualitative technique, comparable enhancement of GFAP immunoreactivity was observed after continuous morphine treatment for 6 days (L5-txd, morphine group) compared with the L5-txd, saline group (see Table 2). In contrast, OX-42 immunoreactivity after 6 days of morphine in L5 spinal nerve-transected rats was less robust than after L5 spinal nerve transection alone.

To fully characterize the effect of the method of administration on astrocytic activation, we performed Western blot analysis to determine whether there were translational alterations in astrocytic or microglial markers and, if so, where such changes were observed in the spinal cord. As shown in Fig. 6, L5 spinal nerve transection alone (L5-txd, saline group) caused increased GFAP and OX-42 immunoreactivity in the dorsal horn of the lumbar spinal cord. Using this qualitative technique, comparable enhancement of GFAP immunoreactivity was observed after continuous morphine treatment for 6 days (L5-txd, morphine group) compared with the L5-txd, saline group (see Table 2). In contrast, OX-42 immunoreactivity after 6 days of morphine in L5 spinal nerve-transected rats was less robust than after L5 spinal nerve transection alone.

To fully characterize the effect of the method of administration on astrocytic activation, we performed Western blot analysis to determine whether there were translational alterations in GFAP after s.c. or i.t. morphine administration for 3 or 6 days. L5 spinal nerve transection alone clearly elevated GFAP protein expression at both time points (Fig. 7), which confirms our previous findings (Sweitzer et al., 2001b) and our current immunohistochemistry findings (Fig. 4; L5-txd, L5 spinal nerve-transected, saline group). At days 9 and 12, L5 spinal nerve-transected rats receiving s.c. morphine (Fig. 7; L5 spinal nerve-transected, low-dose morphine group and L5 spinal nerve-transected, high-dose morphine group) showed an even greater increase in GFAP protein than the L5 spinal nerve-transected saline group. These results indicate that s.c. morphine treatment differentially regulates astrocytic activation, having a significant effect on translation but not on transcription of marker genes for astrocytes.

As noted earlier, i.t. catheterization alone can lead to glial activation; our Western blot data confirm this at the protein level. We observed increases in GFAP in normal rats receiving i.t. saline (Fig. 7, Ns group) compared with normal rats receiving s.c. saline (N group). Again, we observed increases in GFAP after L5 spinal nerve transection alone (L5) and exaggeration of this response with high-dose i.t. morphine treatment (LM group). Finally, high-dose s.c. morphine entrained less robust GFAP immunoreactivity as compared with high-dose i.t. morphine treatment indicating that i.t. opioid treatment enhanced astrocytic activation more than s.c. treatment.

**Discussion**

The hastened development of tolerance to antiallodynia in neuropathic pain states suggests an interaction between mechanisms of tolerance and hypersensitivity, yet few studies have looked at this dual paradigm. Instead, most reports...
determine mechanisms in either tolerance or chronic pain models. In the present study, we sought to uncover the role of CNS neuroimmune activation in a combined rodent model of neuropathic pain and morphine tolerance.

Behavioral Responses to Systemic and Central Morphine after L5 Spinal Nerve Transection. We utilized a modified Chung model of neuropathic pain (Kim and Chung, 1992) that has previously been shown to cause reproducible mechanical allodynia in rats (Sweitzer et al., 2001a, b), namely, transection (rather than tight ligation) of the L5 spinal nerve. Our results indicate a rapid increase in the number of paw withdrawals in response to non-noxious mechanical stimulation starting within 1 day after surgery, which was maintained throughout the study in the absence of pharmacological intervention. With the onset of continuous morphine treatment, mechanical allodynia was significantly attenuated for 3 days (with i.t. dosing) and 6 days (with s.c. dosing) before tolerance became evident. Tolerance may have developed more rapidly with i.t. administration in part due to the delivery of higher doses of agonist to CNS receptor sites; however, we postulate here that spinal glial activation may also play a role.

Interestingly, both spinal and systemic morphine were initially effective in treating neuropathic pain as measured by relief of mechanical allodynia. This finding is consistent with some previous studies (Yu et al., 1997; Suzuki et al., 1999; Christensen and Kayser, 2000) but not all (Lee et al., 1995; Ossipov et al., 1995a, b). In the former group, an initial analgesic effect of morphine in neuropathic pain-induced behaviors followed by tolerance development after 4 to 6 days of daily s.c. (Christensen and Kayser, 2000) or i.t. morphine administration was observed (Yu et al., 1997). Suzuki et al. (1999) demonstrated that i.t. morphine exhibited greater potency in inhibiting both natural and evoked spinal cord dorsal horn neuronal responses after spinal nerve ligation as compared with systemic morphine. These results suggest that ectopic firing leading to central sensitization in neuropathic pain can be attenuated by opioids and that the administration route is a crucial factor in treatment efficacy. These results directly support our current finding that centrally administered morphine more potently attenuates tactile allodynia following spinal nerve transaction than does systemically administered morphine; however, tolerance develops more quickly.

In contrast, a lack of effect of i.t. morphine in spinal nerve ligation has also been demonstrated (Ossipov et al., 1995b). This may be related to glutamatergic afferent drive because prolonged treatment with MK-801, an N-methyl-D-aspartate receptor antagonist, reinstated the acute effectiveness of centrally administered morphine. We have previously demonstrated that acute morphine has decreased efficacy in the treatment of tactile and thermal hypersensitivity in our L5

<table>
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<th>TABLE 2</th>
<th>Immunohistochemistry results for microglial (OX-42) and astrocytic (GFAP) activation in L5 spinal cord at day 12 post-transection in animals receiving s.c. saline or 10 mg/kg morphine</th>
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</table>

DH-ipsi, dorsal horn, ipsilateral to nerve transection; DH-contra, dorsal horn, contralateral to nerve injury.
spinal nerve transection rodent model (Raghavendra et al., 2003). The important difference in the present study is that a chronic dosing paradigm was used to assess the development of tolerance. We conclude that although the comparative potency of morphine may decrease, it is marginally useful for a limited time, which is supported by clinical evidence (Dworkin et al., 2003).

**Neuroimmune Involvement in Morphine Tolerance.**

Our results suggest that i.t. administration of morphine hastens the development of tolerance in neuropathic pain states by affecting both transcription and translation of glial activation genes. This conclusion follows from two observations. We did not find significant changes in GFAP or ITGAM mRNA in the L5 spinal nerve-transected group receiving s.c. morphine, and tolerance development lagged in these animals as compared with those receiving i.t. morphine. We did, however, observe significant increases in GFAP and ITGAM mRNA in the group receiving i.t. morphine. This suggests that i.t. morphine, but not s.c. morphine, enhances transcription. Increased GFAP protein was demonstrated by Western blot analysis in both the s.c. and i.t. morphine groups. This underlines the importance of distinguishing between mRNA and protein regulation of glial activation markers. Enhancement by s.c. morphine of translational processes is suggested by the fact that protein was increased in the s.c. group even though mRNA was not. This observation is consistent with that of Song and Zhao (2001), who demonstrated enhanced GFAP immunoreactivity in the spinal cord following induction of morphine tolerance by s.c. or i.t. administration. GFAP protein increased more after i.t. morphine than after s.c. morphine. We hypothesize that i.t. morphine treatment recruits both transcriptional and translational processes, thus having a stronger effect on glial cell activation and further development of antiallodynic tolerance.

We observed biphasic responses to i.t. morphine administration at the transcriptional level. That is, expression of mRNA for GFAP and ITGAM was enhanced in rats after 3 days of i.t. morphine (day 9 of study) and again after 14 days of i.t. morphine (day 20 of study) but was comparable with that in untreated controls after 6 days, at which time behavioral tolerance (i.e., increased allodynia) was also observed in the morphine-treated rats. In contrast, GFAP protein was enhanced above control levels after 6 days of morphine administration, when the animals were tolerant, indicating increased translation in the absence of transcriptional changes at this time. This may indicate a delayed functional effect of transcriptional alterations. The observed response of glial activation markers at the transcriptional level may, therefore, set up astrocytes and possibly microglia to contribute functionally to the expression of morphine antiallodynic tolerance and long-term maintenance. In addition, our data suggest an enhanced contribution of microglia to the development phase of morphine tolerance as we observed a larger fold change in ITGAM mRNA compared with GFAP mRNA at day 9 post-transection. This supports our previous findings that demonstrate a role for microglia in initiation of hypersensitivity (Tanga et al., 2004). Protein level assessment of microglial markers in future studies would enable us to further determine the distinct roles of each glial cell population.

We have observed enhanced glial activation at the protein level correlated with behavioral tolerance in an earlier study (Raghavendra et al., 2002). The designs of that study and the present one differ in two important ways. First, rats received intermittent morphine doses in the prior study, whereas in the present study, a continuous administration protocol was used to prevent daily variations in plasma drug levels, which have been shown to enhance tolerance (Ibuki et al., 1997). Second, rats were euthanized 16 h after the last morphine injection in the prior study and, therefore, may have been in withdrawal at the time of glial assessment, whereas in the present study, rats received drug up to the time of euthanasia. Despite these design differences, after 6 days of morphine administration, which corresponds to the expression phase of tolerance, we obtained similar protein level increases for GFAP in both studies. This suggests an important role for spinal astrocytes in mediating both tolerance to morphine and, possibly, withdrawal-induced hyperalgesia.

The specific role of glial cells in the development of tolerance remains unknown. However, we can speculate that due to their importance in both homeostasis and pathophysiology, they may contribute in several ways (Liberto et al., 2004). One role for glial cells is the maintenance of excitatory amino acid levels in the spinal cord through glial glutamate transporters (Danbolt, 2001). It has been shown that following morphine tolerance (Mao et al., 2002) or neuropathy (Sung et al., 2003), the expression of glutamate transporters in the spinal cord is altered. Given that transmission through neuronal glutamate receptors is likely to affect signal transmission and the coupling of m-opioid receptors (Trujillo and Akil, 1991), it is reasonable to postulate that changes in the synaptic milieu will have profound effects on morphine antiallodynia. In addition, glia can express cytokines, which have been shown to decrease the function of glutamate transporters in vitro (Ye and Sontheimer, 1996; Hu et al., 2000). However, a role for these cytokines in vivo on glutamate homeostasis remains to be determined. Finally, glia are capable of releasing nociceptive mediators such as reactive oxygen species and nitric oxide, implicating a direct action of glial-derived substances on neuronal sensitization.

Taken together, our results suggest that s.c. morphine does not transcriptionally modulate glial activation but does affect astrocytic translational mechanisms in tolerance or opioid-induced pain and that i.t. morphine modulates astrocytic activation both transcriptionally and translationally. Intrathecal morphine results in elevated activation of astrocytes, which contributes to the hastened development of morphine tolerance and may contribute to the expression of opioid-induced hypersensitivity. Given this and other emerging evidence of the key role of neuroimmune activation in aberrant pain processes and morphine tolerance, it can be anticipated that a more thorough understanding of the specific contributions of CNS glia would enhance treatment options for patients suffering from neuropathic pain.

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