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6 **Differential induction of c-Fos and phosphorylated ERK by a noxious**  
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8 **stimulus after peripheral nerve injury**  
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## Abstract

Induction of c-Fos and phosphorylated extracellular signal-regulated kinase (p-ERK) has been demonstrated in spinal dorsal horn neurons by noxious stimuli. In this study, we compared induction of c-Fos and p-ERK in the spinal dorsal horn after peripheral nerve injury. We examined the spinal dorsal horn for noxious heat-induced c-Fos and p-ERK protein-like immunoreactive (c-Fos- and p-ERK-IR) neuron profiles after tibial nerve injury. A large number of c-Fos- and p-ERK-IR neuron profiles were induced by noxious heat stimulation to the hindpaw in sham-operated animals. A marked reduction in the number of c-Fos- and p-ERK-IR neuron profiles was observed in the medial 1/3 (tibial territory) of the superficial dorsal horn at 3 and 7 days after nerve injury. Although c-Fos-IR neuron profiles had reappeared in large numbers by 14 days after injury, the number of p-ERK-IR neuron profiles remained decreased in the tibial territory of the superficial dorsal horn. Double immunofluorescence labeling for c-Fos and p-ERK induced by noxious heat stimulation to the hindpaw at different time points revealed that a large number of c-Fos-IR, but not p-ERK-IR, neuron profiles were distributed in the tibial territory 14 days after injury. Although administration of a MEK 1/2 inhibitor (PD98059) to the spinal cord suppressed noxious heat-induced c-Fos expression in the peroneal territory, this treatment did not alter c-Fos induction in the tibial territory after nerve injury. We conclude that ERK phosphorylation may be

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involved in c-Fos induction in normal nociceptive responses, but not in exaggerated c-Fos induction after nerve injury.

**Keywords:** c-Fos; ERK phosphorylation; nerve injury; spinal dorsal horn; neuropathic pain

For Peer Review Only

## Introduction

Peripheral nerve injuries often cause neuropathic pain, including abnormal pain sensations such as hyperalgesia and allodynia [1]. These abnormal pain sensations have been reported outside, as well as within, the peripheral territory of the injured nerve. Disruption of the normal arrangement of spinal dorsal horn neurons caused anomalous responses to stimulation of the skin outside their original receptive fields, from which they had now been disconnected [2-5].

c-Fos, the protein product of the immediate early gene (IEG) *c-fos*, has long been known as a marker for the activation of nociceptive neurons in the spinal and medullary dorsal horns [6, 7]. It has been shown that induction of c-Fos in the spinal and medullary dorsal horns is enhanced in response to stimulation of the surrounding areas innervated by the injured nerve [8-10]. Our recent studies indicated that anomalous convergent nociceptive inputs from the uninjured nerves were activated after nerve injury, suggesting that these changes may contribute to the expansion of hyperalgesia to the adjacent areas innervated by the injured nerve [11-13].

Extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family, is activated via phosphorylation of upstream kinase MEK (MAP/ERK kinase). ERK activation requires a cascade that involves sequential activation of Ras, Raf, and MEK [14]. Previous studies have shown that

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6 phosphorylation of ERK is involved in the neuronal plasticity responsible for learning  
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8 and memory, and may have a significant role in pain hypersensitivity [15, 16].  
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10 Induction of phosphorylated ERK (p-ERK) by noxious stimuli was also reported in the  
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12 spinal and medullary dorsal horns [17, 18]. However, the time courses of c-Fos and p-  
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14 ERK induction in dorsal horn neurons following noxious stimulation have been shown  
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16 to be quite different. Following noxious stimulation, c-Fos induction begins after 30  
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18 min, peaks at 2 h, and returns to basal levels after 8-24 h. On the other hand, p-ERK  
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20 induction begins within 1 min, peaks at around 5 min, and returns to basal levels by 2 h  
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22 after noxious stimulation [17, 19].  
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30 It has been documented that p-ERK translocates into the nucleus and results in the  
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32 induction of IEG expression [20]. Intrathecal pre-treatment with a MEK 1/2 inhibitor  
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34 attenuated ERK phosphorylation induced by mechanical pressure and led to decreased  
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36 expression of c-Fos [21]. Therefore, the phosphorylation of ERK can be considered to  
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38 regulate c-Fos expression. However, intracellular signaling mechanisms regulating c-  
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40 Fos expression in a neuropathic pain state remain to be elucidated. In this study, we  
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42 investigated noxious heat-induced c-Fos and p-ERK protein-like immunoreactivities in  
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44 the spinal dorsal horn after tibial nerve injury.  
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## Materials and Methods

### *Surgery*

The study was reviewed and approved by the Animal Care and Use Committee, Okayama University. The animal treatments were performed according to the guidelines of the International Association for the Study of Pain. Animals were housed under conditions of optimum light and temperature (12 h light-dark cycle,  $20 \pm 2$  °C) with food and water provided *ad libitum*. The number of rats and the intensity of noxious stimuli used were the minimum necessary to produce consistent effects, and every attempt was made to reduce their suffering at all stages of the study.

Male Sprague-Dawley rats, each weighing 200-220 g at the time of surgery, were used in this study. A tibial nerve injury was performed under anesthesia with an intraperitoneal (i.p.) injection of pentobarbital sodium (Somnopentyl, 50 mg/kg), as previously described [12, 13, 22]. To expose the right sciatic nerve, a skin incision between the mid-thigh level and the popliteal fossa and a blunt dissection through the biceps femoris muscle were made. The three major divisions of this nerve (the tibial, sural, and common peroneal nerves) could be identified by their individual perineuria. The tibial nerve was then ligated firmly at two separate points with 7-0 silk sutures, and the nerve bundle between the two ligatures was transected with fine scissors. Sham-

operated animals were used as controls. The contralateral side was left intact in all rats.

### ***Induction of c-Fos and p-ERK by noxious heat stimulation after nerve injury***

After a survival time of 3, 7, or 14 days after nerve injury or sham surgery, animals were deeply anesthetized with pentobarbital sodium (50 mg/kg i.p.) and the right hindpaw was dipped in hot water (55 °C) for 10 seconds for noxious heat stimulation. Rats stimulated by non-noxious water (20 °C) at each time point after nerve injury or sham surgery were used as sham-stimulated controls (n = 3 in each group). Five min (for p-ERK; n = 5 for each survival time after nerve injury) or 2 h (for c-Fos; n = 5 for each survival time after nerve injury) after noxious heat stimulation, rats were perfused transcardially with saline, and then with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Under a dissecting microscope, the spinal cord, including the 4th and 5th lumbar (L4 and L5) segments with the dorsal and ventral roots and the dorsal root ganglia attached, was removed and post-fixed in the same fixative solution for 24 h at 4 °C, and then immersed in 0.02 M phosphate-buffered saline (PBS, pH 7.4) containing 20% sucrose for 48 h at 4 °C. The frozen sections from the L4-L5 segments were cut on a transverse plane at 50- $\mu$ m thickness, and 40 alternate series of free-floating sections were collected serially in PBS. Ten consecutive sections were assigned to 1 of 4 blocks; i.e., each of the L4 and L5 segments was subdivided into the

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6 rostral and caudal blocks. Sections were immersed in 0.3% hydrogen peroxide in 80%  
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8 methanol to quench endogenous peroxidase activity for 1 h at room temperature (RT).  
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10 The sections were blocked with 3% normal goat serum for 1 h at RT, and then incubated  
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12 in a rabbit polyclonal anti-c-Fos antibody (1:8,000; Santa Cruz Biotechnology, Santa  
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14 Cruz, CA, USA) or a rabbit polyclonal anti-p-ERK antibody (1:8,000; Cell Signaling,  
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16 Beverly, MA, USA) for 72 h at 4 °C. The sections were sequentially incubated with  
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18 peroxidase conjugated goat anti-rabbit IgG (1:600; Rockland, Gilbertsville, PA, USA)  
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20 for 1 h at RT and peroxidase anti-peroxidase (PAP) complex (1:3,000; Rockland) for 1 h  
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22 at RT. Immunoreaction was visualized with 0.002% 3,3'-diaminobenzidine-  
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24 tetrahydrochloride (DAB), 0.08% nickel ammonium sulfate, and 0.006% hydrogen  
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26 peroxide in 0.05 M Tris buffer (pH 7.4). All sections were mounted on gelatin-coated  
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28 glass slides, air-dried, dehydrated in graded alcohol solutions, cleared in xylene, and  
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30 coverslipped.  
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40 As shown in Figure 1, c-Fos protein-like immunoreactivity was found in the  
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42 neuronal nucleus. Therefore, the c-Fos protein-like immunoreactive profiles are  
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44 referred to as c-Fos protein-like immunoreactive (c-Fos-IR) neuron profiles, hereafter.  
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46 Immunoreactivity for p-ERK appeared as staining spread over the neuronal perikaryon  
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48 or scattered as filiform labeling (Fig. 1). Since filiform labeling was considered to be  
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50 derived from dendrites or axons, we focused on neuronal perikaryal labeling for p-ERK-  
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52 IR neuron profiles. The analysis was based on previous findings [8, 23, 24]. The  
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6 central terminals of primary neurons in the sciatic nerve were most abundant in the  
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8 caudal L4 and rostral L5. Within the caudal L4 and rostral L5, the medial 1/3 of the  
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10 spinal dorsal horn (tibial territory) received inputs from the tibial nerve, which  
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12 innervates the plantar surface of the hindpaw, whereas the lateral 1/4 received inputs  
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14 from primary neurons supplying the posterior cutaneous nerve of the thigh. The area  
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16 between the medial 1/3 and the lateral 1/4 was defined as the peroneal territory, which  
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18 received a primary projection from the superficial peroneal and sural nerves that  
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20 innervate the dorsal and lateral aspects of the hindpaw. Using a camera lucida drawing  
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22 tube, a dark field image of the spinal dorsal horn and the border between the superficial  
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24 laminae I and II (I/II) and the deep laminae III and IV (III/IV) were traced on white  
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26 paper. Based on the width of the spinal dorsal horn, delineations for the tibial and  
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28 peroneal territories were outlined. Then, the c-Fos-IR or p-ERK-IR neuron profiles in a  
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30 bright field image were plotted. The number of c-Fos- or p-ERK-IR neuron profiles  
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32 distributed in laminae I/II of the dorsal horn ipsilateral to the injury and stimulation was  
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34 counted separately for tibial and peroneal territories. The average of the 20 sections  
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36 from the caudal L4 and the rostral L5 blocks was recorded for each rat.  
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### ***Double immunofluorescence labeling for c-Fos and p-ERK***

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53 Double immunofluorescence labeling for c-Fos and p-ERK induced by noxious  
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55 heat stimulation at different time points was performed in rats with chronically injured  
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6 tibial nerves (n = 5) and the corresponding sham-operated rats (n = 5). Fourteen days  
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8 after the tibial nerve injury or sham surgery, re-anesthetized rats received a noxious heat  
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10 stimulation to the hindpaw 2 h and 5 min before perfusion. Rats were perfused and the  
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12 spinal cord at the L4 and L5 segments was removed, post-fixed in the same fixative for  
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14 24 h, and then immersed in 20% sucrose in 0.02 M PBS for 48 h. The boundaries of  
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16 each segment were confirmed by verifying the dorsal root entry zone and each of the L4  
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18 and L5 segments was divided into the rostral and caudal halves thus yielding 4 tissue  
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20 blocks.  
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27 Frozen 10- $\mu$ m-thick sections of the spinal cord were cut on a cryostat and mounted  
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29 onto silane-coated slides. Sections were incubated for 24 h at RT with a mixture of the  
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31 rabbit polyclonal anti-c-Fos antibody (1:2,000) and the mouse monoclonal anti-  
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33 phospho-p44/42 MAP kinase antibody (1:2,000; Cell Signaling). Alexa-488 and Alexa-  
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35 568 (1:1,000; Molecular Probes, Eugene, OR, USA)-conjugated secondary antibodies  
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37 were used to visualize primary antibody binding. The primary antibody was omitted for  
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39 negative controls. Sections were coverslipped with DAKO Fluorescent Mounting  
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41 Medium. The labeled sections were examined with a Nikon fluorescence microscope,  
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43 and images were captured with a CCD spot camera. The numbers of c-Fos-IR, p-ERK-  
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45 IR, and c-Fos/p-ERK double-labeled neuron profiles were counted separately for tibial  
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47 and peroneal territories in laminae I/II of the spinal dorsal horn. Three sections within 1  
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49 mm rostral or caudal to the junction of the L4 and L5 segments were randomly selected  
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6 for statistical analyses, and the average of the 3 sections was recorded for each rat.  
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### 10 11 *Administration of a MEK 1/2 inhibitor* 12

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16 Another group of rats with chronically injured tibial nerves was used to examine  
17 the effect of a MEK 1/2 inhibitor, PD98059 (Calbiochem, San Diego, CA, USA), on  
18 the effect of a MEK 1/2 inhibitor, PD98059 (Calbiochem, San Diego, CA, USA), on  
19 noxious heat-induced c-Fos or p-ERK expression. For intrathecal administration of  
20 drugs, a non-laminectomized catheterization method was performed [25]. A  
21 polyurethane tube (No. 0007740; Alzet, Durect Co., Cupertino, CA, USA) was inserted  
22 through the intervertebral space at the level between the L4 and L5 vertebrae, then  
23 moved cephalad into the subdural space 2.0 cm upward, with the tip of the tube located  
24 near the L4-L5 spinal cord level. A micro-osmotic infusion pump (Model 2002; 0.5  
25  $\mu\text{l/h}$ , 14-day capacity; Alzet) was connected to the tube and placed under the dorsal  
26 skin. This pump releases a solution in the reservoir lineally for 14 days when it is  
27 implanted in living tissue. PD98059 (0.1  $\mu\text{g}/\mu\text{l}$  or 0.2  $\mu\text{g}/\mu\text{l}$ ) dissolved in 10% dimethyl  
28 sulfoxide (DMSO) or vehicle (DMSO) was infused for 14 days at a rate of 1.2 or 2.4  
29  $\mu\text{g}/\text{day}$ . The dose of PD98059 for pump infusion was chosen on the basis of previous  
30 studies [21, 26]. Infusion of PD98059 or vehicle was started immediately before tibial  
31 nerve injury (n = 5 in each group). Fourteen days after surgery, including nerve injury  
32 (n = 5 in each group), animals underwent noxious heat stimulation of the hindpaw and  
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6 were processed for c-Fos or p-ERK immunohistochemistry as described above.  
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### 10 11 *Statistical analysis* 12

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16 Results are presented as the mean value  $\pm$  SEM. Statistical analyses were  
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18 performed using a one-way analysis of variance (ANOVA), followed by pairwise  
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20 multiple comparison procedures (*post-hoc* Tukey-Kramer tests). The criterion used for  
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22 significance was  $p < 0.05$ .  
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## Results

### *Induction of c-Fos and p-ERK protein-like immunoreactivities by noxious heat stimulation after nerve injury*

A small number of c-Fos or p-ERK-IR neuron profiles (less than 5 per section) were found in the spinal dorsal horn after non-noxious sham stimulation (20 °C) of the hindpaw (Fig. 1). No significant differences were found in the distributions of IR neuron profiles between sides ipsi- and contralateral to the stimulation (data not shown). On the other hand, noxious heat stimulation (55 °C) of the hindpaw induced a large number of c-Fos and p-ERK-IR neuron profiles in superficial laminae I/II of the spinal dorsal horn ipsilateral to the stimulation (Figs. 1-3). Consistent with the previously reported central terminal field of primary neurons in the sciatic nerve [23], a large number of c-Fos- and p-ERK-IR neuron profiles were distributed in the caudal L4 and rostral L5 (data not shown). These IR neuron profiles were largely restricted to the medial 3/4 of the dorsal horn, and the lateral 1/4 of the dorsal horn, which received inputs from the posterior cutaneous nerve of the thigh, was almost devoid of immunoreactivity. Noxious heat stimulation also produced a small number of c-Fos- and p-ERK-IR neuron profiles in deeper laminae III/IV. Only a small number of c-Fos or p-ERK-IR neuron profiles (less than 5 per section) were found in the spinal dorsal

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6 horn contralateral to the stimulation (data not shown). These results suggest that most  
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9 of these immunoreactivities had been induced by the noxious heat stimulation of the  
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11 hindpaw.  
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14 Although a large number of noxious heat-induced c-Fos-IR neuron profiles were  
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16 also observed in the spinal dorsal horn ipsilateral to stimulation after nerve injury, the  
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18 number of these varied depending on the territories and postinjury interval (Fig. 2). In  
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20 sham-operated animals, noxious heat stimulation of the hindpaw induced a large  
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22 number of c-Fos-IR neuron profiles in both the tibial and peroneal territories. A marked  
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24 reduction in the number of c-Fos-IR neuron profiles induced in the tibial territory was  
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26 found, and c-Fos-IR neuron profiles were distributed only in the peroneal territory 3 and  
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28 7 days after tibial nerve injury. However, induced c-Fos-IR neuron profiles reappeared  
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30 in the tibial territory 14 days after nerve injury, and the number of these profiles  
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32 returned to a level similar to that in sham-operated rats and was significantly higher  
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34 than those 3 and 7 days after the injury. Noxious heat stimulation induced a large  
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36 number of c-Fos-IR neuron profiles in the peroneal territory of the superficial dorsal  
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38 horn, and the number of these neuron profiles was not affected by the nerve injury  
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40 (Figs. 2 and 4).  
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51 The distribution of p-ERK-IR neuron profiles in the spinal dorsal horn also varied  
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53 according to the territories and postinjury interval (Fig. 3). Consistent with the results  
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55 of c-Fos induction, noxious heat-induced p-ERK-IR neuron profiles were distributed in  
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6 both the tibial and peroneal territories in sham-operated animals. Tibial nerve injury  
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8 reduced the number of p-ERK-IR neuron profiles in the tibial territory, and the IR  
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10 neuron profiles induced were restricted to the peroneal territory (Fig. 3). Unlike c-Fos-  
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12 IR neuron profiles, the number of p-ERK-IR neuron profiles induced in the tibial  
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14 territory did not increase at 14 days compared with 3 and 7 days after injury. Noxious  
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16 heat stimulation induced a large number of p-ERK-IR neuron profiles in the peroneal  
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18 territory, and the number of these neuron profiles was unchanged after nerve injury  
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24 (Figs. 3 and 4).  
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### ***Double immunofluorescence labeling for c-Fos and p-ERK***

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35 We examined double immunofluorescence labeling for c-Fos and p-ERK induced  
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37 by noxious heat stimulation at different time points in nerve-injured and sham-operated  
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39 animals. c-Fos and p-ERK-IR neuron profiles were induced by noxious heat  
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41 stimulation to the hindpaw 2 h and 5 min before perfusion, respectively. In sham-  
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43 operated animals, a large number of single- and double-labeled neuron profiles were  
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45 distributed in the tibial and peroneal territories in the spinal dorsal horn (Fig. 5). A large  
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47 number of single- and double-labeled neuron profiles were also distributed in the  
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49 peroneal territory 14 days after nerve injury. However, p-ERK-IR and double-labeled  
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51 neuron profiles were rarely found in the tibial territory, while a large number of c-Fos-  
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6 IR neuron profiles were distributed in this territory (Fig. 5). The number of p-ERK and  
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8 double-labeled neuron profiles in the tibial territory in the nerve-injured group was  
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10 significantly lower than that in the sham-operated group. No significant differences  
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12 were noted in the number of single- and double-labeled neuron profiles in the peroneal  
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14 territory between sham-operated and nerve-injured groups (Fig. 6).  
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22 ***Effect of administration of a MEK 1/2 inhibitor on c-Fos and p-ERK induction by***  
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24 ***noxious heat stimulation after nerve injury***  
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30 To investigate the contribution of ERK activation to c-Fos expression after nerve  
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32 injury, a MEK 1/2 inhibitor (PD98059, 0.1  $\mu\text{g}/\mu\text{l}$  or 0.2  $\mu\text{g}/\mu\text{l}$ ) was administered  
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34 intrathecally for 14 days, beginning on the day of nerve injury. Treatment with the  
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36 larger dose of PD98059 (0.2  $\mu\text{g}/\mu\text{l}$ ), but not with the smaller dose (0.1  $\mu\text{g}/\mu\text{l}$ ) and  
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38 vehicle, resulted in a significant decrease in the number of noxious heat-induced c-Fos-  
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40 IR neuron profiles in the peroneal territory of the superficial dorsal horn. Despite the  
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42 same treatment, however, the number of c-Fos-IR neuron profiles induced in the tibial  
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44 territory was unaffected (Figs. 7 and 8). The larger dose of PD98059 also resulted in a  
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46 significant decrease in the number of noxious heat-induced p-ERK-IR neuron profiles in  
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48 the tibial and peroneal territories (Figs. 7 and 8).  
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## Discussion

The present study examined the spinal dorsal horn for c-Fos and p-ERK induction by noxious heat stimulation after nerve injury. The number of noxious heat-induced c-Fos-IR neuron profiles in the tibial territory was markedly decreased at 3 and 7 days after injury, and then increased, returning to a level similar to sham-operated controls by day 14. Although the number of p-ERK-IR neuron profiles induced in the tibial territory was also significantly reduced 3 and 7 days after injury, it did not return to sham-operated levels by 14 days after nerve injury. Administration of a MEK 1/2 inhibitor to the spinal cord suppressed noxious heat-induced c-Fos expression in the peroneal, but not in the tibial, territory of the superficial dorsal horn 14 days after nerve injury.

Previous studies have demonstrated that noxious stimulation of the skin induced c-Fos in the spinal dorsal horn and the distribution of the c-Fos-IR neuron profiles has been shown to be consistent with the somatotopic representation within the spinal dorsal horn of the stimulated area [7, 9, 27-30]. Consistent with the central terminal fields of the sciatic nerve's primary neurons [23], noxious heat-induced c-Fos-IR neuron profiles in sham-operated rats were largely restricted to the medial 3/4 of the dorsal horn in L4 and L5 segments. In contrast, a prominent reduction in the number of noxious heat-induced c-Fos-IR neuron profiles in the tibial territory was found, and the c-Fos-IR

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6 neuron profiles induced were restricted to the peroneal territory 3 and 7 days after injury  
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9 to the tibial nerve. This induction can be considered to be the basal level of c-Fos  
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11 induction by the nociceptive signal conveyed by the peroneal nerve [8]. However, a  
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13 significant number of noxious heat-induced c-Fos-IR neuron profiles reappeared in the  
14  
15 tibial territory 14 days after injury. The number of c-Fos-IR neuron profiles returned to  
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17 a level similar to that in sham-operated rats and was significantly higher than those 3  
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19 and 7 days after injury. Double immunofluorescence labeling for c-Fos and p-ERK  
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21 induced by noxious heat stimulation to the hindpaw at different time points also  
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23 indicated that a large number of c-Fos-IR neuron profiles were distributed in the tibial  
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25 territory 14 days after nerve injury. The time course of changes in the number of c-Fos-  
26  
27 IR neuron profiles was consistent with our previous study showing that nocifensive  
28  
29 responses to mechanical and noxious heat stimulation were attenuated at 3 days and  
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31 exaggerated at 14 days after nerve injury [13, 22], suggesting that the increase in the  
32  
33 number of c-Fos-IR neuron profiles reflected neuropathic pain states including  
34  
35 allodynia and thermal hyperalgesia. The increase in the number of c-Fos-IR neuron  
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37 profiles at 14 days, compared with that at 3 and 7 days, might be involved in the  
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39 exaggerated response of secondary neurons to the nociceptive signal conveyed by the  
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41 spared nerves. Our previous studies using double immunofluorescence labeling for c-  
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43 Fos and p-ERK revealed that peripheral nerve injury activated convergent nociceptive  
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45 inputs to spinal dorsal horn neurons from spared intact nerves [11, 12]. In addition to  
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6 changes in the spinal dorsal horn, nerve injury might cause sprouting of surrounding  
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8 intact nerves to denervated peripheral areas.  
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11 Induction of p-ERK has also been shown following noxious peripheral stimuli [18,  
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13 31-34]. The similarity in the distribution of IR neuron profiles between c-Fos and p-  
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15 ERK, especially in sham-operated controls, suggests that both of these are involved in  
16  
17 nociceptive transmission by second order neurons in the spinal dorsal horn. Similar to  
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19 c-Fos, a marked reduction in the number of noxious heat-induced p-ERK-IR neuron  
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21 profiles in the tibial territory was found in the tibial territory 3 and 7 days after nerve  
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23 injury. Unlike c-Fos, however, the number of p-ERK-IR neuron profiles remained  
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25 decreased until 14 days after nerve injury without any sign of recovery in the tibial  
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27 territory. Double immunofluorescence labeling for c-Fos and p-ERK induced by  
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29 noxious heat stimulation at different time points also confirmed that p-ERK-IR neuron  
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31 profiles were only distributed in the peroneal territory and the number of p-ERK neuron  
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33 profiles in the tibial territory in the nerve-injured group was significantly lower than that  
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35 in the sham-operated group. These results suggest that c-Fos induction is involved in  
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37 the exaggerated response of secondary neurons after nerve injury as well as in the  
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39 nociceptive response under normal conditions, while p-ERK induction is mainly  
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41 involved in the normal nociceptive response.  
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53 Induction of double-labeled neuron profiles for c-Fos and p-ERK by noxious heat  
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55 stimulation at different time points indicates that these dorsal horn neurons responded to  
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6 noxious stimulation by ERK phosphorylation and c-Fos expression. It has been  
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8 reported that ERK phosphorylation is essential for cAMP-response element binding  
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10 protein (CREB) phosphorylation in spinal dorsal horn neurons after noxious stimulation  
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12 [32]. CREB has been shown to regulate many genes in spinal dorsal horn neurons  
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14 including *c-fos* [35]. Intrathecal pre-treatment with a MEK 1/2 inhibitor attenuated  
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16 ERK phosphorylation induced by mechanical pressure and led to decreased expression  
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18 of c-Fos [21]. We also found that intrathecal infusion of a MEK 1/2 inhibitor resulted in  
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20 a decrease in the number of c-Fos- as well as p-ERK-IR neuron profiles in the peroneal  
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22 territory of the dorsal horn after nerve injury. These previous and present results  
23  
24 indicate that the phosphorylation of ERK may regulate c-Fos expression in normal  
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26 nociceptive responses. However, the same treatment was less effective on c-Fos  
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28 induction, which reappeared in the tibial territory 14 days after nerve injury, suggesting  
29  
30 that exaggerated c-Fos induction after nerve injury may not be mediated by ERK  
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32 phosphorylation. Although further studies will be needed to elucidate the relationship  
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34 between c-Fos and p-ERK induction, other signal pathways, such as the protein kinase  
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36 A (PKA) or Ca<sup>2+</sup>/calmodulin kinases (CaMKs) pathways may be involved in CREB  
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38 phosphorylation, which leads to exaggerated c-Fos induction after peripheral nerve  
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40 injury [36, 37].  
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54 In conclusion, we have demonstrated that peripheral nerve injury caused  
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56 differential changes in the distribution of noxious heat-induced c-Fos- or p-ERK-IR  
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6 neuron profiles. ERK phosphorylation may contribute to c-Fos induction in normal  
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9 nociceptive responses, but not in exaggerated c-Fos induction after nerve injury.  
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### 11 12 13 14 **Acknowledgments**

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18  
19 Society for the Promotion of Science (16K11440).  
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### 21 22 23 24 **Conflict of interest**

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27 The authors do not have any conflict of interest.  
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## Figure legends

### Figure 1

Distribution of c-Fos- and p-ERK-IR neuron profiles in L4-L5 levels of the spinal dorsal horn after non-noxious sham (20 °C) or noxious heat (55 °C) stimulation of the hindpaw in sham-operated rats. Photomicrographs show the spinal dorsal horn ipsilateral to the stimulation. A small number of c-Fos- and p-ERK-IR neuron profiles were found in the spinal dorsal horn after non-noxious stimulation. Noxious heat stimulation induced a large number of c-Fos- and p-ERK-IR neuron profiles in the medial 3/4 of laminae I/II of the dorsal horn ipsilateral to the stimulation. Insets show high-magnification views of boxed regions. I/II, laminae I and II; III/IV, laminae III and IV. *Scale bar* 100  $\mu$ m.

### Figure 2

Induction of c-Fos immunoreactivity in L4-L5 levels of the spinal dorsal horn by noxious heat (55 °C) stimulation of the hindpaw 3, 7, or 14 days after tibial nerve injury or sham surgery. The number of c-Fos-IR neuron profiles in the tibial territory was decreased at 3 and 7 days after nerve injury, but increased by 14 days after nerve injury. Tibial, tibial territory; Peroneal, peroneal territory. *Scale bar* 100  $\mu$ m.

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6 Figure 3  
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8 Induction of p-ERK immunoreactivity in L4-L5 levels of the spinal dorsal horn by  
9 noxious heat (55 °C) stimulation of the hindpaw 3, 7, or 14 days after tibial nerve injury  
10 or sham surgery. A decrease in the number of p-ERK-IR neuron profiles in the tibial  
11 territory was noted at 3, 7, and 14 days after nerve injury. *Scale bar* 100 μm.  
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22 Figure 4  
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24 The number per section of c-Fos-IR (top) and p-ERK-IR (bottom) neuron profiles  
25 induced in the tibial territory (left column) and the peroneal territory (right column) of  
26 laminae I/II of the dorsal horn ipsilateral to injury and stimulation 3, 7, and 14 days  
27 after tibial nerve injury and sham surgery. A significant decrease in the number of c-  
28 Fos-IR neuron profiles in the tibial territory was noted at 3 and 7 days after injury,  
29 whereas the number of these profiles returned to a level similar to that in sham-operated  
30 rats. A significant decrease in the number of p-ERK-IR neuron profiles in the tibial  
31 territory was noted at 3, 7, and 14 days after injury. Noxious heat stimulation induced a  
32 large number of c-Fos and p-ERK-IR neuron profiles in the peroneal territory of the  
33 superficial dorsal horn; however, the number of these neuron profiles remained  
34 unchanged after nerve injury.  $**P < 0.01$ ;  $***P < 0.001$ , compared to the sham-  
35 operated group.  $\#P < 0.05$ ;  $\#\#P < 0.01$ , comparisons between 3 and 14 days or between  
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Figure 5

Merged images of double immunofluorescence for c-Fos (red) and p-ERK (green) in the spinal dorsal horn following noxious heat stimulation to the hindpaw at different time points (2 h and 5 min before perfusion) 14 days after tibial nerve injury or the corresponding sham-surgery. Representative data from 1 animal in each group are shown. Insets show high-magnification views of boxed regions. *Scale bar* 100  $\mu$ m.

Figure 6

The number per section of c-Fos-IR (top), p-ERK-IR (middle), and double-labeled cell profiles (bottom) in the tibial territory and peroneal territory of the superficial laminae in the spinal dorsal horn following noxious heat stimulation to the hindpaw at different time points (2 h and 5 min before perfusion) 14 days after tibial nerve injury or the corresponding sham-surgery. Double-labeled cell profiles were included in the populations of both c-Fos-IR and p-ERK-IR cell profiles. The number of p-ERK and double-labeled neuron profiles in the tibial territory in the nerve-injured group was significantly lower than that in the sham-operated group. No significant differences were noted in the number of single- and double-labeled neuron profiles in the peroneal territory between sham-operated and nerve-injured groups. Each bar represents the mean value  $\pm$  SEM of 5 individual experiments. \*\*\* $P < 0.001$ , compared with the sham-

operated group.

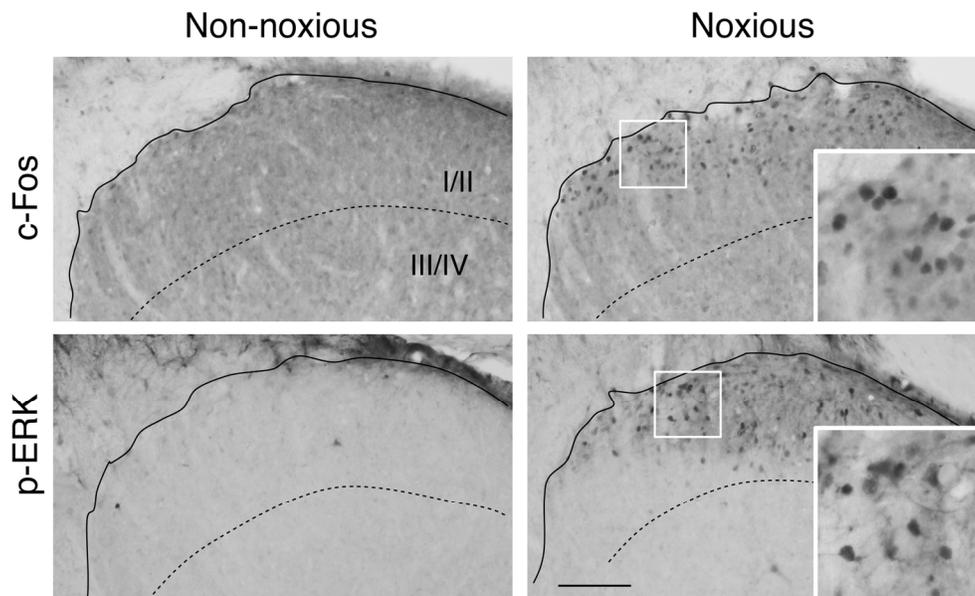
### Figure 7

The effect of MEK inhibitor on noxious heat-induced c-Fos or p-ERK expression 14 days after the tibial nerve injury. Induction of c-Fos- (top) and p-ERK-IR (bottom) neuron profiles in L4-L5 levels of the spinal dorsal horn by noxious heat (55 °C) stimulation of the hindpaw 14 days after the tibial nerve injury with intrathecal infusion of vehicle (left column) or PD98059 (right column). Treatment with a high dose of PD98059 (0.2 µg/µl) attenuated c-Fos expression in the peroneal, but not in the tibial, territory of the spinal dorsal horn. Treatment with a high dose of PD98059 also attenuated p-ERK expression in the tibial and peroneal territories of the spinal dorsal horn. *Scale bar* 100 µm.

### Figure 8

The number per section of c-Fos-IR (top) and p-ERK-IR (bottom) neuron profiles induced in the tibial territory (left column) and the peroneal territory (right column) in the superficial laminae of the spinal dorsal horn 14 days after the tibial nerve injury with intrathecal infusion of vehicle or PD98059. Treatment with a high dose of PD98059 (0.2 µg/µl), but not with a low dose (0.1 µg/µl) or vehicle, resulted in a significant decrease in the number of c-Fos-IR neuron profiles in the peroneal territory of the

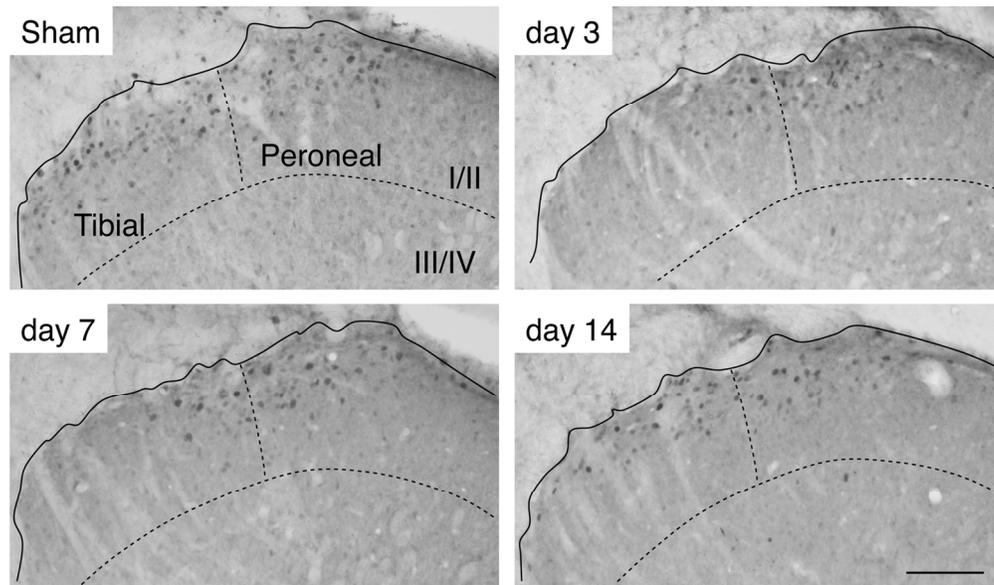
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6 superficial dorsal horn. Treatment with a high dose of PD98059 (0.2  $\mu\text{g}/\mu\text{l}$ ), but not  
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9 with a low dose (0.1  $\mu\text{g}/\mu\text{l}$ ) or vehicle, also resulted in a significant decrease in the  
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11 number of p-ERK-IR neuron profiles in the tibial and peroneal territories.  $*P < 0.05$ ;  
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Distribution of c-Fos- and p-ERK-IR neuron profiles in L4-L5 levels of the spinal dorsal horn after non-noxious sham (20 °C) or noxious heat (55 °C) stimulation of the hindpaw in sham-operated rats.

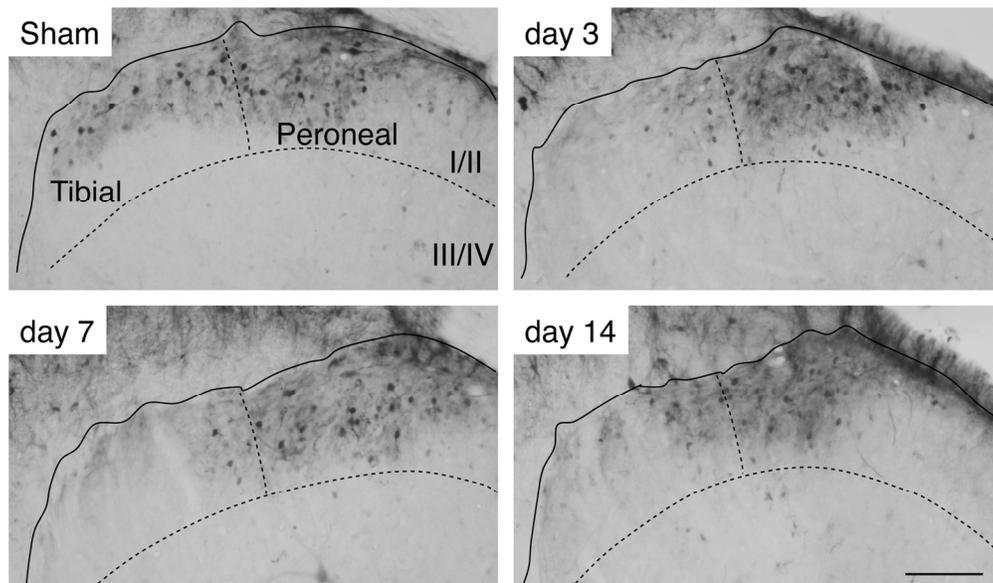
149x91mm (300 x 300 DPI)

Review Only



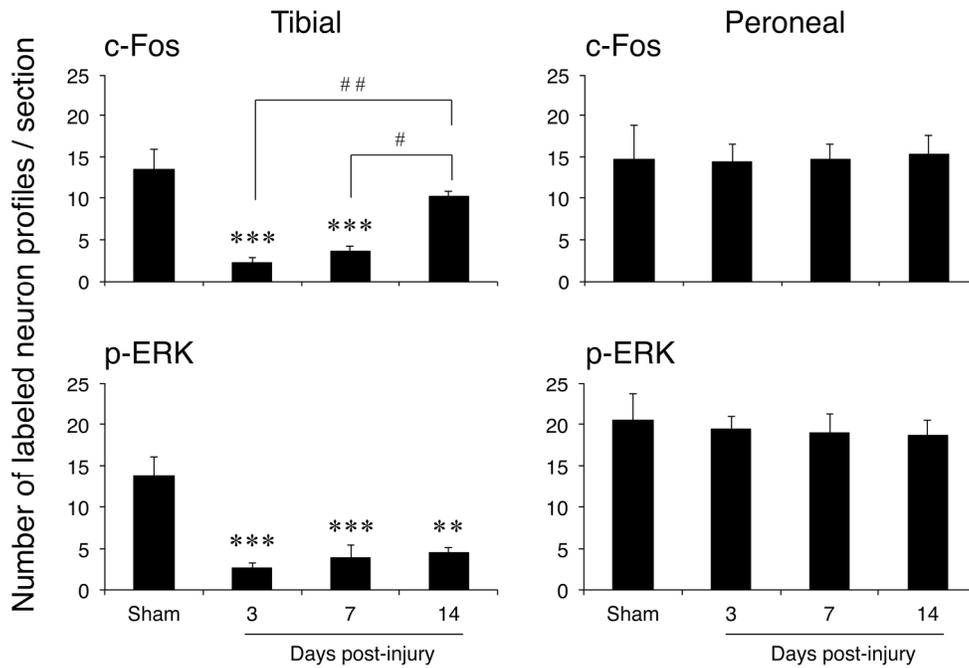
Induction of c-Fos immunoreactivity in L4-L5 levels of the spinal dorsal horn by noxious heat (55 °C) stimulation of the hindpaw 3, 7, or 14 days after tibial nerve injury or sham surgery.

134x78mm (300 x 300 DPI)



Induction of p-ERK immunoreactivity in L4-L5 levels of the spinal dorsal horn by noxious heat (55 °C) stimulation of the hindpaw 3, 7, or 14 days after tibial nerve injury or sham surgery.

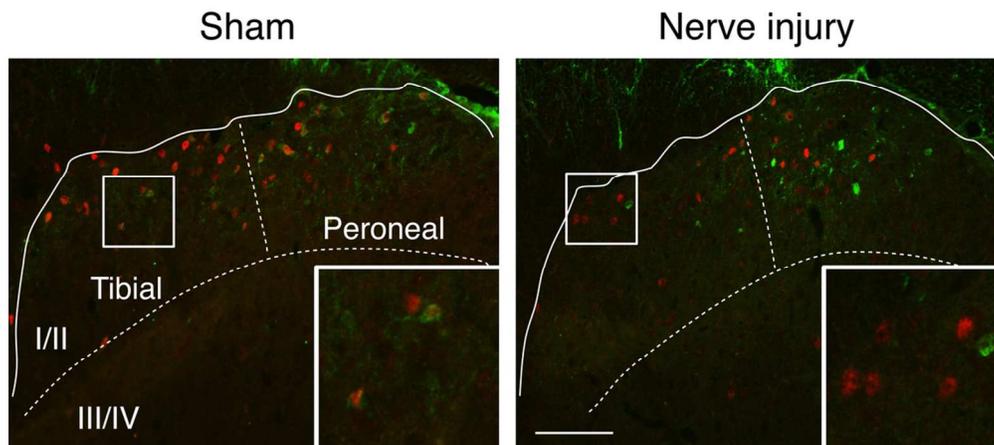
134x78mm (300 x 300 DPI)



The number per section of c-Fos-IR (top) and p-ERK-IR (bottom) neuron profiles induced in the tibial territory (left column) and the peroneal territory (right column) of laminae I/II of the dorsal horn ipsilateral to injury and stimulation 3, 7, and 14 days after tibial nerve injury and sham surgery.

188x127mm (300 x 300 DPI)

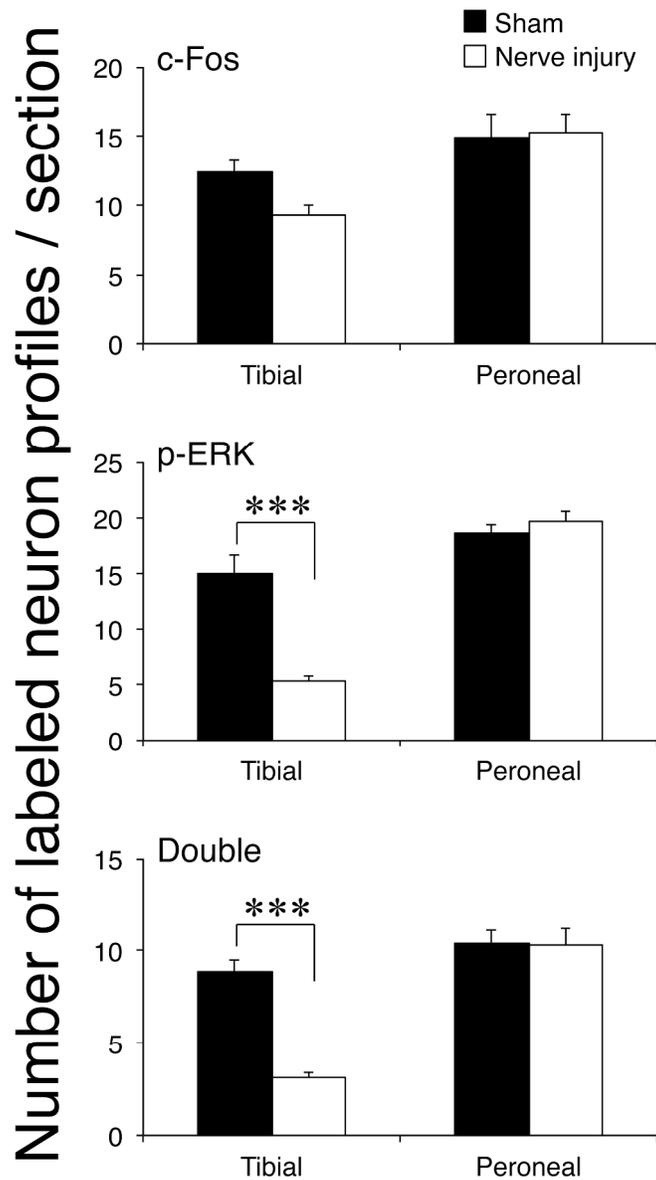
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Merged images of double immunofluorescence for c-Fos (red) and p-ERK (green) in the spinal dorsal horn following noxious heat stimulation to the hindpaw at different time points (2 h and 5 min before perfusion) 14 days after tibial nerve injury or the corresponding sham-surgery.

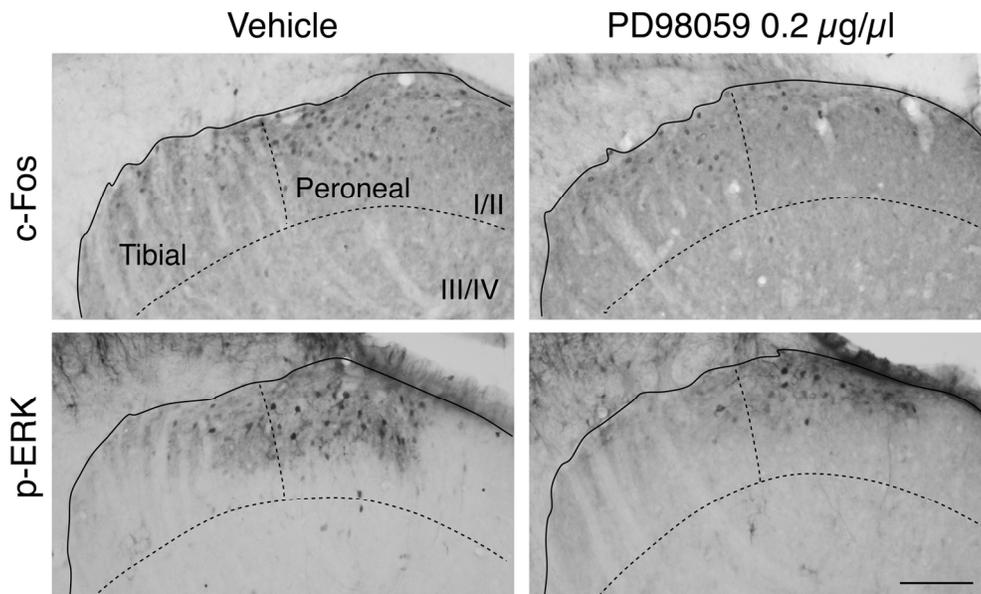
104x47mm (300 x 300 DPI)

Review Only



The number per section of c-Fos-IR (top), p-ERK-IR (middle), and double-labeled cell profiles (bottom) in the tibial territory and peroneal territory of the superficial laminae in the spinal dorsal horn following noxious heat stimulation to the hindpaw at different time points (2 h and 5 min before perfusion) 14 days after tibial nerve injury or the corresponding sham-surgery.

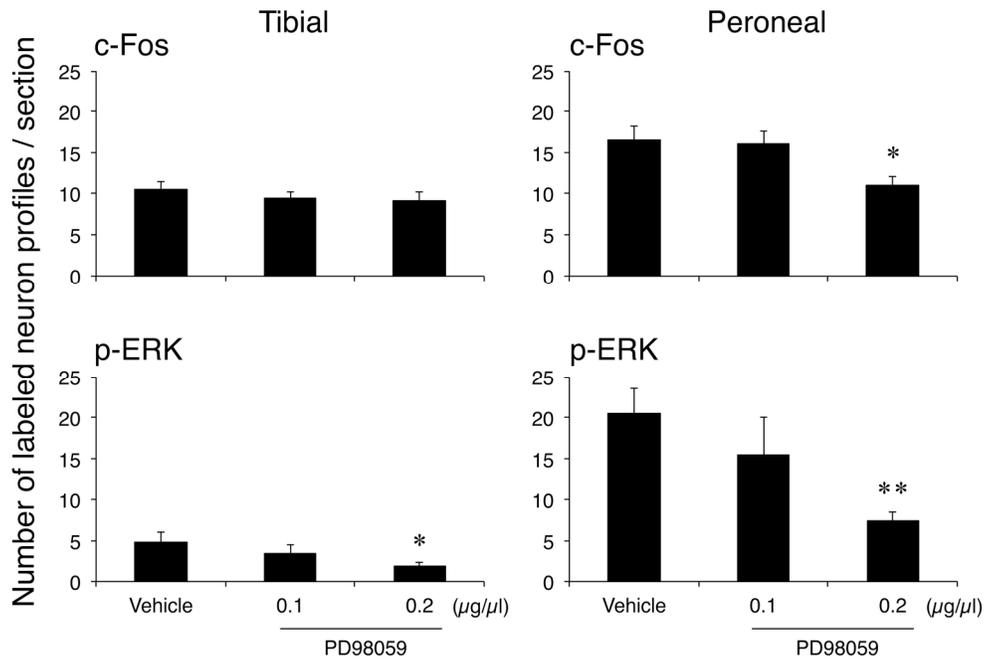
254x440mm (300 x 300 DPI)



The effect of MEK inhibitor on noxious heat-induced c-Fos or p-ERK expression 14 days after the tibial nerve injury.

147x89mm (300 x 300 DPI)

Review Only



The number per section of c-Fos-IR (top) and p-ERK-IR (bottom) neuron profiles induced in the tibial territory (left column) and the peroneal territory (right column) in the superficial laminae of the spinal dorsal horn 14 days after the tibial nerve injury with intrathecal infusion of vehicle or PD98059.

188x126mm (300 x 300 DPI)