

Role of Mitogen-Activated Protein Kinase Activation in Injured and Intact Primary Afferent Neurons for Mechanical and Heat Hypersensitivity after Spinal Nerve Ligation

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To investigate whether activation of mitogen-activated protein kinase (MAPK) in damaged and/or undamaged primary afferents participates in neuropathic pain after partial nerve injury, we examined the phosphorylation of extracellular signal-regulated protein kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK) in the L4 and L5 dorsal root ganglion (DRG) in the L5 spinal nerve ligation (SNL) model. We first confirmed, using activating transcription factor 3 and neuropeptide Y immunoreactivity, that virtually all L4 DRG neurons are spared from axotomy in this model. In the injured L5 DRG, the L5 SNL induced the activation of ERK, p38, and JNK in different populations of DRG neurons. In contrast, in the uninjured L4 DRG, the L5 SNL induced only p38 activation in tyrosine kinase A-expressing small- to medium-diameter neurons. Intrathecal ERK, p38, and JNK inhibitor infusions reversed SNL-induced mechanical allodynia, whereas only p38 inhibitor application attenuated SNL-induced thermal hyperalgesia. Furthermore, the L5 dorsal rhizotomy did not prevent SNL-induced thermal hyperalgesia. We therefore hypothesized that p38 activation in the uninjured L4 DRG might be involved in the development of heat hypersensitivity in the L5 SNL model. In fact, the treatment of the p38 inhibitor and also anti-nerve growth factor reduced SNL-induced upregulation of brain-derived neurotrophic factor and transient receptor potential vanilloid type 1 expression in the L4 DRG. Together, our results demonstrate that the L5 SNL induces differential activation of MAPK in injured and uninjured DRG neurons and, furthermore, that MAPK activation in the primary afferents may participate in generating pain hypersensitivity after partial nerve injury.

Key words: extracellular signal-regulated protein kinase; p38 mitogen-activated protein kinase; c-Jun N-terminal kinase/stress-activated protein kinase; brain-derived neurotrophic factor; TRPV1; neuropathic pain

Introduction

Peripheral nerve injury often results in spontaneous pain sensation, hyperalgesia, and allodynia, which are associated with neuropathic pain. A number of animal models of chronic pain after nerve injury have been introduced. Chronic constriction injury (CCI) of the sciatic nerve (Bennett and Xie, 1988), partial sciatic nerve ligation (Seltzer et al., 1990), L5 and L6 spinal nerve ligation (SNL) (Kim and Chung, 1992), and spared nerve injury (Decosterd and Woolf, 2000) have been widely used. All of these neuropathic pain models are made by partial nerve injury, in which some primary afferents are axotomized and the others are spared. Among them, the L5 SNL model is unique because the L4 dorsal root ganglion (DRG) neurons are clearly separated from the axotomized L5 DRG neurons. In previous studies, much at-

tention has been focused on the directly damaged primary afferents and their influence on the activity of dorsal horn neurons. However, there is increasing evidence suggesting that DRG neurons with intact axons also show an alteration of excitability and gene expression, and these changes might have functional roles in neuropathic pain (Ali et al., 1999; Gold, 2000; Michaelis et al., 2000; Wu et al., 2001; Ma et al., 2003; Schafers et al., 2003c). For example, substance P (SP), calcitonin gene-related peptide (CGRP), brain-derived neurotrophic factor (BDNF), and transient receptor potential vanilloid type 1 (TRPV1) increase in the intact DRG neurons in several neuropathic pain models (Ma and Bisby, 1998a,b; Hudson et al., 2001; Fukuoka and Noguchi, 2002).

Mitogen-activated protein kinases (MAPKs) transduce diverse extracellular stimuli to mitogenic and differentiation signals (Lewis et al., 1998; Widmann et al., 1999). The MAPK family includes extracellular signal-regulated protein kinases (ERKs), p38 MAPK, and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). Activation of ERK in DRG neurons contributes to pain hypersensitivity by increasing gene transcription, as well as by posttranslational modifications of target proteins (Aley et al., 2001; Averill et al., 2001; Dai et al., 2002; Dina et al., 2003; Obata et al., 2003b). p38 MAPK activation in DRG neurons has

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also been implicated in exaggerated pain states (Ji et al., 2002; Jin et al., 2003; Schafers et al., 2003b). However, there has been no study examining MAPK activation in adjacent uninjured DRG neurons after partial nerve injury. Furthermore, the contribution of JNK activation in sensory neurons to pain is unknown, although peripheral axotomy has been shown to induce JNK/SAPK activation in DRG neurons (Kenney and Kocsis, 1998).

In this study, we precisely identified the axotomized sensory neurons in the L4 and L5 DRG in the L5 SNL model and then set out to investigate whether ERK, p38, and JNK are activated in both damaged and undamaged primary afferents and participate in the development of mechanical and/or heat hypersensitivity. We now show that in the nearby uninjured L4 DRG, the L5 SNL induces activation of p38 but not ERK or JNK, and, furthermore, this p38 activation regulates BDNF and TRPV1 expression and contributes to thermal hyperalgesia, partially through alterations in the target-derived nerve growth factor (NGF).

Materials and Methods

Animals. A total of 130 male Sprague Dawley rats weighing 200–250 gm was used. All animal experimental procedures were approved by the Hyogo College of Medicine Committee on Animal Research and were performed in accordance with National Institutes of Health guidelines on animal care.

Surgical procedures. All experimental procedures were done on rats that were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Additional doses of the anesthetics were given as needed. In all rats, no surgery was performed on the right side. Special care was taken to prevent infection and to minimize the influence of inflammation. The hair of the rat's lower back and thigh was shaved, and the skin was sterilized with 0.5% chlorhexidine and covered with clean paper. Sterile operating instruments were used. Rats without surgery [$n = 4$ for immunohistochemistry and $n = 3$ for reverse transcription (RT)-PCR] were used as naive controls.

To produce an L5 SNL, a skin incision (3–4 cm) was made in the midline lumbar region (L4–S1). The L6 transverse process was identified, freed of muscular attachments, and partially removed with the help of bone ronguers. This exposed the L5 spinal nerve. The L5 ventral ramus was isolated, freed from the adjacent nerves, and then the L5 spinal nerve was tightly ligated with silk suture and transected distal to the ligature. After surgery, the wound was washed with saline and closed in layers (fascia and skin) with 3-0 silk thread. Animals were allowed to survive for 3, 7, or 14 d after surgery ($n = 4$ for immunohistochemistry for each time point).

For an additional group of rats that received the L5 SNL and the L4 or L5 dorsal rhizotomy surgery ($n = 8$), hemilaminectomy of the L4 and/or L5 vertebra after a similar skin incision was performed. After opening the dura and arachnoid membranes with a sharp needle, the left L4 or L5 dorsal root was exposed and cut 2–3 mm proximal to the DRG, and ~2 mm of the root was removed, taking care not to damage the ventral root. The distal and proximal stumps of the transected L4 or L5 dorsal root were then gently separated from each other to avoid nerve regeneration between them. Immediately after the L4 or L5 dorsal rhizotomy, the L5 SNL surgery was performed, as described above. Animals were allowed to survive for 14 d after surgery.

The intrathecal delivery of MAPK inhibitors or anti-NGF was performed basically as described previously (Fukuoka et al., 2001; Obata et al., 2003b). A laminectomy of the L5 vertebra was performed under adequate anesthesia with sodium pentobarbital. The dura was cut, and a soft tube (Silascon; outer diameter, 0.64 mm; Kaneka Medix, Osaka, Japan) was inserted into the subarachnoid space of the spinal cord at the L4/5 DRG level. For sustained intrathecal drug delivery, a mini-osmotic pump (Alzet type 2001; Durect, Cupertino, CA) that operates at a rate of 1 μ l/hr for a period of 7 d was filled with the MAPK kinase (MEK) 1/2 inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene (U0126; 0.5 μ g/ μ l; Calbiochem, La Jolla, CA), the p38 inhibitors 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)

imidazole (SB203580; 0.5 μ g/ μ l; Calbiochem) and FR167653 (10 or 50 μ g/ μ l; a gift from Fujisawa Pharmaceutical, Osaka, Japan), the JNK inhibitor anthra[1,9-cd]pyrazol-6(2H)-one (SP600125; 2.5 μ g/ μ l; Calbiochem), in 50% DMSO, or sheep anti-NGF antibody (1 μ g/ μ l; Chemicon, Temecula, CA), in normal saline. The catheter of the pump was implanted intrathecally at least 3 hr before the L5 SNL. DMSO (50%) or normal saline was used as the vehicle control. Rats were allowed to survive for up to 7 d after surgery [$n = 4$ for immunohistochemistry; $n = 4$ for *in situ* hybridization histochemistry (ISHH); $n = 3$ for RT-PCR].

The local application of anti-NGF was performed basically as described previously (Fukuoka et al., 2001). A soft tube (Silascon; outer diameter, 0.64 mm; Kaneka Medix) was placed on the surface of the L4 spinal nerve. For sustained peripheral drug delivery, a mini-osmotic pump (Alzet type 2001; Durect) that operates at a rate of 1 μ l/hr for a period of 7 d was filled with sheep anti-NGF antibody (1 μ g/ μ l; Chemicon), in normal saline. The catheter of the pump was implanted at least 3 hr before the L5 SNL. Normal saline was used as the vehicle control. Rats were allowed to survive for up to 7 d after surgery ($n = 4$ for immunohistochemistry).

Behavioral tests. All rats were tested for mechanical allodynia and thermal hyperalgesia of the plantar surface of the hindpaw 1 d before surgery and 3, 7, or 14 d after surgery. Mechanical allodynia was assessed with a dynamic plantar aesthesiometer (Ugo Basile, Comerio, Italy), which is an automated von Frey-type system (Kalmar et al., 2003; Lever et al., 2003). To measure rat hindpaw mechanical thresholds, rats were placed in plastic cages with a wire mesh floor and allowed to acclimate for 15 min before each test session. A paw-flick response was elicited by applying an increasing force (measured in grams) using a plastic filament (0.5 mm diameter) focused on the middle of the plantar surface of the ipsilateral hindpaw. The force applied was initially below detection threshold, increased from 1 to 50 gm in 1 gm steps over 20 sec, and was then held at 50 gm for an additional 10 sec. The rate of force increase was 2.5 gm/sec. The force applied to elicit a reflex removal of the ipsilateral hindpaw was monitored. This was defined as the mean of three measurements at 1 min intervals. The variability between trials was ~2 gm.

Heat hypersensitivity was tested using the plantar test (7370; Ugo Basile). A radiant heat source beneath a glass floor was aimed at the plantar surface of the hindpaw. Three measurements of latency were taken for each hindpaw in each test session. The hindpaws were tested alternately, with intervals between consecutive tests of >5 min. The three measurements of latency per side were averaged, and a difference score was computed by subtracting the average latency of the contralateral side from that of the ipsilateral side. Negative difference scores indicated a hyperalgesic response on the ipsilateral side. An assistant who was unaware of the treatment group performed all of the behavioral experiments.

Data are expressed as mean \pm SEM. Differences in changes of values over time of each group were tested using one-way ANOVA, followed by individual *post hoc* comparisons (Fisher's exact test). One-way ANOVA, followed by individual *post hoc* comparisons (Fisher's exact test) or pairwise comparisons (*t* test), was used to assess differences of values between the intrathecal groups. A difference was accepted as significant if $p < 0.05$.

Immunohistochemistry. The rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with 1% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, followed by 4% paraformaldehyde in 0.1 M PB 3, 7, or 14 d after surgery ($n = 4$ at each time point). The left L4/5 DRGs were dissected out and processed for phosphorylated-ERK (p-ERK) 1/2, p-p38, p-SAPK/JNK, activating transcription factor 3 (ATF3), neuropeptide Y (NPY), neurofilament (NF) 200, glial fibrillary acid protein (GFAP), tyrosine kinase (trk) A, BDNF, and TRPV1 immunohistochemistry according to the procedure used in our previous study (Noguchi et al., 1995). The polyclonal primary antibody for p-ERK1/2 (1:400; Cell Signaling Technology, Beverly, MA), p-p38 (1:400; Cell Signaling Technology), p-SAPK/JNK (1:400; Cell Signaling Technology), ATF3 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), NPY (1:2000; Amersham Biosciences, Little Chalfont, UK), GFAP (1:400; Dako, Glostrup, Denmark), trkA (1:500; Chemicon), BDNF (1:400; Chemicon), and TRPV1 (1:400; Oncogene, San Diego, CA) and the monoclonal

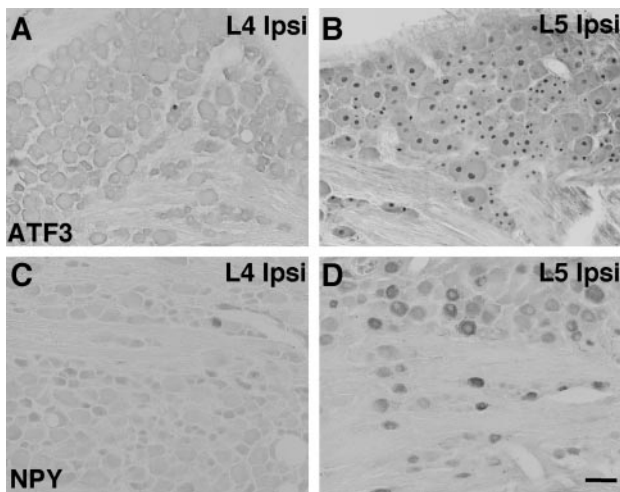


Figure 1. L4 DRG neurons are predominantly spared neurons in the L5 SNL model. Photomicrographs showing the ATF3 immunoreactivity (A, B) and NPY immunoreactivity (C, D) in the ipsilateral L4 and L5 DRG, respectively, at 7 d after L5 SNL surgery. A very small number of ATF3- and NPY-IR neurons was seen in the ipsilateral L4 DRG. In contrast, increased ATF3 and NPY immunoreactivity was clearly apparent in the ipsilateral L5 DRG. Scale bar: (in D) A–D, 100 μ m.

primary antibody for NF200 (1:400; Sigma, St. Louis, MO) were used for DAB staining. For the double immunofluorescent staining for p-p38 and NF200, GFAP, trkA, BDNF, or TRPV1, the tyramide signal amplification (PerkinElmer Life Sciences, Boston, MA) fluorescence procedures

(Michael et al., 1997; Obata et al., 2002) were used to detect staining for rabbit anti-p-p38 polyclonal antibody (1:10,000; Cell Signaling Technology).

The number of p-ERK1/2-, p-p38-, p-SAPK/JNK-, BDNF-, and TRPV1-immunoreactive (IR) neurons per section was counted in the left DRG. The proportion of p-ERK1/2-, p-p38-, p-SAPK/JNK-, BDNF-, and TRPV1-expressing DRG neurons was determined by counting the neuronal profiles that showed distinctive labeling in the DRG sections. In each rat, four to six sections of the L4/5 DRG at each time point were selected randomly, and 2000–3000 profiles were counted. An average percentage of p-ERK1/2-, p-p38-, p-SAPK/JNK-, BDNF-, and TRPV1-IR neurons, relative to the total number of neurons, was obtained for each animal across the different tissue sections, then the mean \pm SD across animals was determined. For the size-frequency histogram data, measurements of the area of positive neurons over selected tissue profiles were performed using a computerized image analysis system (NIH Image), and only neurons with clearly visible nuclei were used for quantification. To distinguish cell size-specific changes, we divided the DRG neurons into small (<600 μ m²), medium (600–1200 μ m²), and large (>1200 μ m²) neurons according to their cross-sectional area. At least 300 p-ERK1/2-, p-p38-, or p-SAPK/JNK-IR neuron profiles were measured in each group. Because a stereological approach was not used in this study, quantification of the data may represent a biased estimate of the actual number of cells and neurons. An assistant who was unaware of the treatment group of the tissue sections performed all counting.

Differences in changes of values over time were tested using one-way ANOVA, followed by individual *post hoc* comparisons (Fisher’s exact test). Pairwise comparisons (*t* test) were used to assess differences of values between the intrathecal groups. A difference was accepted as significant if *p* < 0.05.

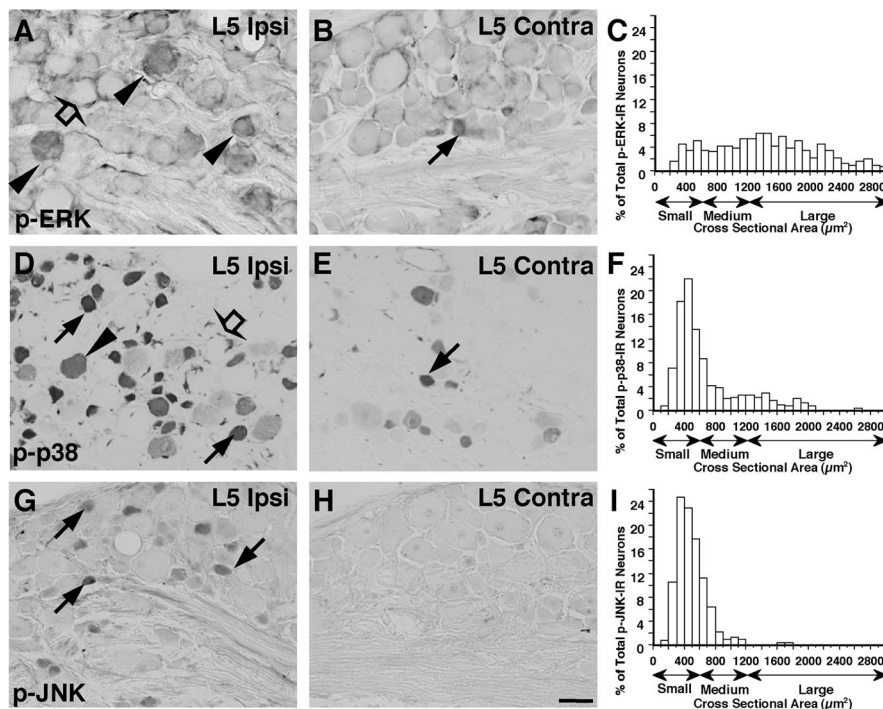


Figure 2. In the injured L5 DRG, L5 SNL induces an increase in p-ERK, p-p38, and p-JNK expression in different populations of DRG neurons and satellite glial cells. Photomicrographs show p-ERK (A, B), p-p38 (D, E), and p-JNK (G, H) immunoreactivity in the ipsilateral and contralateral L5 DRG, respectively, at 7 d after L5 SNL surgery. The L5 SNL increased p-ERK and p-p38 expression in both neurons and surrounding satellite cells (open arrows) in the ipsilateral L5 DRG. The arrowheads indicate medium-sized to large neurons, whereas the arrows indicate small sensory neurons. In contrast, the L5 SNL increased p-JNK expression only in small neurons (arrows). C, F, I, Size-distribution histogram of p-ERK-IR (C), p-p38-IR (F), and p-JNK-IR (I) neuron profiles in the ipsilateral L5 DRG at 7 d after the L5 SNL surgery. At least 300 neuron profiles with visible nuclei were counted in four to six randomly chosen sections from two rats (2–3 sections/rat). The increase in p-ERK and p-JNK was seen in large and small neurons, respectively, whereas the increase in p-p38 was detected in small to large-diameter neurons. Scale bar: (in H) A, B, D, E, G, H, 50 μ m.

ISHH. For the ISHH, the tissue was sectioned (16 μ m thick) with a cryostat, thaw-mounted onto Vectabond (Vector Laboratories, Burlingame, CA)-coated slides, and stored at -80° C until ready for use. The procedure for ISHH was basically the same as that used in previous studies (Yamanaka et al., 1999; Hashimoto et al., 2001). Briefly, the rat BDNF and TRPV1 cRNA probe corresponding to nucleotides 2273–2579 and 149–505, respectively, was prepared. The sections were treated with 10 μ g/ml proteinase K in 50 mM Tris-HCl and 5 mM EDTA for 3 min and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, then ³⁵S-labeled RNA probe (5×10^6 cpm/ml) was placed on these sections overnight at 55°C. Hybridized sections were rinsed in 5 \times SSC and 5 mM DTT for 30 min at 65°C, washed in high-stringency buffer for 30 min at 65°C, and treated with 2 μ g/ml RNase A for 30 min at 37°C. Sections were rinsed, dehydrated in an ascending ethanol series, and air dried. For autoradiography, the sections were coated with NTB-3 emulsion (Eastman Kodak, Rochester, NY), diluted 6:4 with distilled water at 45°C, and exposed for 2 weeks in light-tight boxes at 4°C. After development in D19 (Eastman Kodak) and fixation in 24% sodium thiosulfate, the sections were rinsed in distilled water, stained with hematoxylin–eosin, dehydrated in a graded ethanol series, cleared in xylene, and coverslipped.

Measurements of the density of silver grains over randomly selected tissue profiles were performed by a blinded assistant using the NIH Image program, in which only neuronal profiles that contained nuclei were used for quantification. At a magnification of 200 \times and with bright-field illumination, the upper and lower thresholds of gray level density were set such

that only silver grains were accurately discriminated from the background in the outlined cell or tissue profile and read by the computer pixel-by-pixel. Subsequently, the area of discriminated pixels was measured and divided by the area of the outlined profile, giving a grain density for each cell or tissue profile. To reduce the risk of biased sampling of the data because of varying emulsion thickness, we used a signal/noise (S/N) ratio for each cell in each tissue section. The S/N ratio of an individual neuron and its cross-sectioned area, which was computed from the outlined profile, was plotted. Based on this scattergram, neurons with a grain density of 10-fold the background level or higher ($10 \leq S/N$ ratio) were considered positively labeled for BDNF and TRPV1 mRNA. At least 250 neurons from each DRG of each rat were measured. The number of positively labeled DRG neurons was divided by the number of neuronal profiles counted in each DRG.

One-way ANOVA, followed by individual *post hoc* comparisons (Fisher's exact test) or pairwise comparisons (*t* test), was used to assess differences of values between the intrathecal groups. A difference was accepted as significant if $p < 0.05$.

RT-PCR. For the RT-PCR, the rats were killed by decapitation under deep anesthesia at 7 d after surgery, and the left L4 DRG was removed and rapidly frozen with powdered dry ice and stored at -80°C until ready for use. The procedure of extraction of total RNA using an RNA extraction reagent ISOGEN (Nippon Gene, Tokyo, Japan) was described in our previous study (Fukuoka et al., 2001). PCR primers for BDNF, TRPV1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA were designed corresponding to the coding region of the genes as follows: BDNF primers, sense 5'-GGCAG-GTTCGAGAGGTCTGA-3' and antisense 5'-CGCTGTGACCCACTCGCTAA-3'; TRPV1 primers, sense 5'-CCCTCCAGACAGAGAC-CCTA-3' and antisense 5'-CGCTTCTTGCTCCTCTGCAG-3'; GAPDH primers, sense 5'-TGCTGGTGCTGAGTATGTCG-3' and antisense 5'-GCATGTCAGATCCACAACGG-3'. Subsequent PCR was performed in a 50 μl solution of 1 \times PCR buffer (PerkinElmer Life Sciences), 0.2 mM deoxyNTP, and 1.25 U of AmpliTaq (PerkinElmer Life Sciences) with a pair of 50 pmol BDNF primers, 20 pmol TRPV1 primers, or 20 pmol GAPDH primers on a Perkin DNA Thermal Cycler (PerkinElmer Life Sciences), and the PCR program was 15 sec at 94°C , 15 sec at 57°C , and 45 sec at 72°C . The intensity of stained bands was measured with a computer-assisted imaging analysis system (ATTO Densitograph, version 4.02; ATTO, Tokyo, Japan). The density of PCR product bands of BDNF, TRPV1, and GAPDH mRNAs was increased between 25 and 35 PCR cycles, depending on the number of cycles; therefore the number of PCR cycles used was 30. The ratio of BDNF or TRPV1 to GAPDH mRNAs was considered to indicate the level of each transcript. The mRNA level was expressed as a percentage of the mRNA level in the normal control ganglia. Samples without the addition of reverse transcriptase or without the addition of RNA (negative controls) revealed no detectable product.

Data are expressed as mean \pm SD. Differences in changes of values between the intrathecal groups were tested using one-way ANOVA, followed by individual *post hoc* comparisons (Fisher's exact test) or pairwise comparisons (*t* test). A difference was accepted as significant if $p < 0.05$.

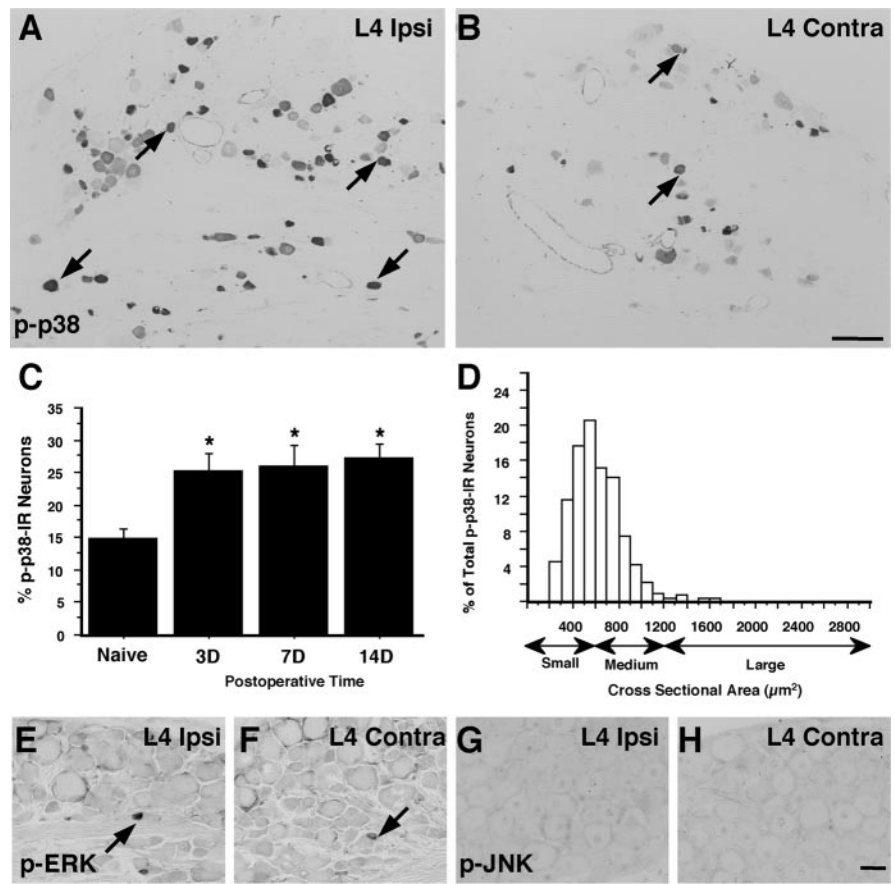


Figure 3. In the uninjured L4 DRG, L5 SNL induced a sustained increase in p-p38, but not p-ERK or p-JNK, mainly in small and medium-sized neurons. *A, B*, Photomicrographs showing p-p38 immunoreactivity in the ipsilateral (*A*) and contralateral (*B*) L4 DRG at 7 d after the L5 SNL surgery. There was an increase in the number of p-p38-IR neurons (arrows), but not satellite glial cells, in the ipsilateral L4 DRG. *C*, Time course of the mean percentages of p-p38-IR neurons relative to the total number of neurons in the ipsilateral L4 DRG. * $p < 0.05$ compared with the naive control. *D*, Size-distribution histogram of p-p38-IR neuron profiles in the ipsilateral L4 DRG at 7 d after the L5 SNL surgery. *E–H*, Photomicrographs showing p-ERK (*E, F*) and p-JNK (*G, H*) immunoreactivity in the ipsilateral and contralateral L4 DRG, respectively, at 7 d after the L5 SNL surgery. The L5 SNL did not alter p-ERK and p-JNK expression in the ipsilateral L4 DRG. Scale bar: (in *B*) *A, B*, 100 μm ; (in *H*) *E–H*, 50 μm .

Results

Virtually all L4 DRG neurons are spared from axotomy in the L5 SNL model

To identify the axotomized sensory neurons in the L4 and L5 DRG after the L5 SNL, we examined the immunoreactivity of ATF3, which has been suggested to be a neuronal injury marker (Tsujino et al., 2000; Suzuki et al., 2001). ATF3 immunoreactivity was expressed exclusively in the nuclei of virtually all ipsilateral L5 DRG neurons (Fig. 1*B*), whereas there was only occasional ATF3 immunoreactivity in the naive and ipsilateral L4 DRG throughout the 2 week time course of the experiment (Fig. 1*A*). We also assessed the expression of NPY in the L4 and L5 DRG because the increase in NPY expression occurs in the axotomized medium- to large-diameter DRG neurons (Wakisaka et al., 1991; Hokfelt et al., 1994). NPY was dramatically upregulated in the large-diameter neurons in the ipsilateral L5 DRG (Fig. 1*D*), but there were few NPY-IR neurons in the naive and ipsilateral L4 DRG (Fig. 1*C*), consistent with previous studies (Fukuoka et al., 1998; Ossipov et al., 2002). There was also no evidence of an upregulation of galanin immunoreactivity in the ipsilateral L4 DRG after the L5 SNL (data not shown). These observations indicate that L4 DRG neurons are essentially uninjured in the L5 SNL model.

L5 SNL induces differential activation of MAPK in injured L5 DRG neurons

To examine the activation of ERK, p38, and JNK in injured L5 DRG neurons, we used antiserum to the phosphorylated forms. In the ipsilateral L5 DRG, the L5 SNL induced an increase in the phosphorylation of ERK, mainly in large DRG neurons and in satellite glial cells (Fig. 2*A,B*), consistent with a previous study (Obata et al., 2003b). Seven days after L5 SNL surgery, 46 of 313 p-ERK-IR neurons counted (14.7%) were small, 76 (24.3%) were medium, and 191 (61.0%) were large in size (mean \pm SD of the cross-sectional area, $1414 \pm 673 \mu\text{m}^2$) (Fig. 2*C*). The L5 SNL also induced a sustained increase in p38 phosphorylation in small- to large-diameter DRG neurons and in satellite glial cells (Fig. 2*D,E*), as reported previously (Jin et al., 2003). Seven days after surgery, 192 of 312 p-p38-IR neurons counted (61.5%) were small, 73 (23.4%) were medium, and 47 (15.1%) were large in size (mean \pm SD of the cross-sectional area, $688 \pm 441 \mu\text{m}^2$) (Fig. 2*F*).

Furthermore, we examined the expression of p-JNK in the injured L5 DRG. p-JNK immunoreactivity was never detected in the naive and contralateral ganglia after the lesion (Fig. 2*H*); however, we found the activation of JNK mainly in small DRG neurons and not in satellite glial cells in the ipsilateral L5 DRG (Fig. 2*G*). Seven days after surgery, 241 of 315 p-JNK-IR neurons counted (76.5%) were small, 72 (22.9%) were medium, and 2 (0.6%) were large in size (mean \pm SD of the cross-sectional area, $496 \pm 204 \mu\text{m}^2$) (Fig. 2*I*). The p-JNK immunoreactivity in the ipsilateral L5 DRG was evident by 3 d after the L5 SNL, and the levels of p-JNK immunoreactivity persisted throughout the 2 week time course of the experiment, as did p-ERK and p-p38 immunoreactivity (data not shown).

L5 SNL induces the activation of p38, but not ERK or JNK, in the uninjured L4 DRG neurons

We first examined p-p38 immunoreactivity in the uninjured L4 DRG after L5 SNL. The number of p-p38-IR neurons in the ipsilateral L4 DRG markedly increased at 7 d after surgery compared with that in the naive and contralateral ganglia; the increase in p-p38 immunoreactivity was seen mainly in small and medium-sized neurons (Fig. 3*A,B*). There was no increase in the number of p-p38-IR satellite glial cells in the ipsilateral L4 DRG (Fig. 2*D*, open arrow). The time course of change in the percentage of p-p38-IR neurons is shown in Figure 3*C*. In the L4 DRG of naive rats, $15.0 \pm 1.4\%$ of the neurons were p-p38 immunoreactive. There was no significant change in the percentage of p-p38-IR neurons in the contralateral DRG (data not shown). The increase in the percentage of p-p38-IR neurons in the ipsilateral L4 DRG was first evident at 3 d after surgery ($25.6 \pm 3.0\%$; $n = 4$) and remained significantly elevated at 14 d after surgery ($27.3 \pm 2.4\%$) compared with those of naive control rats. Seven days after L5 SNL surgery, 169 of 311 p-p38-IR neurons counted (54.3%) were small, 137 (44.1%) were medium, and 5 (1.6%) were large in size (mean \pm SD of the cross-sectional area, $607 \pm 219 \mu\text{m}^2$) (Fig. 3*D*). The size of neurons labeled for p-p38 immunoreactivity in the L4 DRG was much smaller than that in the L5 DRG ($688 \pm 441 \mu\text{m}^2$; $p < 0.005$) (Fig. 2*F*), indicating that L4 DRG neurons are clearly different from damaged L5 DRG neurons. The total nonphosphorylated p38 levels did not change after the L5 SNL (data not shown).

Next, to determine whether ERK and JNK activation occurs in the ipsilateral L4 DRG after L5 SNL, we assessed p-ERK and p-JNK immunoreactivity. However, there was no apparent change in p-ERK and p-JNK immunoreactivity in the ipsilateral

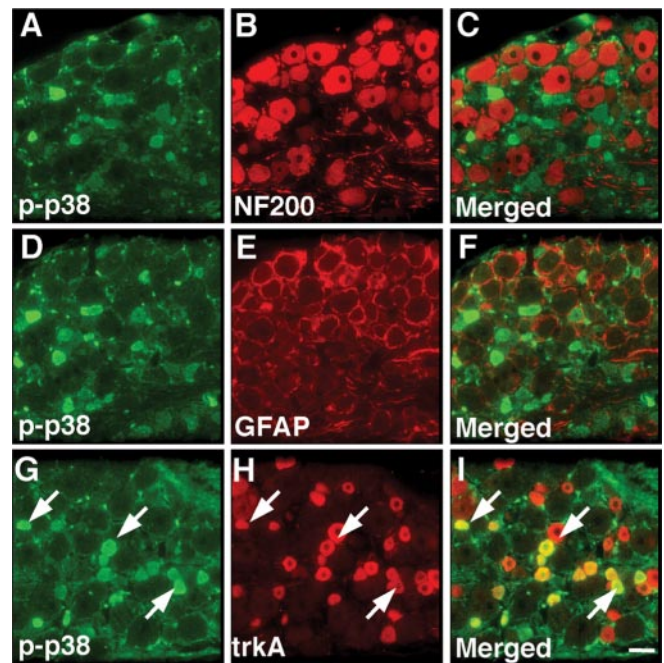


Figure 4. p38 is activated in small, presumably nociceptive DRG neurons, but not satellite glial cells in the uninjured L4 DRG after L5 SNL. Immunohistochemical colocalization of green reaction product for p-p38 (*A, D, G*) and red product for NF200 (*B*), GFAP (*E*), or trkA (*H*) in the ipsilateral L4 DRG at 7 d after the L5 SNL surgery. *A–C*, Double staining of p-p38 with NF200, a marker for myelinated A-fibers, showed no colocalization in the L4 DRG neurons, indicating that p38 is predominantly activated in unmyelinated C-fiber nociceptors. *D–F*, Double labeling of p-p38 with GFAP, a marker for activated satellite glial cells, indicates that p38 is not phosphorylated in activated satellite glial cells in the L4 DRG. *G–I*, Double staining for p-p38 and trkA revealed a heavy colocalization in the L4 DRG neurons. The arrows indicate double-labeled neurons for p-p38 and trkA. Scale bar: (in *I*) *A–I*, 50 μm .

L4 DRG compared with that in the naive and contralateral ganglia (Fig. 3*E–H*).

In the uninjured L4 DRG, p-p38 is predominantly expressed in trkA-containing small sensory neurons

To investigate whether an increase in p-p38 in the uninjured L4 DRG after the L5 SNL is seen in a subpopulation of the DRG neurons with myelinated fibers, we examined the immunohistochemical colocalization of p-p38 and NF200, a marker for myelinated A-fibers (Michael and Priestley, 1999). The results of the colocalization study with p-p38 and NF200 in the ipsilateral L4 DRG at 7 d after surgery are shown in Figure 4*A–C*. p-p38- and NF200-IR neurons were clearly distinguishable, indicating that p-p38 is predominantly expressed in neurons with unmyelinated axons, the C-fibers. Next, to determine whether p38 was phosphorylated in activated satellite glial cells in the L4 DRG, colocalization of the activated satellite glial cell marker GFAP with p-p38 was performed (Fig. 4*D–F*). There was no staining of p-p38 in activated glial cells that stained positively for GFAP. These findings again indicate that L4 DRG neurons are primarily undamaged/spared neurons, because peripheral axotomy induces p38 activation not only in large neurons but also in GFAP-IR glial cells, as described above (Fig. 2*D,F*). Furthermore, we performed double immunofluorescence with p-p38 and trkA in the L4 DRG (Fig. 4*G–I*). p-p38 heavily colocalized with trkA at 7 d after surgery, indicating that these p-p38-IR neurons were NGF-responsive small sensory neurons.

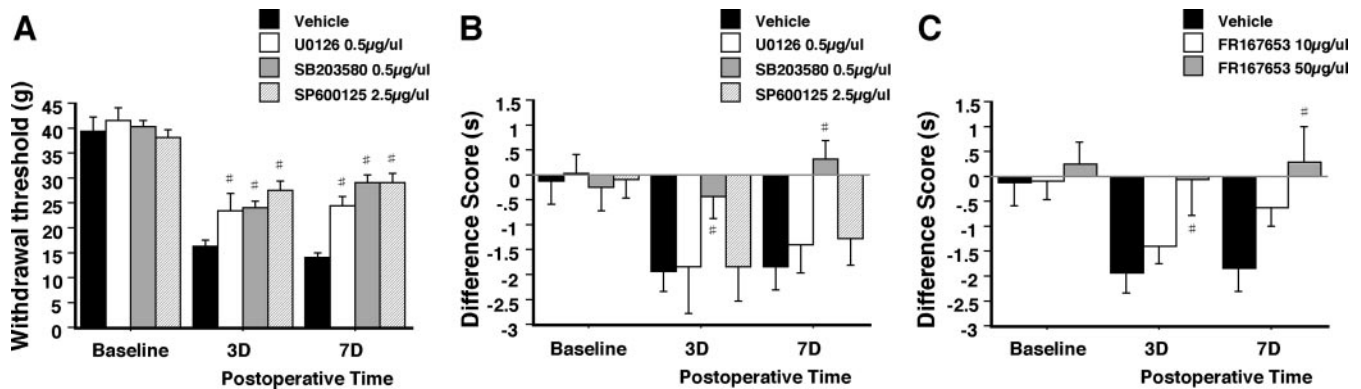


Figure 5. Effects of the MEK1/2 inhibitor U0126, the p38 inhibitors SB203580 and FR167653, or the JNK inhibitor SP600125 delivered intrathecally on L5 SNL-induced neuropathic pain behaviors. *A*, Mechanical allodynia was examined using an apparatus called a dynamic plantar aesthesiometer. Rats in the vehicle group developed mechanical hypersensitivity of the ipsilateral hindpaw at 3 and 7 d after surgery, but intrathecal U0126, SB203580, or SP600125 infusion significantly changed the threshold of response to mechanical stimulation. *B*, The difference score (latency on the operated side minus latency on the contralateral side) to the radiant heat stimuli was obtained from the same rats that received the mechanical stimuli. The mean difference score decreased and became a negative value at 3 and 7 d after surgery in the vehicle, U0126, and SP600125 groups, but only rats in the SB203580 group did not show a hyperalgesic response to thermal stimulation. Data from the vehicle group (■), the $0.5 \mu\text{g} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$ U0126 group (□), the $0.5 \mu\text{g} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$ SB203580 group (▨), and the $2.5 \mu\text{g} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$ SP600125 group (▩) are shown (mean \pm SEM; $n = 8/\text{group}$). $\#p < 0.05$ compared with vehicle control. *C*, Intrathecal administration of FR167653 also attenuated the L5 SNL-induced thermal hyperalgesia compared with the vehicle group, with the difference being dose dependent. Data from the vehicle group (■), the FR167653 ($10 \mu\text{g} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$) group (□), and the FR167653 ($50 \mu\text{g} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$) group (▨) are shown (mean \pm SEM; $n = 8/\text{group}$). $\#p < 0.05$ compared with vehicle control.

Inhibition of p38 activation attenuates L5 SNL-induced thermal hyperalgesia

To elucidate whether the inhibition of MAPK activation altered neuropathic pain behaviors, such as mechanical allodynia and thermal hyperalgesia, the MEK1/2 inhibitor U0126, the p38 inhibitor SB203580, or the JNK inhibitor SP600125 was delivered intrathecally before L5 SNL surgery and maintained for 7 d via a catheter, the tip of which was positioned close to the L4/5 DRG to target MAPK activity in the DRG. To obtain a sustained drug infusion, the drug was delivered by an osmotic pump connected to a catheter. Intrathecal administration of U0126, SB203580, or SP600125 into naive animals produced no significant changes in basal pain sensitivity (data not shown). Intrathecal U0126 ($0.5 \mu\text{g} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$) and SB203580 ($0.5 \mu\text{g} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$) significantly reduced the nerve injury-induced allodynia at 3 and 7 d after surgery, consistent with previous reports (Ciruela et al., 2003; Jin et al., 2003; Schafers et al., 2003b) (Fig. 5*A*). Furthermore, we found that the JNK inhibitor SP600125 ($2.5 \mu\text{g} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$) also inhibited mechanical hypersensitivity at 3 and 7 d after surgery (Fig. 5*A*). In contrast, rats in the U0126 and SP600125 groups did not show a significant change in thermal hyperalgesia compared with rats in the vehicle group (Fig. 5*B*). However, the p38 inhibitor SB203580 completely reversed the L5 SNL-induced hyperalgesia not only at 3 d but also at 7 d after surgery (Fig. 5*B*).

The p38 inhibitor SB203580 does not inhibit the phosphorylation of p38 but rather binds to the ATP pocket in the enzyme, thereby inhibiting its activity (Koistinaho and Koistinaho, 2002). On the other hand, FR167653 was first discovered as a potent inhibitor of tumor necrosis factor (TNF)- α and interleukin-1 β production (Yamamoto et al., 1996, 1997). Recent reports have shown that FR167653 inhibits the activation of p38 MAPK by

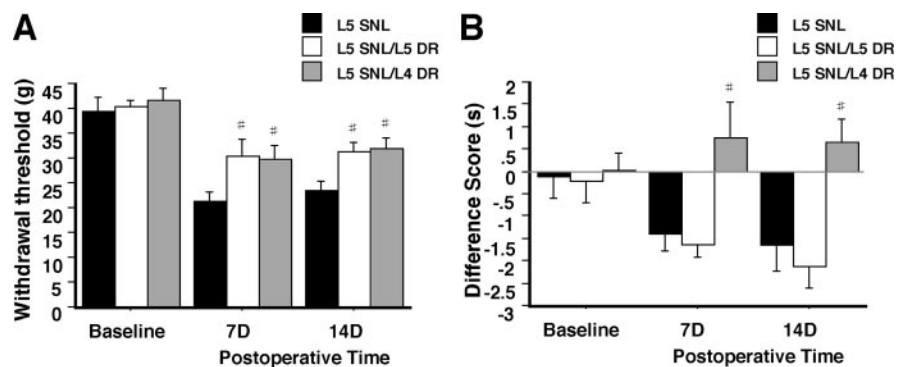


Figure 6. Effects of L4 or L5 dorsal rhizotomy (DR) on L5 SNL-induced mechanical (*A*) and heat (*B*) hypersensitivity at 7 and 14 d after surgery. *A*, Mechanical allodynia was examined using an apparatus called a dynamic plantar aesthesiometer. Rats in the SNL group developed mechanical hypersensitivity on the ipsilateral hindpaw at 7 and 14 d after surgery. In contrast, L5 dorsal rhizotomy significantly reduced this L5 SNL-induced mechanical allodynia. *B*, The difference score (latency on the operated side minus latency on the contralateral side) to the radiant heat stimuli was obtained from the same rats that received the mechanical stimuli. L5 dorsal rhizotomy was not able to prevent L5 SNL-induced thermal hyperalgesia. Data from the L5 SNL group (■), the L5 SNL/L5 DR group (□), and the L5 SNL/L4 DR group (▨) are shown (mean \pm SEM; $n = 8/\text{group}$). $\#p < 0.05$ compared with the SNL group.

suppressing the phosphorylation of p38, preferentially in the α isoform, but not in the γ isoform (Kawashima et al., 2001; Takahashi et al., 2001; Yoshinari et al., 2001). We therefore investigated whether not only SB203580 but also FR167653 blocks the SNL-induced thermal hyperalgesia (Fig. 5*C*). Intrathecal administration of FR167653 ($50 \mu\text{g} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$) significantly reduced the L5 SNL-induced heat hypersensitivity measured at 3 and 7 d. Rats in the FR167653 ($10 \mu\text{g} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$) group showed a trend toward decreased heat hyperalgesia compared with the vehicle control, although there was no significant change (Fig. 5*C*).

L5 dorsal rhizotomy did not block the SNL-induced thermal hyperalgesia

To ascertain whether p38 activation in the uninjured L4 DRG might be involved in the development of heat hypersensitivity in the L5 SNL model, we first assessed the effects of L4 dorsal rhizotomy on pain hypersensitivity produced by L5 SNL (Fig. 6). Rats in the L5 SNL and L4 dorsal rhizotomy group showed a signifi-

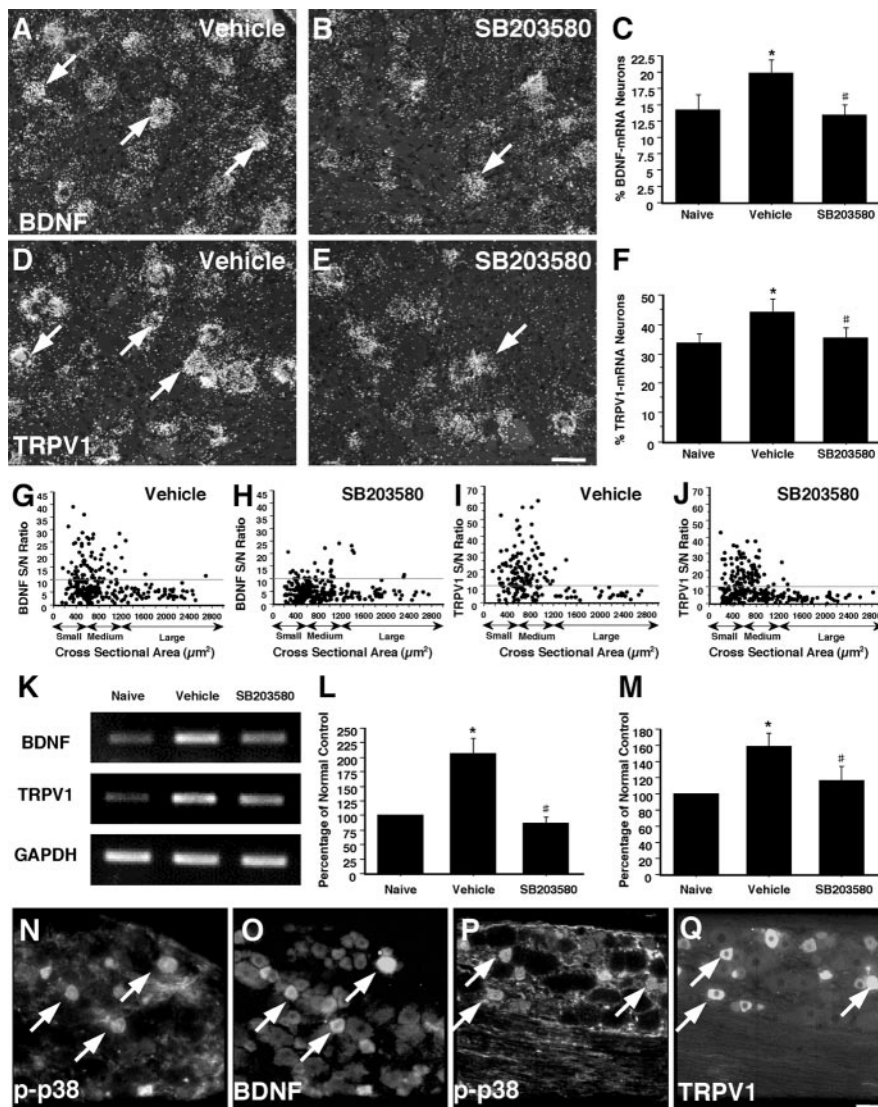


Figure 7. Effects of the p38 inhibitor SB203580 delivered intrathecally on L5 SNL-induced BDNF and TRPV1 expression in the uninjured L4 DRG. *A, B*, Photomicrographs showing the expression of BDNF mRNA (*A, B*) and TRPV1 mRNA (*D, E*) in the L4 DRG in the vehicle and SB203580 groups, respectively, at 7 d after L5 SNL surgery. *C, F*, Quantification of the percentage of BDNF mRNA-positive (*C*) and TRPV1 mRNA-positive (*F*) neurons at 7 d after the L5 SNL. **p* < 0.05 compared with the naive control; #*p* < 0.05 compared with vehicle control. *G–J*, The scatterplot diagrams of BDNF mRNA (*G, H*) and TRPV1 mRNA (*I, J*) were made by plotting the individual cell profiles according to the cross-sectional area (in micrometers squared; along the x-axis) and the S/N ratio (along the y-axis) in the vehicle and SB203580 groups, respectively. The dotted lines represent the borderlines between the negatively and positively labeled neurons (S/N ratio, 10). *K–M*, The levels of BDNF and TRPV1 mRNA in the ipsilateral L4 DRG as determined by the RT-PCR technique. Gel panels show PCR products from the L4 DRG taken at 7 d after surgery (*K*). The mRNA levels of BDNF (*L*) and TRPV1 (*M*) were expressed as a percentage of the mRNA level in the normal control ganglia (mean ± SD). **p* < 0.05 compared with the naive control; #*p* < 0.05 compared with vehicle control. *N–Q*, Double labeling for p-p38 (*N, P*) and BDNF (*O*) or TRPV1 (*Q*) immunoreactivity in the L4 DRG at 7 d after the L5 SNL. Double labeling of neurons with p-p38 and BDNF or TRPV1 was observed (*N–Q*; arrows). Scale bar: (in *E*) *A, B, D, E*, 50 μm; (in *N–Q*), 50 μm.

cant change in mechanical and heat hypersensitivity compared with rats in the L5 SNL group, indicating that the L4 spinal nerve and DRG is the main route through which the impulses evoked in the periphery are transferred to the spinal dorsal horn in the L5 SNL model (Takahashi et al., 1994; Prats-Galino et al., 1999; Decosterd and Woolf, 2000). Indeed, the difference scores shifted to positive values at 7 and 14 d after surgery (i.e., thermal hypoalgesia). Next, we examined whether L5 dorsal rhizotomy could reverse the L5 SNL-induced mechanical and heat hypersensitivity (Fig. 6). L5 dorsal rhizotomy that was made immediately before L5 SNL prevented the development of mechanical allodynia;

however, it was not able to block the SNL-induced thermal hyperalgesia at 7 d, and also at 14 d, after L5 SNL surgery, suggesting that the uninjured L4 DRG neurons might contribute to the development of thermal hyperalgesia. L4 or L5 dorsal rhizotomy, by itself, did not induce neuropathic pain behaviors across the testing period (data not shown).

p38 activation regulates BDNF and TRPV1 expression in the uninjured L4 DRG

Previous reports have demonstrated up-regulation of BDNF and TRPV1 expression in the uninjured L4 DRG after L5 SNL surgery (Ha et al., 2001; Hudson et al., 2001; Fukuoka et al., 2002). To examine whether BDNF and TRPV1 expression in the ipsilateral L4 DRG is regulated by p38 activation, the mRNA for BDNF and TRPV1 was compared in the vehicle and SB203580 groups using ISHH (Fig. 7*A–J*). The p38 inhibitor SB203580 (0.5 μg · μl⁻¹ · hr⁻¹) significantly reduced the SNL-induced increase in BDNF mRNA, which was seen mainly in small and medium-sized neurons (Fig. 7*G, H*). In the L4 DRG of naive rats, 14.4 ± 2.2% of the neurons were BDNF mRNA-positive neurons. The percentage of BDNF mRNA-positive neurons in the vehicle group at 7 d after the SNL was markedly increased to 20.0 ± 1.9%, but SB203580 was able to block the effects induced by the SNL (13.5 ± 1.4%; *n* = 4) (Fig. 7*C*). Furthermore, SB203580 suppressed the SNL-induced elevation of TRPV1 mRNA, which was also observed mainly in small- to medium-diameter sensory neurons (Fig. 7*D, E, I, J*). In the L4 DRG of naive rats, 34.1 ± 2.7% of the neurons were TRPV1 mRNA-positive neurons. The number of neurons labeled for TRPV1 mRNA in the vehicle group 7 d after the lesion was clearly increased (44.5 ± 4.1%; *n* = 4), but the increase in the percentage of TRPV1 mRNA-positive neurons was blocked significantly by the p38 inhibitor (35.6 ± 3.4%) (Fig. 7*F*). The changes in BDNF and TRPV1 mRNA/protein were

also confirmed by RT-PCR and immunohistochemistry (Fig. 7*K–M*, Table 1). The levels of BDNF and TRPV1 mRNA/protein in the vehicle group at 7 d after surgery were significantly greater than the preoperative value, but these increases were prevented by SB203580.

Next, to determine whether the p-p38-IR neurons and BDNF- or TRPV1-expressing neurons belonged to the same subset of DRG neurons, we performed double labeling for p-p38 and BDNF or TRPV1 in the ipsilateral L4 DRG (Fig. 7*N–Q*). Seven days after surgery, both BDNF and TRPV1 were detected in ~80% of p-p38-labeled neurons.

Table 1. Quantification of the percentage of BDNF- and TRPV1-IR neurons in the ipsilateral L4 DRG at 7 d after the L5 SNL ($n = 4/\text{group}$)

	Naive	Intrathecal injection		
		Vehicle	SB203580	Anti-NGF
BDNF	11.7 ± 0.9%	18.5 ± 2.5%*	11.3 ± 2.8%**	12.8 ± 4.0%**
TRPV1	33.0 ± 2.4%	39.9 ± 0.5%*	31.6 ± 2.0%**	34.0 ± 3.2%**

* $p < 0.05$ compared with the naive control; ** $p < 0.05$ compared with vehicle control.

NGF, p38 activation, and BDNF and TRPV1 expression in the uninjured L4 DRG

NGF injection in a peripheral target induces p38 activation in small DRG neurons (Ji et al., 2002; Delcroix et al., 2003), and NGF also increases the production of BDNF and TRPV1 mRNA through trkA receptors (Apfel et al., 1996; Winston et al., 2001). To elucidate whether alterations in endogenous NGF can trigger changes in L5 SNL-induced thermal hyperalgesia, activation of p38, and, furthermore, upregulation of BDNF and TRPV1 in the ipsilateral L4 DRG, we administered anti-NGF into the intrathecal space via a catheter, the tip of which was positioned close to the L4/5 DRG. Intrathecal anti-NGF application ($1 \mu\text{g} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$) reversed the development of thermal hyperalgesia at 3 and 7 d after surgery (Fig. 8A). Next, we assessed the effect of anti-NGF on L5 SNL-induced neurochemical changes in the L4 DRG. L5 SNL induced a substantial increase in the percentage of p-p38-IR neurons in the vehicle group at 7 d after surgery (Fig. 8B), but this increase was prevented by anti-NGF (Fig. 8C). Furthermore, anti-NGF significantly reduced the L5 SNL-induced increase in the percentage of BDNF mRNA- and TRPV1 mRNA-positive neurons in the L4 DRG at 7 d after surgery (Fig. 8D,E). The decreases in BDNF and TRPV1 mRNA/protein were also confirmed by RT-PCR and immunohistochemistry (Fig. 8F, Table 1). SP and CGRP expression in the ipsilateral L4 DRG was also decreased after intrathecal SB203580 or anti-NGF infusion (data not shown).

NGF is a neurotrophic factor that is retrogradely transported to the DRG from the periphery. Therefore, we aimed to block the NGF being transported to the L4 DRG using local application of anti-NGF antibody ($1 \mu\text{g} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$) on the surface of the L4 spinal nerve. Thermal hyperalgesia was apparent in the vehicle group at 3 and 7 d after surgery, whereas the anti-NGF group showed the significant reversal of heat hypersensitivity at 7 d after surgery (Fig. 8G). Furthermore, local anti-NGF application significantly reduced the L5 SNL-induced increase in BDNF and TRPV1 protein in the L4 DRG at 7 d after surgery (Table 2).

Discussion

In this study, we have shown that in the injured L5 DRG, the L5 SNL induced an increase in the phosphorylation of ERK, p38, and JNK in different populations of DRG neurons. Intrathecal ERK, p38, and JNK inhibitor infusions reversed the L5 SNL-induced mechanical allodynia. In contrast, in the uninjured L4 DRG, the L5 SNL induced the activation of p38, but not ERK and JNK, in trkA-expressing small- to medium-diameter neurons. Only p38 inhibitor treatment attenuated the L5 SNL-induced thermal hyperalgesia. L5 dorsal rhizotomy prevented the L5 SNL-induced mechanical allodynia but not thermal hyperalgesia. Furthermore, both p38 inhibitor and anti-NGF application blocked the SNL-induced increase in BDNF and TRPV1 expression in the L4 DRG.

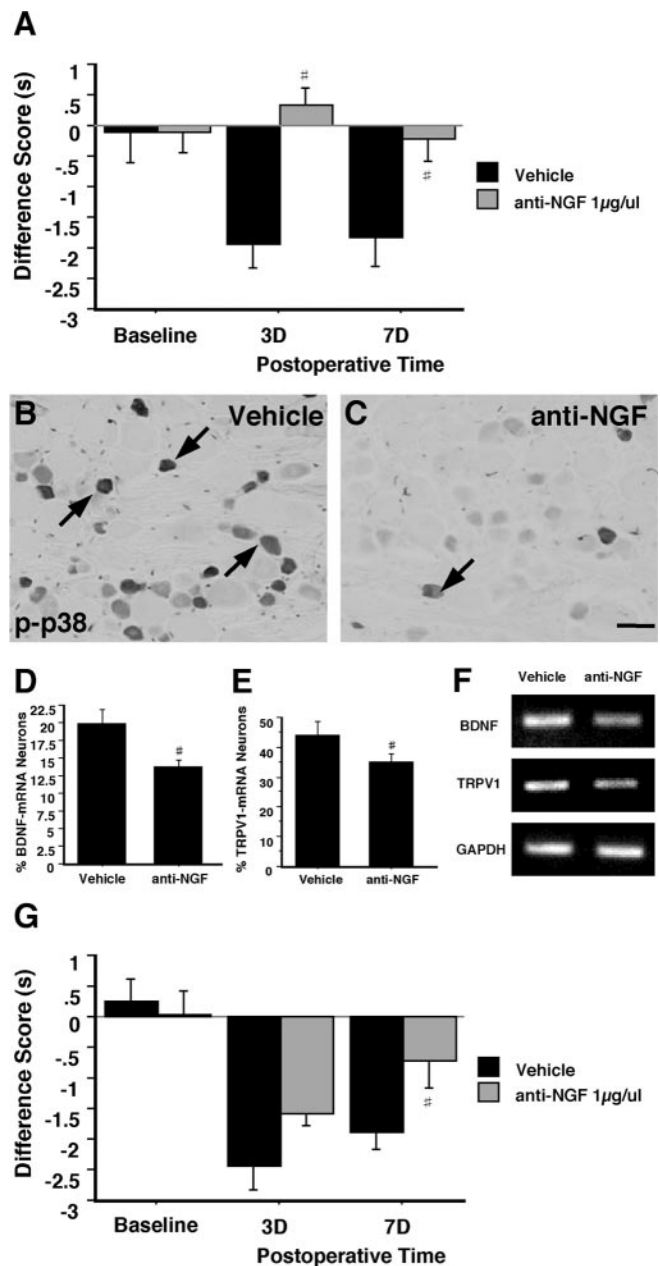


Figure 8. A–F, Effects of intrathecal infusion of anti-NGF on the L5 SNL-induced heat hyperalgesia and increase in p-p38, BDNF, and TRPV1 expression in the L4 DRG. A, Bar graph showing the difference scores to the radiant heat stimuli. Data from the vehicle group (■) and the $1 \mu\text{g} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$ anti-NGF group (▨) are shown (mean ± SEM; $n = 8/\text{group}$). * $p < 0.05$ compared with vehicle control. B, C, Photomicrographs showing p-p38 immunoreactivity in the L4 DRG in the vehicle (B) and anti-NGF (C) groups at 7 d after the L5 SNL surgery. D, E, Quantification of the percentage of BDNF mRNA-positive (D) and TRPV1 mRNA-positive (E) neurons at 7 d after the L5 SNL. * $p < 0.05$ compared with the vehicle group. F, The levels of BDNF and TRPV1 mRNA in the ipsilateral L4 DRG as determined by the RT-PCR technique. Gel panels show PCR products from the L4 DRG taken at 7 d after surgery. G, Effects of local application of anti-NGF on the L5 SNL-induced heat hypersensitivity. A bar graph showing the difference scores to the radiant heat stimuli is shown. Data from the vehicle group (■) and the $1 \mu\text{g} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$ anti-NGF group (▨) are shown (mean ± SEM; $n = 7/\text{group}$). * $p < 0.05$ compared with vehicle control. Scale bar, (in C) B, C, 50 μm .

p38 activation in the adjacent intact L4 DRG neurons and Wallerian degeneration

Recently, electrophysiological experiments have suggested that the uninjured primary afferents are functionally important in the maintenance of neuropathic pain (Ali et al., 1999; Michaelis et al.,

Table 2. Quantification of the percentage of BDNF- and TRPV1-IR neurons in the ipsilateral L4 DRG at 7 d after the L5 SNL (n = 3/group)

	Local application	
	Vehicle	Anti-NGF
BDNF	19.3 ± 2.3%	15.8 ± 2.2%*
TRPV1	40.1 ± 0.5%	35.7 ± 1.6%*

*p < 0.05 compared with the vehicle group.

2000; Wu et al., 2001). In addition, changes in the molecular phenotype of undamaged DRG neurons after partial nerve injury have been reported. For example, the expression of SP, CGRP, BDNF, and TRPV1 increases in the spared neurons (Fukuoka and Noguchi, 2002; Fukuoka et al., 2002; Obata et al., 2003a, 2004). In contrast, recent reports demonstrated that peripheral nerve injury induces the activation of p38 in axotomized DRG neurons (Jin et al., 2003; Schafers et al., 2003b). Furthermore, Kim et al. (2002) showed p38 activation in small sensory neurons after CCI; however, they did not identify whether these neurons were injured or spared. In the present study, using the L5 SNL model, we demonstrated for the first time that p38, but not ERK and JNK, was activated in the adjacent intact DRG, mainly trkA-containing small neurons. We also found that anti-NGF blocked the L5 SNL-induced p38 activation in the L4 DRG.

Wallerian degeneration, after nerve lesion, leads to an increase in cytokines and growth factors (Myers et al., 1996; Ramer et al., 1997; Sommer and Schafers, 1998; Cui et al., 2000; Shamash et al., 2002). Among them, NGF is suggested to be a major contributor to the persistent pain states, and NGF injection in a peripheral target induces p38 activation in small DRG neurons (Ji et al., 2002). Therefore, these findings suggest that after L5 SNL, NGF, synthesized and released in the degenerative nerve fibers, acts on nearby sensory fibers along the course of the nerve and induces p38 activation in the adjacent intact L4 DRG neurons. However, considering that not only p38 but also ERK and JNK could be activated by NGF (Averil et al., 2001; Mamet et al., 2003) and, furthermore, that local application of anti-NGF did not completely block the L5 SNL-induced thermal hyperalgesia, there is a possibility that other cytokines and growth factors, such as TNF- α , also may be involved in selective p38 activation in the L4 DRG (Schafers et al., 2003a).

BDNF and TRPV1 expression in adjacent intact L4 DRG neurons and thermal hyperalgesia

An enhanced transport of NGF to the DRG leads to an increase in the production of SP, CGRP, BDNF, and TRPV1 expression. In the present study, not only the p38 inhibitor but also anti-NGF reduced SNL-induced thermal hyperalgesia and upregulation of BDNF and TRPV1 expression in the intact L4 DRG. Furthermore, L5 dorsal rhizotomy did not prevent the development of heat hypersensitivity. Therefore, we believe that p38 activation by NGF in intact L4 DRG neurons participates in thermal hyperalgesia by regulating BDNF and TRPV1 expression after the L5 SNL.

BDNF that is synthesized in the DRG is transported to the central terminals of the primary afferents in the dorsal horn, is released, and acts on the trkB receptor on second-order sensory neurons (Kafitz et al., 1999; Mannion et al., 1999; Thompson et al., 1999). Several studies have reported that intrathecal injection of trkB-IgG and anti-BDNF prevented the development of thermal hyperalgesia in the peripheral inflammation and neuropathic pain models (Kerr et al., 1999; Fukuoka et al., 2001; Groth and Aanonsen, 2002), although the action of BDNF in the dorsal

horn is likely to be complex (Pezet et al., 2002; Malcangio and Lessmann, 2003). In contrast, TRPV1, one of the transducer proteins, can generate depolarizing currents in response to noxious thermal stimuli. Our data are very consistent with previous electrophysiological and gene knock-out studies of TRPV1 function (i.e., TRPV1 is required for peripheral sensitization to noxious thermal but not mechanical stimuli) (Caterina et al., 2000; Davis et al., 2000). The present study showed the upregulation in TRPV1 mRNA/protein in the uninjured DRG, although several studies have demonstrated an increase in the TRPV1 protein but not in its mRNA, after peripheral inflammation (Tohda et al., 2001; Ji et al., 2002). The reasons for this discrepancy are not clear, but other reports have shown that inflammation and/or NGF increased TRPV1 mRNA in adult DRG neurons (Winston et al., 2001; Amaya et al., 2003).

MAPK activation in the injured L5 DRG neurons and mechanical allodynia

Nerve injury-induced pain has been closely linked to activation of spontaneous and persistent abnormal discharge from ectopic foci, primarily observed in amyloid β (Devor, 1991; Koltzenburg et al., 1994; Devor and Seltzer, 1999), and A-fibers have been suggested to drive central sensitization (Scholz and Woolf, 2002). The present study demonstrated that in the injured L5 DRG, most of the p-ERK-IR neurons and a subpopulation of p-p38-IR neurons were large in size and that the ERK and p38 inhibitors reversed the L5 SNL-induced mechanical allodynia. Furthermore, L5 dorsal rhizotomy prevented the development of mechanical allodynia, as reported previously (Yoon et al., 1996; Sukhotinsky et al., 2004). These observations support the possibility that large-diameter A-fibers mediate mechanical allodynia, whereas nociceptive C-fibers mediate thermal hyperalgesia (Ossipov et al., 1999; Pan et al., 2003). A recent report showed that nerve injury-induced tactile but not thermal hypersensitivity is mediated by upregulated NPY via large fibers projecting to the gracile nucleus (Ossipov et al., 2002). Therefore, we speculate that MAPK activation in injured large DRG neurons participates in allodynia development by regulating NPY expression after the L5 SNL.

It is well known that withdrawal of growth factors activates the JNK pathway, which could underlie the negative regulation. In addition, JNK is also activated by positive factors such as the cytokine TNF- α in cultured sensory neurons (Pollock et al., 2002). An unexpected finding in the present study was that the JNK inhibitor SP600125 inhibited SNL-induced mechanical allodynia, whereas the increase in p-JNK was found predominantly in injured small neurons. Several studies revealed spontaneous activity only in A-fiber afferents but not in injured C-fiber afferents (Boucher et al., 2000; Liu et al., 2000); however, other reports showed a crucial role for small sensory neurons in the development of mechanical allodynia (Nichols et al., 1999; Porreca et al., 1999). Therefore, we believe that no matter what the cell size, the activation of MAPK in the injured L5 DRG plays an important role in allodynia development after nerve injury by increasing gene transcription, although the mechanisms of MAPK activation in different populations of L5 DRG neurons are not clearly characterized at this point. Alternatively, we cannot exclude the possibility that p38 activation in the uninjured L4 DRG contributes to the development of mechanical allodynia.

MAPK signaling in the spinal cord and pain hypersensitivity Sheen and Chung (1993) demonstrated that L5 dorsal rhizotomy attenuated L5 SNL-induced thermal hyperalgesia. Furthermore,

others reported that L5 dorsal rhizotomy did not attenuate L5 SNL-induced mechanical allodynia and that L5 dorsal rhizotomy itself can lead to pain behaviors (Colburn et al., 1999; Tabo et al., 1999; Eschenfelder et al., 2000; Li et al., 2000; Sheth et al., 2002). Therefore, various methodological factors, such as the type of injury, the extent of the lesion, the distance of the injury from the DRG, time courses, and behavioral testing techniques, may contribute to the discrepancy between our observations and those of others.

A number of pathophysiological changes in the spinal cord and the higher CNS might occur and participate in producing neuropathic pain. Indeed, recent studies have demonstrated that the activation of MAPK in dorsal horn neurons and glial cells in the spinal cord contributes to persistent inflammatory and neuropathic pain (Ji, 2004; Obata and Noguchi, 2004). For example, the reduction in neuropathic pain by p38 inhibition occurs before the appearance of p38 activation in DRG neurons (Jin et al., 2003). Therefore, the effects of MAPK inhibitors on pathological pain could be mediated by the inhibition of MAPK activation, not only in the DRG, but also in the spinal cord. However, these pathological changes in the CNS are secondary to pathophysiological changes in first-order sensory neurons and require altered afferent input for their maintenance (Devor and Seltzer, 1999). We believe that the differential activation of MAPK in injured and intact primary afferents is likely to have a substantial role in the pathogenesis of neuropathic pain after partial nerve injury, and MAPK pathways in nociceptors may be potential targets for the development of novel analgesics.

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