The Journal of Neuroscience, March 1, 1997, 17(5):1776–1785

Phosphorylation of Transcription Factor CREB in Rat Spinal Cord after Formalin-Induced Hyperalgesia: Relationship to *c-fos* Induction

Ru-Rong Ji and Fabio Rupp

Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The involvement of cAMP-responsive element-binding protein (CREB) signaling in tissue injury-induced inflammation and hyperalgesia has been characterized by measuring phosphorylation of CREB at serine-133 (CREB Ser133) using a specific antibody. In the unstimulated state, unphosphorylated CREB was observed in most nuclei of spinal neurons except for motor neurons, where only a small portion of neurons were stained. A few dorsal root ganglion (DRG) neurons were also CREB-positive. After a unilateral injection of formalin into the hindpaw, a strong and bilateral phosphorylation of CREB Ser133 was induced, as assessed by both immunohistochemistry and Western blot. PhosphoCREB (pCREB)-positive neurons were found in laminae I, II, V, and X of spinal cord on both sides. CREB phosphorylation was very rapid and reached peak levels within 10 min of formalin treatment, whereas few pCREB-positive neurons were seen in unstimulated spinal cord. The induction of pCREB was predominantly postsynaptic, because only 5% of DRG neurons were labeled after inflammation. In contrast to CREB phosphorylation, the induction of c-Fos expression reached peak levels 2 hr after formalin treatment and c-Fos induction was mainly ipsilateral. Both formalin-evoked CREB phosphorylation and c-Fos expression in the spinal cord were suppressed by pretreatment with the NMDA receptor antagonist MK-801 (3.5 mg/kg, i.p.) or halothane anesthesia.

These results suggest that CREB signaling may play a role in the long-term facilitation of spinal cord neurons after hyperalgesia. Furthermore, our results indicate that CREB phosphorylation may be necessary but not sufficient for *c-fos* induction.

Key words: phosphorylation; CREB; c-fos*; spinal cord; dorsal root ganglia; hyperalgesia; NMDA receptor; formalin; plasticity; inflammation*

Injection of irritative chemicals, such as carrageenan, complete Freund's adjuvant, or formalin, into the hindpaw of the rat produces an intense inflammatory pain that comprises three components: a spontaneous pain related to the site of inflammation, an increased sensitivity to subsequent noxious stimuli (hyperalgesia), and the generation of pain by innocuous stimuli (allodynia). This activity-dependent plasticity is mediated by neurons located in the spinal cord and dorsal root ganglia (DRG) that use different types of neurotransmitters/neuromodulators such as substance P and glutamate (see Dubner and Ruda, 1992; Levine et al., 1993). In particular, electrophysiological studies have demonstrated that NMDA receptors play a role in the induction and maintenance of hyperalgesia. (Davies and Lodge, 1987; Woolf and Thompson, 1991; Coderre and Melzack, 1992; Ma and Woolf, 1995). Additionally, long-term potentiation produced by C-fiber stimulation in the rat spinal dorsal horn is prevented by NMDA receptor blockade (Randic et al., 1993; Liu and Sandkuhler, 1995). Analyses of gene expression indicate that immediate-early genes such as *c-fos* (Hunt et al., 1987; Presley et al., 1990; Hylden et al., 1992; Ji et al., 1994a,b) and several late-response genes such as dynorphin (Ruda et al., 1988; Noguchi et al., 1991, Dubner and Ruda, 1992, Ji et al., 1994a), neuropeptide Y (NPY) (Ji et al., 1994a), galanin (Ji et al., 1995a), neurokinin-1 receptor (Schafer et al., 1993), NPY (Y1) receptor (Ji et al., 1994a), and the μ-opioid receptor (Ji et al., 1995b) are induced in spinal cord by peripheral inflammation. It has been proposed that the induction of *c-fos* plays an important role in mediating the neuronal response to peripheral inflammation because *c-fos* antisense oligonucleotides could inhibit the upregulation of dynorphin in spinal cord neurons both *in vitro* and *in vivo* (Lucas et al., 1993; Hunter et al., 1995).

The cAMP response element-binding protein CREB is a transcription factor that has been implicated in the transcriptional regulation of many genes (Sheng et al., 1991). CREB-binding sites have been found in the promoter regions of immediate-early genes such as *c-fos* (Sassone-Corsi et al., 1988; Ginty et al., 1992) and Zif/268 (Sakamoto et al., 1991) and genes encoding synapsin I (Sauerwald et al., 1990), somatostatin (Gonzalez and Montminy, 1989), dynorphin (Cole et al., 1995), and enkephalin (Borsook et al., 1994). It has been shown that the phosphorylation of CREB at serine-133 is required for CREB-mediated transcription (Gonzalez and Montminy, 1989; Sheng et al., 1991; Ginty et al., 1994). For example, CREB phosphorylation mediates *c-fos* expression in response to agents that increase intracellular concentrations of cAMP or Ca^{2+} (Sheng and Greenberg, 1990; Ginty et al., 1992). Similarly, nerve growth factor appears to stimulate *c-fos* transcription via phosphorylation of CREB (Ginty et al., 1994). Recently, it has been proposed that CREB-mediated signaling is necessary for the establishment of a late phase of long-term facilitation in *Aplysia* C. (Dash et al., 1990) or long-term potentiation in mammalian hippocampus (Bourtchuladze et al., 1994).

Received July 1, 1996; revised Dec. 6, 1996; accepted Dec. 11, 1996.

This work was supported by grants to F.R. from National Institutes of Health (MH51158), the Muscular Dystrophy Association, the E. A. and J. Klingenstein Fund, and the Council for Tobacco Research. We thank Dr. Qin Zhang, Department of Anesthesiology, The Johns Hopkins School of Medicine, for valuable technical assistance, and Drs. David Ginty and David Linden for helpful suggestions and discussions.

Correspondence should be addressed to Fabio Rupp, Department of Neuroscience, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205.

We have used a specific antibody (Ginty et al., 1993) to measure phosphorylation of CREB Ser133 in neurons within the spinal cord and DRG after peripheral inflammation. Peripheral inflammation evoked a rapid and strong phosphorylation of CREB Ser133 in spinal cord, which was dependent on the activation of NMDA receptors. Furthermore, we showed a strong correlation between CREB phosphorylation and *c-fos* induction after inflammation.

MATERIALS AND METHODS

Animals. Adult male Sprague Dawley rats weighing 200–230 gm were used. Animals were kept in cages (3–4 animals per cage) at an ambient temperature of 20–25°C under a 12 hr light/dark cycle and had free access to food and water. Because CREB phosphorylation and *c-fos* expression are very sensitive to sensory stimuli, uncontrolled variables were minimized. We adhered to the ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983). One-hundred microliters of a 5% formalin solution (dissolved in saline) were injected into the plantar surface of the left hindpaw. In some cases, the NMDA receptor antagonist MK-801(RBI, Natick, MA) was administrated (3.5 mg/kg, i.p.) 20 min before formalin injection. Some animals were briefly anesthetized with halothane (2%) immediately before formalin injection.

Immunohistochemistry. CREB phosphorylation and *c-fos* expression were analyzed in animals 10 min, 30 min, or 2 hr after formalin injection. This analysis in the MK-801- and halothane-treated animals was performed 40 min after formalin injection. At this time point, both CREB phosphorylation and c-Fos induction are obvious, allowing the selection of adjacent spinal cord sections for pCREB and c-Fos staining. Formalin-injected animals and control animals (4–5 per group) were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and transcardially perfused with 60 ml of warm saline, followed by 400 ml of 4% paraformaldehyde with 0.4% picric acid in 0.16 M phosphate buffer solution, pH 7.2. L4–L5 segments of the spinal cord and L5 DRGs were removed, post-fixed in the same fixative for 3 hr, and placed in 15% sucrose solution at 4°C overnight. Spinal cords and DRGs from different animal groups were embedded in OCT compound (Miles, Elkhardt, IN) on the same blocks so as to allow identical conditions of further processing. Tissues were cut coronally in a cryostat at 20 μm thickness and mounted onto gelatin-coated slides. Tissue sections were placed in a humid chamber and processed for immunohistochemistry according to the ABC method (Hsu et al., 1981). Sections were incubated overnight at 4°C in the following primary antisera: CREB (1:2000), pCREB (1:1000), and c-Fos (1:1000). The sections were then incubated for 1 hr at 37°C with the biotinylated secondary antibody (1:200) and subsequently with the ABC complex (1:100; ABC Kit, Vector Laboratories, Burlingame, CA). The reaction product was visualized with 0.05% DAB/0.006% hydrogen peroxide in 0.1 M acetate buffer, pH 6, containing 2% ammonium nickel sulfate for 5 min and then rinsed in acetate buffer, air-dried, dehydrated, and coverslipped.

The anti-pCREB antiserum was obtained from a rabbit immunized with phosphopeptide corresponding to amino acids 123–136 of CREB (Ginty et al., 1993). Anti-CREB polyclonal antiserum was prepared against a TrpE–CREB fusion protein (Ginty et al., 1993). c-Fos polyclonal antiserum was purchased from Oncogene Science (Uniondale, NY). The specificity of these antisera, including preabsorption, has been tested extensively in previous studies (Ginty et al., 1993, 1994; Ji et al., 1994b; Konradi et al., 1994; Dash et al., 1995; Gu et al., 1996).

Quantification. Immunohistochemically stained tissue sections were examined under a light microscope at 20× magnification. Neurons with distinct nuclear staining were counted in several subregions, such as superficial layers (laminae I–II), deeper layers (laminae III–VI), and lamina X of spinal cord. Six to ten sections from L4–L5 spinal cord per animal were counted and averaged, and four to five animals were included in each group. All data were assessed using an ANOVA test followed by a Scheffe *F*-test. The criterion for statistical significance was $p < 0.05$.

Before quantification, immunostained DRG sections were lightly counterstained with toluidine blue; the total number of CREB- or pCREB-immunoreactive nuclei was divided by the total number of neuronal profiles in each DRG section, and the percentage of stained nuclei was calculated.

Western blot. Spinal cords (L4–L5) were dissected from control animals and from animals 20 and 60 min after formalin injection, homogenized in boiling SDS sample buffer (100 mM Tris, pH 6.8, 2% SDS, 20% glycerol), boiled for 5 min, and placed on ice. The amount of protein in each sample was measured using a BCA assay (Pierce, Rockford, IL). 2-Mercaptoethanol and bromophenol blue were added to a final concentration of 10 and 0.1%, respectively. The extracts were separated using SDS-PAGE (10%) using 50 μg of protein per lane and transferred onto nitrocellulose filters. Filters were blocked for 1 hr in 4% BSA and incubated in the primary antiserum (pCREB, 1:5000) for 3 hr at room temperature. After the incubation in the secondary antibody, reactive bands were visualized in ECL solutions (Amersham, Arlington Heights, IL) for 1 min and immediately exposed onto XAR films (Eastman Kodak, Rochester, NY) for 2–10 min.

RESULTS

Distribution of CREB and pCREB in rat spinal cord

The expression of CREB and pCREB in spinal cord neurons was analyzed after a single unilateral injection of formalin in the hindpaw. In control animals, except for motor neurons unphosphorylated CREB-immunoreactive neurons could be detected in all of the spinal cord laminae, in which nearly all neurons were CREB-positive. Only a small portion of motor neurons in the ventral horn were CREB-positive (Fig. 1). As described previously (Ginty et al., 1993; Konradi et al., 1994; Dash et al., 1995), CREB-immunoreactive staining was localized to nuclei. The distribution of CREB-immunoreactive neurons in spinal cord did not vary after formalin injection (Fig. 1).

In contrast to CREB expression, formalin injection induced a robust change in CREB phosphorylation in spinal cord neurons. CREB phosphorylation was detected using a specific antibody raised against the serine-133-phosphorylated form of CREB (pCREB) (Ginty et al., 1993). pCREB immunoreactivity was restricted to nuclei (Fig. 2). In unstimulated control rats, only a few pCREB-positive neurons could be detected (Figs. 3*A*, 8*A*; Table 1). Inflammation and phosphorylation of CREB Ser133 were both noticeable within 10 min after formalin injection. After formalin treatment, pCREB-positive nuclei were found in neurons of the superficial layers (laminae I-II; medial part) and laminae V of the dorsal horn (Figs. 2*A,B*, 3*B*). Many labeled neurons were also detected in the region around the central canal (lamina X), especially the dorsal part of lamina X, and medial part of lamina VI (Fig. 3). Some labeled neurons were seen in laminae I–II (lateral part) and laminae III, IV, and VII (Figs. 2, 3). Surprisingly, inflammation induced CREB phosphorylation bilaterally; pCREB-positive neurons were observed in the corresponding laminae of the contralateral spinal cord (Figs. 2, 3).

Formalin-induced phosphorylation of CREB Ser133 was maximal after 10 min, and it persisted at high level after 30 min. Two hours after injection, however, the number of pCREB-positive neurons declined nearly to background levels (Figs. 2, 3). When a larger volume of formalin (>200 μl) was injected into the left hindpaw, more labeled neurons were observed, especially in the lateral part of laminae I–II (data not shown).

This pattern of pCREB induction was confirmed by Western blot analyses. A pCREB-immunoreactive band with an M_r of 43 kDa (Ginty et al., 1993; Konradi et al., 1994; Dash et al., 1995) could be detected at high levels in extracts obtained from both sides of spinal cord after inflammation, but only very weakly in control spinal cord. Whereas the levels of pCREB immunoreactivity at 20 min remained high, they were distinctively reduced at 60 min (Fig. 4).

Because CREB phosphorylation may regulate transcription of *c-fos* (Sheng et al., 1991; Ginty et al., 1994), and because inflammation results in an increase of c-Fos-positive neurons in the spinal cord (Hunt et al., 1987; Presley et al., 1990; Hylden et al.,

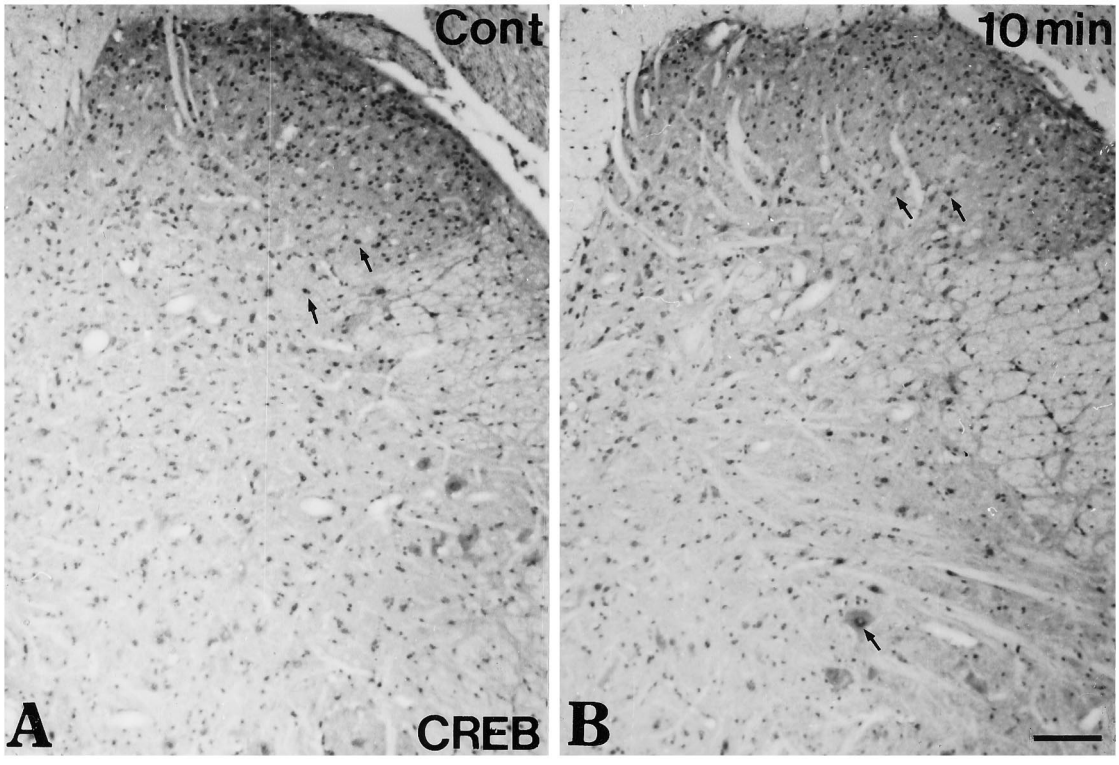

Figure 1. Photomicrographs showing the distribution of CREB-positive neurons in spinal cord (L4–L5) of normal control (*A*) and inflamed animal (*B*) 10 min after unilateral formalin injection. CREB is localized in nuclei of spinal neurons. Almost all spinal neurons are CREB-positive, with the exception of motor neurons. No difference in the pattern of CREB staining was observed between spinal cords of normal and inflamed animals. *Arrows* indicate the labeled neurons. Scale bar, 100 μm. *A* and *B* have the same magnification.

1992; Ji et al., 1994b), we compared formalin induction of c-Fos versus pCREB. Interestingly, although the pattern of c-Fos expression resembled that of pCREB, the kinetics of induction differed; c-Fos expression was detectable in spinal cord neurons as early as 30 min after formalin injection and peaked at ~2 hr. c-Fos-positive neurons were mainly localized in laminae I–II (medial part) and laminae III–VI, as well as lamina X (Fig. 5). However, in contrast to the bilateral induction of pCREB, c-Fos expression was ipsilateral. Only a few labeled neurons were found in the contralateral side. Additionally, the total number of c-Fos-positive neurons in the spinal cord was much lower than that of CREB-positive neurons (Figs. 2, 3, 5). In spinal cord of untreated animals, only a few c-Fos-labeled neurons were observed.

In conclusion, peripheral inflammation induced a rapid phosphorylation of CREB in distinct populations of spinal cord neurons. The same stimulus also induced an increased expression of c-Fos in these neurons. However, CREB phosphorylation was induced both ipsilaterally and contralaterally, and c-Fos induction was ipsilaterally restricted.

CREB phosphorylation in DRG neurons

The robust induction of CREB phosphorylation in spinal cord neurons after peripheral inflammation could originate from a stimulation of presynaptic neurons located in the DRG. For this reason, we examined CREB phosphorylation in the presynaptic neurons (primary sensory neurons located in DRG). In normal control L5 DRG, 9.1% (193/2121) of neurons contained CREB-positive nuclei (Fig. 6*A*) and no pCREB-positive nuclei were detected (Fig. 6*B*). Ten minutes after formalin application, 5.0% (77/1542) and 4.1% (67/1648) of pCREB-positive neurons were found in the ipsilateral and contralateral DRG, respectively (Fig. 6*C,D*). Interestingly, pCREB was localized in small DRG neurons (Fig. 6*C,D*), which mediate pain transmission. No obvious pCREB labeling was seen in DRG 30 min or 2 hr after inflammation (data not shown). No staining for c-Fos-positive nuclei was detected in the normal control DRG and ipsilateral or contralateral DRG of inflamed rats (data not shown). In summary, inflammation induced CREB phosphorylation in a small portion of DRG neurons. This induction was not followed by an increased expression of c-Fos.

Effects of MK-801 and halothane on CREB phosphorylation and c-Fos expression

Hindpaw formalin injection induced a rapid phosphorylation of CREB in spinal cord neurons. To determine whether NMDA receptor-mediated synaptic transmission is involved in this response, we have studied CREB phosphorylation in animals that have been pretreated with the NMDA receptor antagonist MK-801. Additionally, we have studied the effect of a brief exposure to the general anesthetic halothane on the inflammation-induced phosphorylation of CREB (see Materials and Methods for details). Formalin-induced CREB phosphorylation was significantly suppressed by MK-801 and halothane. MK-801 and halothane pretreatments produced a 45.6% ($p < 0.001$) and 61.2% ($p < 0.001$) decrease, respectively, in the number of pCREB-positive neurons in ipsilateral laminae I–II of the dorsal horn 40 min after formalin challenge (Figs. 7*A,C,E*, 8*A*; Table 1).

c-Fos-positive neurons were observed mainly in the ipsilateral superficial layers of the dorsal horn 40 min after inflammation. However, MK-801 and halothane pretreatment induced a 57.3%

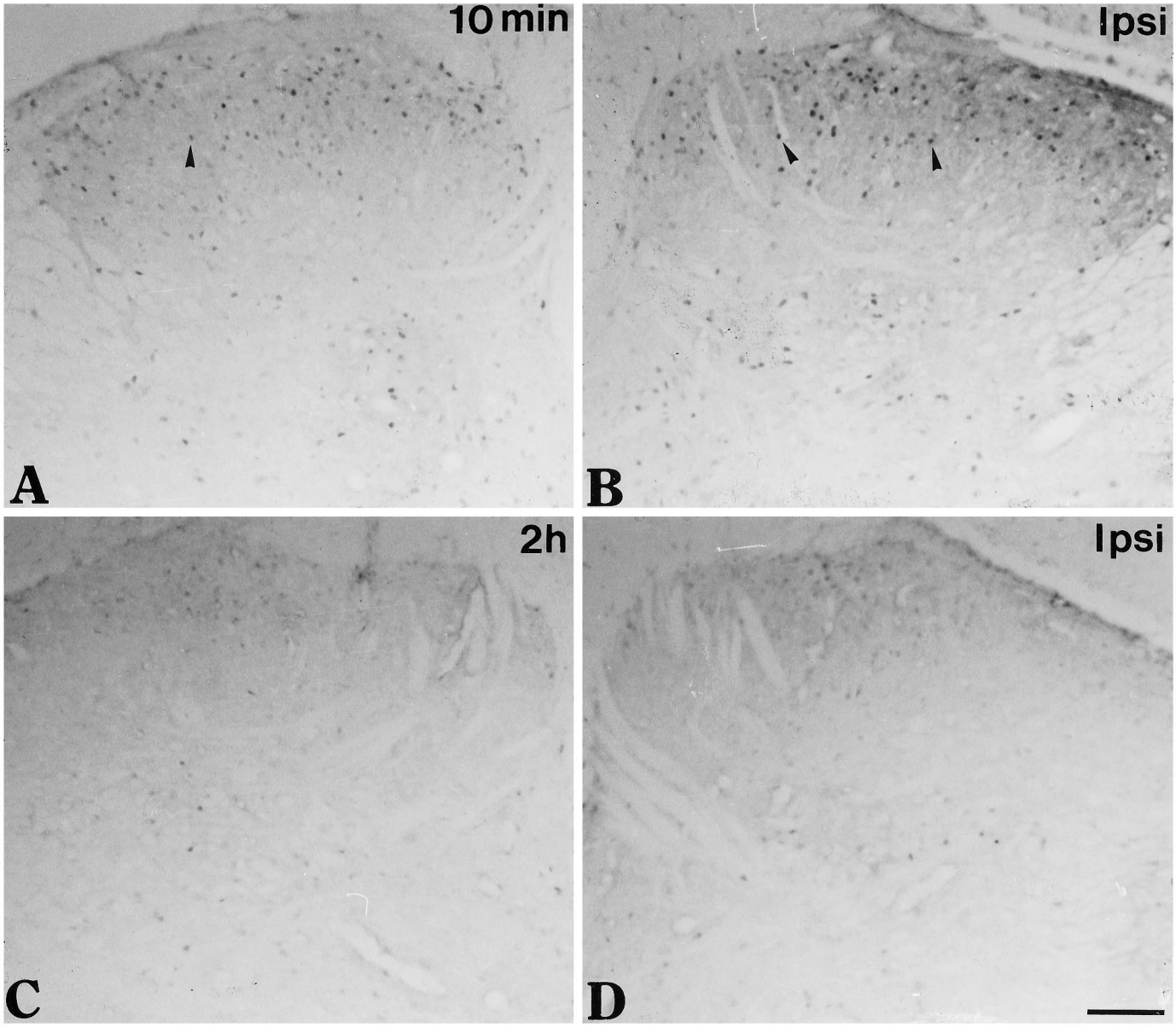

Figure 2. The distribution of phosphoCREB-positive neurons in the ipsilateral (*Ipsi*, *B*, *D*) and contralateral (*A*, *C*) dorsal horn of the spinal cord (L4–L5) of animals 10 min (*A*, *B*) and 2 hr (*C*, *D*) after unilateral formalin injection is shown. pCREB is localized in nuclei of spinal neurons. Numerous pCREB-positive neurons appear in both sides of the dorsal horn 10 min after stimulation (*A*, *B*). In contrast, 2 hr after injection only a few labeled neurons are observed (*C*, *D*). *Arrowheads* indicate labeled neurons. Scale bar, 100 μm. All micrographs have the same magnification.

($p < 0.001$) and 74.4% ($p < 0.001$) reduction in the number of c-Fos-positive neurons in this subregion (Figs. 7*B*,*D*,*F*, 8*B*). A corresponding suppression of pCREB was also found in the following subregions of the spinal cord: contralateral laminae I–II, ipsilateral and contralateral laminae III–VI, and lamina X, as shown in Table 1. In summary, pretreatments of MK-801 and halothane drastically reduced the induction of pCREB and c-Fos by inflammation.

DISCUSSION

The presented results demonstrate that formalin injection into the hindpaw of an adult rat induces, within minutes, the phosphorylation of CREB at the transcriptional regulatory site serine-133 in spinal cord neurons. Our results show that CREB Ser133 phosphorylation is induced predominantly in postsynaptic neurons. Interestingly, unilateral peripheral inflammation induces this phosphorylation event bilaterally in spinal cord neurons. The phosphorylation of CREB in spinal cord neurons, and subsequent induction of c-Fos, can be suppressed by pretreatment with the NMDA antagonist MK-801 or the general anesthetic halothane.

CREB phosphorylation in spinal cord

A majority of neurons containing pCREB-positive nuclei in the spinal cord after formalin stimulation were observed in laminae I–II and V–VI. These are spinal cord regions in which a majority of noxious primary afferents terminate and in which the cell bodies of dorsal horn nociceptive neurons are localized (Sugiura et al., 1986; Besson and Chaouch, 1987). The specificity of this response was also supported by the overlapping, but distinct, pattern of pCREB-positive neurons observed in spinal cord after

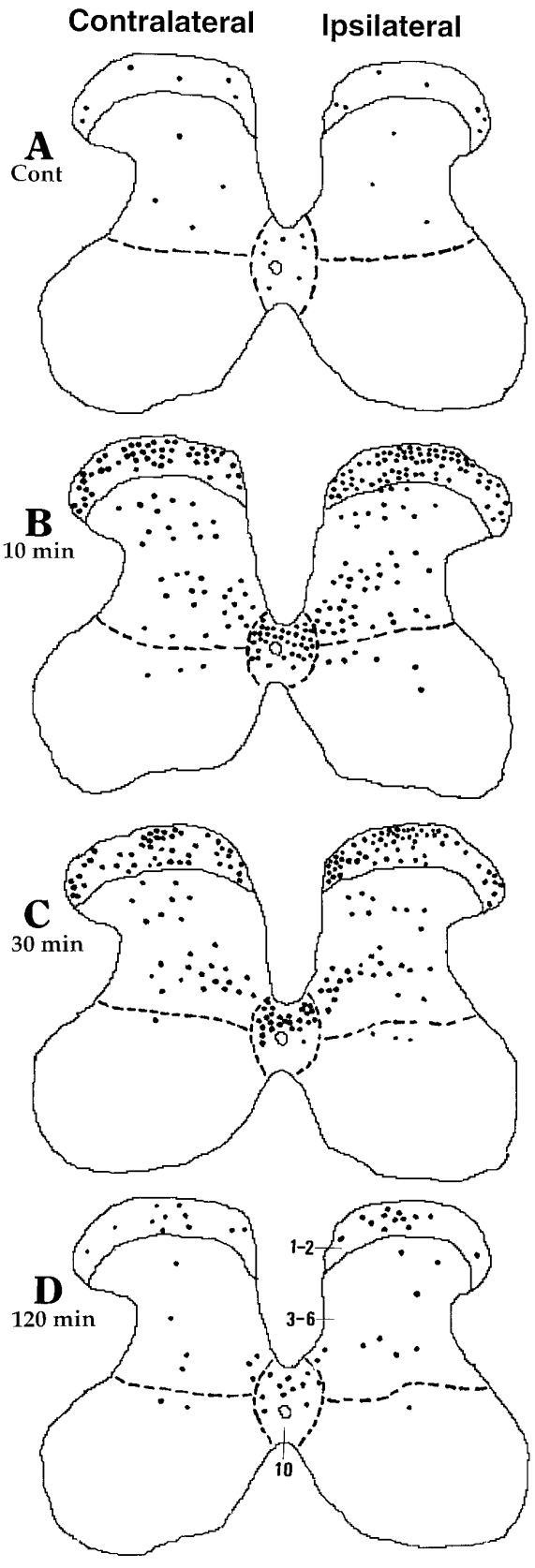

Figure 3. Camera lucida drawings depicting the time course of the induction of phosphoCREB-positive neurons in the ipsilateral and contralateral spinal cord (L4–L5) of control (*A*) and inflamed animals 10 min (*B*), 30 min (*C*), and 120 min (*D*) after unilateral formalin injection. Numerous phosphoCREB-positive neurons appear in laminae I, II, and V of both sides of the dorsal horn, and lamina X 10 min and 30 min after stimulation (*B*, *C*). The number of labeled nuclei 2 hr after injection is much lower. Each *black dot* represents a labeled neuron. *1–2*, Laminae I–II; *3–6*, laminae III–VI; *10*, lamina X.

limited and severe paw inflammation. Whereas a 100 μl formalin challenge affected mainly neurons located in the medial part of the superficial dorsal horn, indicating precise topographic projections, larger volumes of formalin (>200 μl) also induced CREB phosphorylation in neurons located in the lateral part of laminae I–II, suggesting a possible spread of inflammation from the distal part to the proximal part of the hindlimb.

Interestingly, a large number of pCREB-positive neurons were also found in the dorsal part of central canal (lamina X), where dorsal commissures interconnect the two sides of the dorsal horn. Lamina X neurons receive afferent inputs similar to that of laminae I–II (Brown, 1981). This region has been implicated in pain regulation. For example, several neuropeptides thought to mediate nociception, such as enkephalin and substance P, are found in relatively high concentration in the central canal (Gibson et al., 1981). Central branches of primary afferents conducting nociception terminate to this area (Light and Perl, 1979). Neurons found in this region are reminiscent of the noxious-specific cells in the outer-most layers of the dorsal horn (Nahin et al., 1983).

Furthermore, pCREB was observed in a few small DRG neurons after inflammation, which are involved in mediating pain transmission. Taken together, these results indicate that formalin-induced CREB phosphorylation is predominantly confined to neurons involved in pain response.

The bilateral induction of CREB phosphorylation in the spinal cord neurons after peripheral inflammation is surprising, particularly because it appears to occur concomitantly in the contralateral and ipsilateral sides. This is in contrast to the induction of c-Fos expression, which was ipsilateral, in agreement with previously reported results (Presely et al., 1990; Ji et al., 1994a,b; Hunter et al., 1995). The mechanisms underlying the contralateral effect remain to be evaluated, and both peripheral and central mechanisms might be involved (Woolf, 1983). An interesting possibility is that signal strength may differentially affect gene expression versus protein phosphorylation. This hypothesis would predict, for instance, that the threshold of stimulation required for initiation of gene expression is higher than induction of post-translational modifications such as phosphorylation. Ipsilateral spinal cord neurons receive monosynaptic inputs from peripheral sensory neurons, whereas contralateral neurons receive multisynaptic inputs from the ipsilateral site either through interneurons or via descending spinal tracts. It is conceivable, therefore, that a unilateral peripheral stimulation may produce a different response in ipsilateral versus contralateral neurons attributable, for example, to the activation of inhibitory interneurons. It follows that a peripheral stimulation may have sufficient strength to elicit post-translational modifications (phosphorylation) contralaterally, but would only be able to induce gene expression ipsilaterally. Some observations seem to support this hypothesis. For example, a rapid release of nerve growth factor in contralateral hindpaw and a bilateral increase in NADPH-diaphorase have been reported after unilateral inflammation (Solodkin et al., 1992; Woolf et al., 1996), whereas induction of dynorphin, enkephalin, NPY and NPY receptor (Y1), and galanin mRNAs after tissue injury is predominantly ipsilateral (Ruda et al., 1988; Dubner and Ruda, 1992; Ji et al., 1994a, 1995a). However, when a very strong,

Table 1. Number of pCREB-positive neurons in the subregions of ipsilateral laminae I–II (Ipsi I–II) and III–VI (Ipsi III–VI), contralateral laminae I–II (Contra I–II) and III–VI (Contra III–VI), and lamina X in the spinal cord (L4–L5) of control animals and animals subjected to formalin, formalin + MK-801, and formalin + halothane 40 min after injection into the left paw

Subregion	Control	Formalin	Formalin + MK-801	Formalin + halothane
Ipsi I–II	8.2 ± 2.2***	53.8 ± 4.0	29.2 ± 3.9*** (46%)	20.8 ± 4.5*** (61%)
Contra I–II	8.2 ± 2.2***	44.6 ± 1.8	20.4 ± 2.3*** (54%)	20.1 ± 1.9*** (55%)
Ipsi III–IV	6.5 ± 1.8***	30.2 ± 3.9	16.1 ± 2.1** (47%)	15.7 ± 2.1** (48%)
Contra III–VI	6.5 ± 1.8***	26.5 ± 2.8	13.9 ± 2.0** (48%)	15.1 ± 2.0** (43%)
Lamina X	7.9 ± 0.9***	28.5 ± 2.9	13.5 ± 2.7*** (53%)	18.0 ± 1.8** (37%)

Results are presented as the mean ± SEM from 4–5 rats. For each rat, counts from 6–8 sections (20 μm) were averaged. The numbers in the parentheses indicate the percentage of inhibition as compared to the formalin group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the formalin group; ANOVA followed by Scheffe *F*-test. CREB phosphorylation is low in the unstimulated control spinal cord. Formalin-induced pCREB is significantly inhibitied by MK-801 and halothane in all of the spinal subregions examined.

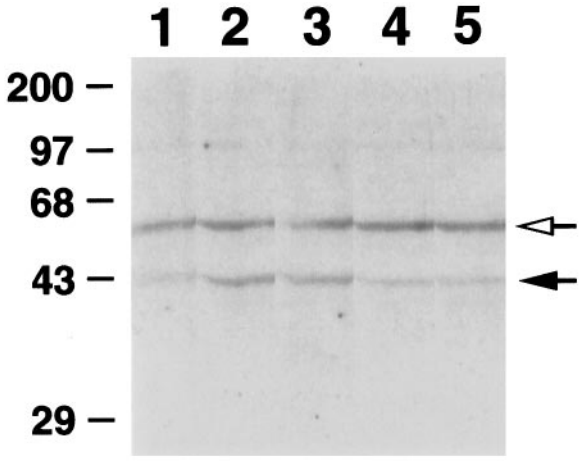

Figure 4. PhosphoCREB immunoblot obtained from the ipsilateral and contralateral sides of the spinal cord of animals 20 and 60 min after unilateral formalin injection together with control animals. The specific phosphoCREB-immunoreactive bands around 43 kDa (*filled arrow*) are clearly induced after inflammation. The bands indicated by the *open arrow* are nonspecific, which are unchangeable after inflammation. *Lane 1*, Control spinal cord; *lanes 2, 3*, ipsilateral and contralateral spinal cord 20 min after formalin injection; *lanes 4, 5*, ipsilateral and contralateral spinal cord 60 min after injection.

long-lasting inflammation is induced by injecting complete Freund's adjuvant unilaterally into the ankle joint, a contralateral increase in neuropeptide synthesis has been described (Donaldson et al., 1995). Our results could be explained, therefore, as follows: a unilateral formalin injection would be sufficiently strong to induce bilateral stimulation of CREB phosphorylation, but only strong enough to produce an ipsilateral induction of c-Fos expression.

NMDA activation of CREB phosphorylation

An important result of our study is that the NMDA receptor antagonist MK-801 markedly suppressed formalin-evoked CREB phosphorylation in several spinal cord subregions. These data are in agreement with previously reported studies demonstrating the role of NMDA receptors for CREB phosphorylation *in vitro* (Ginty et al., 1993; Deisseroth et al., 1996). Expression of glutamate receptors of the NMDA and non-NMDA types has been demonstrated in spinal cord. Results of *in situ* hybridization analyses using probes specific for various NMDA and non-NMDA receptor subunits indicate differential expression of these subunits in spinal cord (Furuyama et al., 1993; Tolle et al., 1993; Watanabe et al., 1994). Additionally, different splice variants of the NMDA receptor subunit NR1 are also found differentially distributed in the dorsal versus ventral horns (Tolle et al., 1995). Immunohistochemical analyses detected high levels of a particular type of NMDA receptor subunit (NR1) in spinal cord (dorsal and ventral horns) and DRG. Interestingly, NMDR1 subunits have been localized both pre- and postsynaptically in neurons localized in the superficial dorsal horn. A majority of these presynaptic terminals contain glutamate, suggesting that receptors containing the NR1 subunit may also function as autoreceptors (Liu et al., 1994).

These results are significant, because functional studies indicate that long-term plastic changes occurring in the spinal cord after peripheral tissue injury are mediated, in part, by NMDA receptors. For example, it has been demonstrated that both the induction and the maintenance of central sensitization produced by repetitive noxious stimulation are dependent on NMDA receptor activation (Woolf and Thompson, 1991; Coderre and Melzack, 1992; Ma and Woolf, 1995). Long-term potentiation in spinal cord produced by supramaximal electrical stimulation of C-fibers is also prevented by the NMDA receptor antagonist (Randic et al., 1993; Liu and Sandkuhler, 1995). Our results show, however, that MK-801 reduces the induction of CREB phosphorylation in spinal cord neurons after inflammation by ~50%. This is consistent with the observation that NMDA antagonists alone only partially block C-fiber excitation produced by capsaicin (Nagy et al., 1993). Moreover, neurokinin also plays an important role in spinal hyperactivity. In fact, a combination of NMDA and neurokinin receptor antagonists produced an almost complete abolition of the capsaicin-evoked depolarization (Nagy et al., 1993). In summary, our results suggest that the induction of CREB phosphorylation in spinal cord neurons may be a component of the cellular response to inflammation that results from the activation of NMDA receptors.

Functional implication of CREB phosphorylation

Our results revealed a similar distribution of pCREB- and c-Fos-positive neurons in different laminae of the spinal cord after peripheral inflammation. The induction of CREB phosphorylation precedes that of c-Fos. Importantly, inhibition of CREB phosphorylation by MK-801 or halothane in spinal cord also prevents the subsequent expression of c-Fos. These data suggest a role for CREB phosphorylation in the induction of *c-fos* expression in a spinal model of hyperalgesia. Several observations implicating CREB phosphorylation as a component for the activation of *c-fos* expression support this hypothesis. For example, *in vitro* studies using PC12 cells show that CREB phosphorylation is crucial for the nerve growth factor-mediated induction of *c-fos* transcription (Ginty et al., 1994). Also, injection of CREB anti-

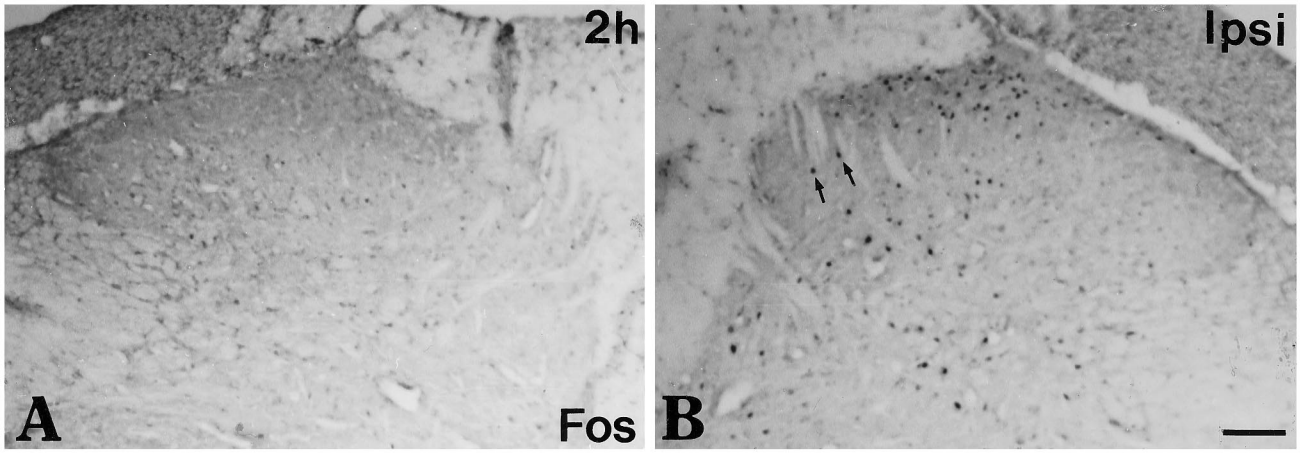

Figure 5. Photomicrographs illustrating the distribution of c-Fos-positive neurons in the contralateral (*A*) and ipsilateral (*Ipsi*, *B*) dorsal horn of the spinal cord (L4–L5) 2 hr (*2h*) after unilateral formalin injection. c-Fos induction is ipsilateral. Many c-Fos-positive neurons are found in the medial part of superficial layers of the spinal cord. Scale bar, 100 μm. *A* and *B* have the same magnification.

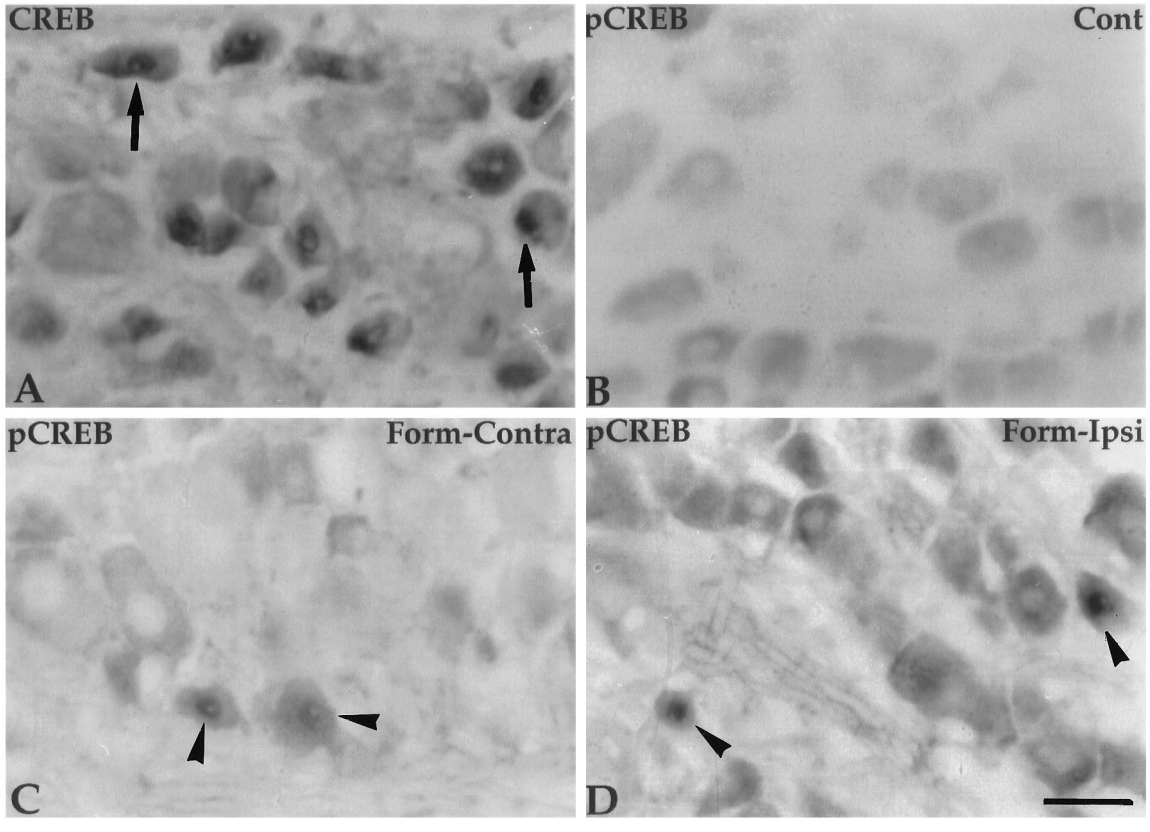

Figure 6. The distribution of CREB-positive (*A*) and phosphoCREB-positive (*pCREB*; *B–D*) neurons in normal control DRG (L5, *A*, *B*) versus contralateral (*C*) and ipsilateral (*D*) DRG (L5) 10 min after unilateral formalin (*Form*) injection is shown. CREB, but not pCREB, is present in many normal DRG neurons (*A*, *B*). Unilateral inflammation induces CREB phosphorylation in a few DRG neurons on both sides (*C*, *D*). *Arrow* and *arrowheads* indicate CREB- and phosphoCREB-positive neurons, respectively. Scale bar, 50 μm. All micrographs have the same magnification.

sense oligonucleotides in the striatum inhibits *c-fos* expression induced by amphetamine (Konradi et al., 1994). Finally, dominant-negative CREB transgenic mice showed that *c-fos* expression was markedly reduced in activated lymphocytes (Barton et al., 1996). Thus, formalin induction of phosphorylation of CREB Ser133 likely contributes to transcriptional activation of *c-fos* in spinal neurons.

Two observations seem to suggest, however, that CREB phosphorylation may be necessary but not sufficient for *c-fos* expression in spinal cord neurons after peripheral inflammation. First, formalin injection induces CREB phosphorylation bilaterally but c-Fos expression ipsilaterally. Second, the number of pCREB-positive nuclei induced in the same spinal cord regions is greater than the number of c-Fos-positive neurons. These results are in agreement with previous *in vitro* observations indicating that CREB phosphorylation is necessary but

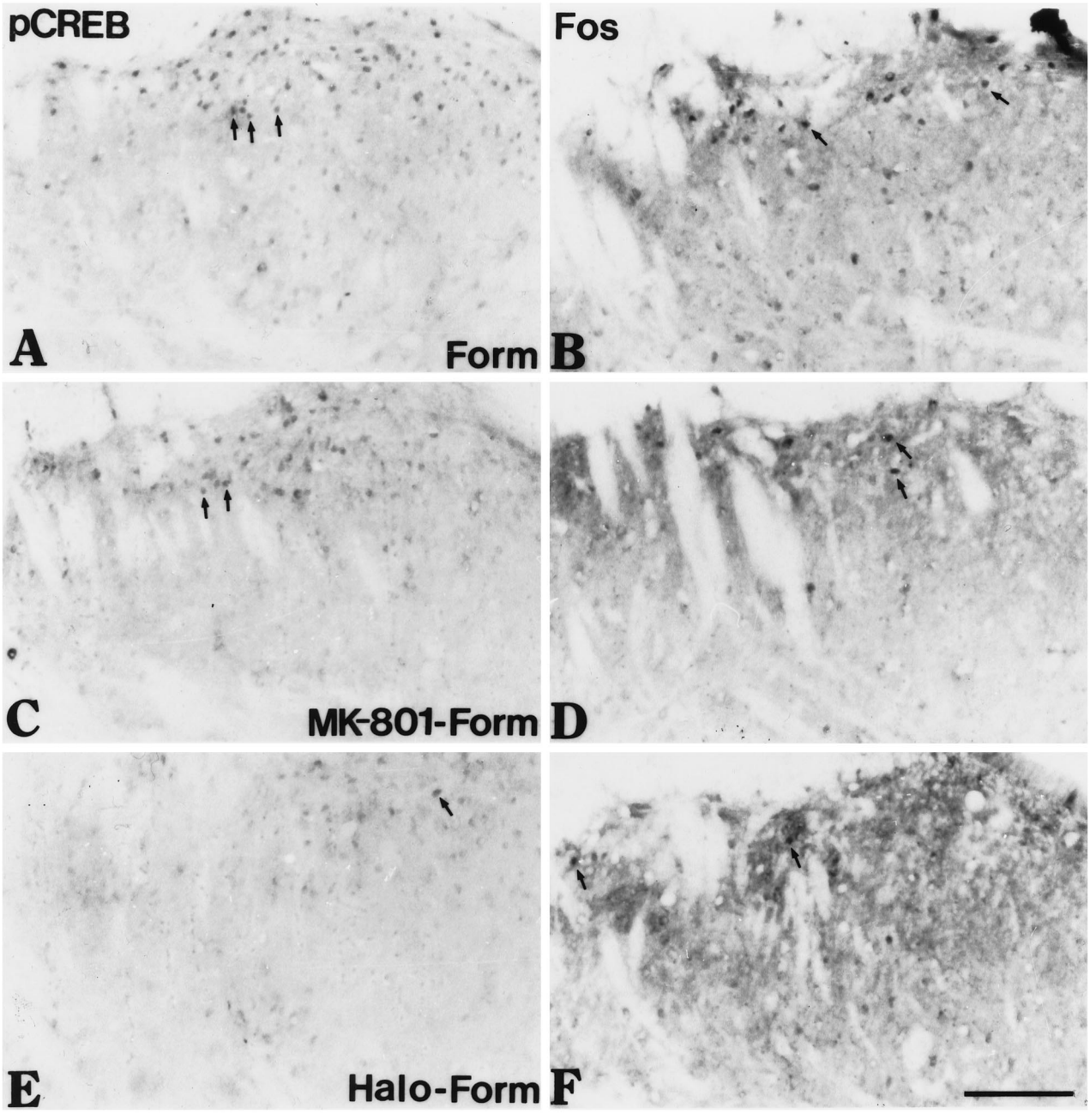

Figure 7. *A–F*, Photomicrographs showing the distribution of phosphoCREB-positive (*A*, *C*, *E*) and c-Fos-positive nuclei (*B*, *D*, *F*) in the medial part of the ipsilateral superficial dorsal horn of the spinal cord (L4–L5) 40 min after formalin (*Form*) injection (*A*, *B*), MK-801 + formalin (*C*, *D*), and halothane (*Halo*) + formalin (*E*, *F*). Induction of phosphoCREB- and c-Fos-positive neurons by formalin is significantly suppressed by MK-801 or halothane. *Arrows* indicate labeled neurons. Scale bar, 100 μm. All micrographs have the same magnification.

not sufficient for immediate-early gene activation (Bonni et al., 1995). Finally, CREB-binding sites have been found in several genes involved in pain (Konradi et al., 1993; Borsook et al., 1994; Cole et al., 1995). It will be interesting to determine whether other hyperalgesia-induced genes are also regulated by CREB phosphorylation.

Concluding remarks

Unilateral injection of formalin into the hindpaw induced a rapid and bilateral phosphorylation of CREB mainly in laminae I, II, V, and X of the spinal cord, with a postsynaptic predominance. This response is in sharp contrast to the ipsilateral induction of c-Fos. The inflammation-evoked CREB phosphorylation and c-Fos expression were markedly suppressed by pretreatment with MK-801 or halothane. We suggest, therefore, that formalin-induced inflammation results in the activation of CREB signaling in spinal cord nociceptive neurons through an NMDA receptor-mediated mechanism and that this signal is an early event in the triggering of sustained hyperalgesia. CREB phosphorylation seems to be crucial for the induction of *c-fos*, and possibly some later response genes, in response to peripheral hyperalgesia. Moreover, CREB phosphorylation may represent a better marker than *c-fos* expres-

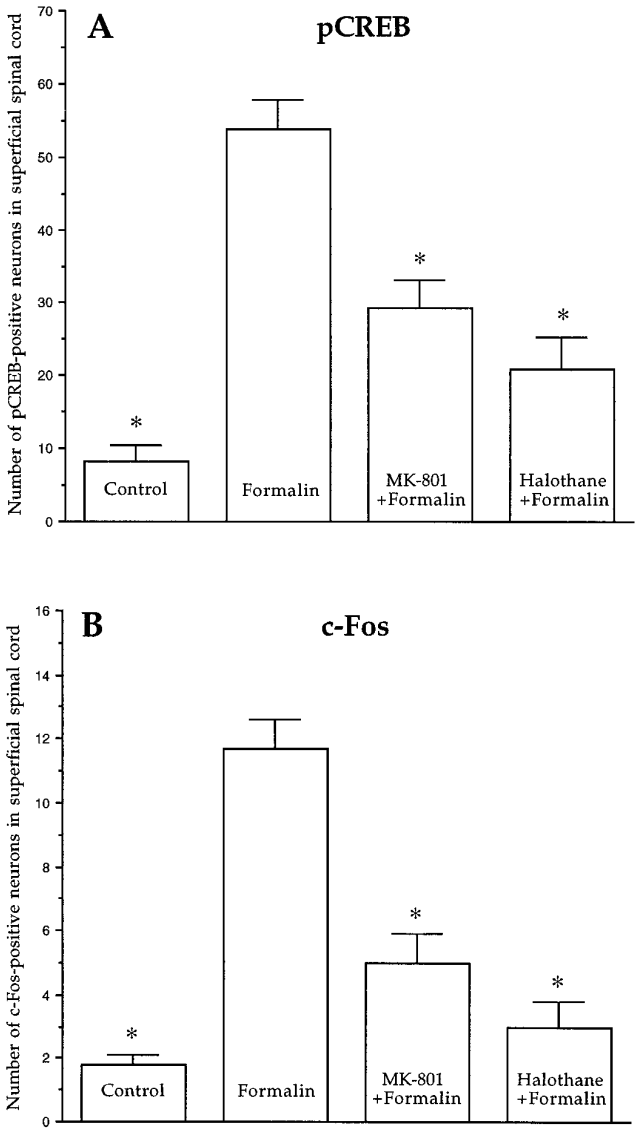

Figure 8. *A*, *B*, Effects of MK-801 and halothane on formalin-evoked phosphoCREB-positive (*A*) and c-Fos-positive (*B*) neurons in the superficial layers (laminae I–II) of the ipsilateral spinal dorsal horn (L4–L5), as indicated by the number of phosphoCREB- or c-Fos-positive neurons in that region. The data are presented as mean ± SEM. Formalin-evoked phosphoCREB- and c-Fos-positive neurons are significantly suppressed by MK-801 or halothane. $^*p < 0.001$ compared with the formalin group; ANOVA test.

sion for neuronal activity after noxious stimulation because its induction is more rapid and more sensitive.

REFERENCES

Barton K, Muthusamy N, Chanyangam M, Fischer C, Clendenin C, Leiden JM (1996) Defective thymocyte proliferation and IL-2 production in transgenic mice expressing a dominant-negative form of CREB. Nature 379:81–85.

Besson JM, Chaouch A (1987) Peripheral and spinal mechanisms of nociception. Physiol Rev 67:67–186.

Bonni A, Ginty DD, Dudek H, Greenberg ME (1995) Serine 133-phosphorylated CREB induces transcription via a cooperative mechanisms that may confer specificity to neurotrophin signals. Mol Cell Neurosci 6:168–183.

Borsook D, Konradi C, Falkowski O, Comb M, Hyman SE (1994) Molecular mechanisms of stress-induced proenkephalin gene regulation: CREB interacts with proenkephalin gene in the mouse hypothalamus and is phosphorylated in response to hyperosmolar stress. Mol Endocrinol 8:240–248.

Bourtchuladze R, Frenguelli B, Blendy J, Cioffi D, Schutz G, Silva AJ (1994) Deficient long-term memory in mouse with a targeted mutation of the cAMP-response element-binding protein. Cell 79:59–68.

Brown AG (1981) Organization in the spinal cord: the anatomy and physiology of identified neurons. New York: Springer.

Coderre TJ, Melzack R (1992) The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. J Neurosci 12:3665–3670.

Cole RL, Konradi C, Douglass J, Hyman SE (1995) Neuronal adaptation to amphetamine and dopamine: molecular mechanisms of prodynorphin gene regulation in rat striatum. Neuron 14:813–823.

Dash PK, Hochner B, Kandel ER (1990) Injection of the cAMP-responsive element into the nucleus of *Aplysia* sensory neurons blocks long-term facilitation. Nature 345:718–721.

Dash PK, Moore AN, Dixon CE (1995) Spatial memory deficits, increased phosphorylation of the transcription factor CREB, and induction of the AP-1 complex following experimental brain injury. J Neurosci 15:2030–2039.

Davies SN, Lodge D (1987) Evidence for involvement of *N*-methyl-D-aspartate receptors in "wind up" of class 2 neurons in the dorsal horn of the rat. Brain Res 424:402–406.

Deisseroth K, Bito H, Tsien RW (1996) Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. Neuron 16:89–101.

Donaldson LF, McQueen DS, Seckl JR (1995) Neuropeptide gene expression and capsaicin-sensitive primary afferents: maintenance and spread of adjuvant arthritis in the rat. J Physiol (Lond) 486:473–482.

Dubner R, Ruda MA (1992) Activity-dependent neuronal plasticity following tissue injury and inflammation. Trends Neurosci 15:96–103.

Furuyama T, Kiyama H, Sato K, Park HT, Maeno H, Takagi H, Tohyama M (1993) Region-specific expression of subunits of ionotropic glutamate receptors (AMPA-type, KA-type and NMDA receptors) in the rat spinal cord with special reference to nociception. Mol Brain Res 18:141–151.

Gibson SJ, Polak JM, Bloom SR, Wall PD (1981) The distribution of nine peptides in rat spinal cord with special emphasis on the substantia gelatinosa and on the area around the central canal (lamina X). J Comp Neurol 210:65–79.

Ginty DD, Bading H, Greenberg ME (1992) Trans-synaptic regulation of gene expression. Curr Opin Neurobiol 2:312–316.

Ginty DD, Kornhauser JM, Thompson MA, Bading H, Mayo KE, Takahashi JS, Greenberg ME (1993) Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. Science 260:238–241.

Ginty DD, Bonni A, Greenberg ME (1994) Nerve growth factor activates a Ras-dependent protein kinase that stimulates *c-fos* transcription via phosphorylation of CREB. Cell 77:713–725.

Gonzalez GA, Montminy MR (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59:675–680.

Gu G, Rojo AA, Zee MC, Yu J, Simerly RB (1996) Hormonal regulation of CREB phosphorylation in the anteroventral periverntricular nucleus. J Neurosci 16:3035–3044.

Hsu SM, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577–580.

Hunt SP, Pini A, Evan G (1987) Induction of *c-fos*-like protein in spinal cord neurons following sensory stimulation. Nature 328:632–634.

Hunter JC, Woodburn VL, Durieux C, Pettersson EK, Poat JA, Hughes J (1995) c-fos antisense oligodeoxynucleotide increases formalin-induced nociception and regulates preprodynorphin expression. Neuroscience 65:485–492.

Hylden JL, Noguchi K, Ruda MA (1992) Neonatal capsaicin treatment attenuates spinal Fos activation and dynorphin gene expression following peripheral tissue inflammation and hyperalgesia. J Neurosci 12:1716–1725.

Ji RR, Zhang X, Wiesenfeld-Hallin Z, Hokelt T (1994a) Expression of neuropeptide Y and neuropeptide Y (Y1) receptor mRNA in rat spinal cord and dorsal root ganglia following peripheral tissue inflammation. J Neurosci 14:6423–6434.

Ji RR, Zhang M, Zhang Q, Han JS (1994b) Effects of capsaicin on Fos

expression evoked by formalin and electroacupuncture stimulation in the rat spinal cord. Pain Research 9:37–47.
Ji RR, Zhang X, Zhang Q, Dagerlind A, Nilsson S, Wiesenfeld-Hallin Z, Hokfelt T (1995a) Central and peripheral expression of galanin in response to inflammation. Neuroscience 68:563–576.
Ji RR, Zhang Q, Law PY, Low HH, Elde R, Hokfelt T (1995b) Expression of μ-, δ-, and κ-opioid receptor-like immunoreactivities in rat dorsal root ganglia after carrageenan-induced inflammation. J Neurosci 15:8156–8166.
Konradi C, Kobierski LA, Nguyen TV, Heckers S, Hyman SE (1993) The cAMP-response-element-binding protein interacts, but Fos protein does not interact, with the proenkephalin enhancer in rat striatum. Proc Natl Acad Sci USA 90:7005–7009.
Konradi C, Cole RL, Heckers S, Hyman SE (1994) Amphetamine regulates gene expression in rat striatum via transcription factor CREB. J Neurosci 14:5623–5634.
Levine JD, Fields HL, Basbaum AI (1993) Peptides and primary afferent nociceptor. J Neurosci 13:2273–2286.
Light AR, Perl ER (1979) Spinal terminations of functionally identified primary afferent neurons with slowly conducting myelinated fibers. J Comp Neurol 186:133–150.
Liu XG, Sandkuhler J (1995) Long-term potentiation of C-fiber-evoked potentials in the rat spinal dorsal horn is prevented by spinal *N*-methyl-D-aspartic acid receptor blockage. Neurosci Lett 191:43–46.
Liu H, Wang H, Sheng M, Jan LY, Jan YN, Basbaum AI (1994) Evidence for presynaptic *N*-methyl-D-aspartate autoreceptors in the spinal cord dorsal horn. Proc Natl Acad Sci USA 91:8383–8387.
Lucas JJ, Mellstrom B, Colado MI, Naranjo JR (1993) Molecular mechanisms of pain: serotonin$_{1a}$ receptor agonists trigger transactivation by *c-fos* of the prodynorphin gene in spinal cord neurons. Neuron 10:599–611.
Ma QP, Woolf CJ (1995) Noxious stimuli induce an *N*-methyl-D-aspartate receptor-dependent hypersensitivity of the flexion withdrawal reflex to touch: implications for the treatment of mechanical allodynia. Pain 61:383–390.
Nagy I, Maggi CA, Dray A, Woolf CJ, Urban L (1993) The role of neurokinin and *N*-methyl-D-aspartate receptors in synaptic transmission from capsaicin-sensitive primary afferents in the rat spinal cord in vitro. Neuroscience 52:1029–1037.
Nahin RL, Madsen AM, Giesler Jr GJ (1983) Anatomical and physiological studies of the gray matter surrounding the spinal cord central canal. J Comp Neurol 220:321–335.
Noguchi K, Kowalski K, Traub R, Solodkin A, Iadarola MJ, Ruda MA (1991) Dynorphin expression and Fos-like immunoreactivity following inflammation induced hyperalgesia are colocalized in spinal cord neurons. Mol Brain Res 10:227–233.
Presley RW, Menetrey D, Levine JD, Basbaum AI (1990) Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord. J Neurosci 10:323–335.
Randic M, Jiang MC, Cerne R (1993) Long-term potentiation and long-term depression of primary afferent neurotransmission in the rat spinal cord. J Neurosci 13:5228–5241.
Ruda MA, Iadarola MJ, Cohen LV, Young WS (1988) In situ hybridization histochemistry and immunohistochemistry reveal an increase in spinal dynorphin biosynthesis in rat model of peripheral inflammation and hyperalgesia. Proc Natl Acad Sci USA 85:622–626.
Sakamoto KM, Bardeleben C, Yates KE, Raines A, Golde DW, Gasson JC (1991) 5′-Upstream sequence and the genomic structure of the human primary responsive gene EGR-1/TIS8. Oncogene 6:867–871.
Sassone-Corsi P, Visvader J, Ferland L, Mellon PL, Verma IM (1988) Induction of proto-oncogene *fos* transcription through adenylate cyclase pathway: characterization of cAMP-responsive element. Genes Dev 2:1529–1538.
Sauerwald A, Hoesche C, Oschwald R, Kilimann MW (1990) The 5′-flanking region of the synapsin I gene. J Biol Chem 265:14932–14937.
Schafer MK, Nohr D, Krause JE, Weihe E (1993) Inflammation-induced upregulation of NK1 receptor mRNA in dorsal horn neurons. NeuroReport 4:1007–1010.
Sheng M, Greenberg ME (1990) The regulation and function of *c-fos* and other immediate-early genes in the nervous system. Neuron 4:477–485.
Sheng M, Thompson MA, Greenberg ME (1991) CREB: a Ca^{2+}-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science 252:1427–1430.
Solodkin A, Traub RJ, Gebhart GF (1992) Unilateral hindpaw inflammation produces a bilateral increase in NADPH-diaphorase histochemical staining in the rat lumbar spinal cord. Neuroscience 51:495–499.
Sugiura Y, Lee CL, Perl ER (1986) Central projections of identified, unmyelinated (C) afferent fibers innervating mammalian skin. Science 234:358–361.
Tolle TR, Berthele A, Zieglgansberger W, Seeburg PH, Wisden W (1993) The differential expression of 16 NMDA and non-NMDA receptor subunits in the rat spinal cord and in periaqueductal gray. J Neurosci 13:5009–5028.
Tolle TR, Berthele A, Laurie DJ, Seeburg PH, Zieglgansberger W (1995) Cellular and subcellular distribution of NMDAR1 splice variant mRNA in the rat lumbar spinal cord. Eur J Neurosci 7:1235–1244.
Watanabe M, Mishina M, Inoue Y (1994) Distinct spatiotemporal distributions of the *N*-methyl-D-aspartate receptor channel subunit mRNA in the mouse cervical cord. J Comp Neurol 345:314–319.
Woolf CJ (1983) Evidence for a central component of post-injury pain hypersensitivity. Nature 306:686–688.
Woolf CJ, Thompson SW (1991) The induction and maintenance of central sensitization is dependent on *N*-methyl-D-aspartic acid receptor activation: implications for the treatment of post-injury pain hypersensitivity states. Pain 44:293–299.
Woolf CJ, Ma QP, Allchorne A, Poole S (1996) Peripheral cell types contributing to the hyperalgesic action of nerve growth factor in inflammation. J Neurosci 16:2716–2723.
Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16:109–110.