

Increased Spinal Dynorphin Levels and Phospho-Extracellular Signal-Regulated Kinases 1 and 2 and c-Fos Immunoreactivity after Surgery under Remifentanil Anesthesia in Mice

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ABSTRACT

In humans, remifentanil anesthesia enhances nociceptive sensitization in the postoperative period. We hypothesized that activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and the expression of c-Fos, prodynorphin (mRNA), and dynorphin in the spinal cord could participate in the molecular mechanisms underlying postoperative opioid-induced sensitization. In a mouse model of incisional pain, we evaluated thermal (Hargreaves test) and mechanical (von Frey) hyperalgesia during the first 21 postoperative days. Moreover, prodynorphin (mRNA, real-time polymerase chain reaction), dynorphin (enzymatic immunoassay), c-Fos expression, and ERK1/2 phosphorylation (both by immunohistochemistry) in the lumbar spinal cord were assessed. Surgery performed under remifentanil anesthesia induced a maximal decrease in nociceptive thresholds between

4 h and 2 days postoperatively ($p < 0.001$) that lasted 10 to 14 days compared with noninjured animals. In the same experimental conditions, a significant increase in prodynorphin mRNA expression (at 2 and 4 days) followed by a sustained increase of dynorphin (days 2 to 10) in the spinal cord was observed. We also identified an early expression of c-Fos immunoreactivity in the superficial laminae of the dorsal horn of the spinal cord (peak at 4 h; $p < 0.001$), together with a partial activation of ERK1/2 (4 h; $p < 0.001$). These findings suggest that activated ERK1/2 could induce c-Fos expression and trigger the transcription of prodynorphin in the spinal cord. This in turn would result in long-lasting increased levels of dynorphin that, in our model, could participate in the persistence of pain but not in the manifestation of first pain.

In humans, exposure to μ -opioid receptor agonists during anesthesia induces delayed hyperalgesia, increasing pain and analgesic requirements in the postoperative period (Angst and Clark, 2006). The pronociceptive effects of opioids have also been reported in human volunteers and in animal models (Li et al., 2001; Angst et al., 2003). In a mouse model of postoperative pain, we have shown that postincisional pain is enhanced when the ultra-short-act-

ing μ -opioid receptor agonist remifentanil is administered during anesthesia (Célérier et al., 2006; Cabañero et al., 2009b). Remifentanil is commonly used in clinical practice to provide analgesia during surgery, although it has been associated with hyperalgesia in the immediate postoperative period (Angst and Clark, 2006; Joly et al., 2005).

The mechanisms and signal transduction pathways that mediate pain sensitization after opioid administration are not well known. Multiple central and peripheral pathways and mechanisms have been implicated, including C-fiber sensitization, protein kinase C, the nitric oxide system, *N*-methyl-D-aspartate (NMDA) receptors, mitogen-activated protein kinases (MAPKs) and spinal dynorphin, among others (Angst and Clark, 2006). It has also been shown that down-regulation of δ -opioid receptor mRNA in the dorsal root ganglia contributes to remifentanil and surgery-induced enhanced nociception (Cabañero et al., 2009b).

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; AAC, area above the curve; CREB, cAMP-responsive element binding protein; ERK1/2, extracellular signal-regulated kinases 1 and 2; L4–L6, lumbar spinal cord segments 4 and 6; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction.

In the spinal cord, increased levels of the prodynorphin-derived endogenous opioid peptide dynorphin A (1–17) participates in the development and maintenance of nociceptive sensitization in different acute and chronic pain models (Schwei et al., 1999; Wang et al., 2001; Ma and Eisenach, 2003) and after prolonged opioid exposure (Vanderah et al., 2001; Gardell et al., 2006). Dynorphin has been shown to have both antinociceptive (binding to κ -opioid receptors) and pronociceptive effects, probably acting at NMDA and/or bradykinin receptors. Dynorphin acting at nonopioid receptors seems to increase intracellular calcium and activate protein kinase signaling pathways and, as a consequence, induce pronociceptive effects (Tan-No et al., 2002; Lai et al., 2006). Moreover, high doses of intrathecal dynorphin induce prominent excitatory effects, including abnormal pain and severe motor dysfunction (Lai et al., 2001).

Prodynorphin transcription in spinal cord is regulated by transactivators such as the cAMP-responsive element binding protein (CREB), binding to the cAMP-responsive element site, and the early genes *c-fos* and *c-jun*, which bind to the activator protein-1 site (Costigan and Woolf, 2002). The expression of the *c-fos* gene and its protein product (c-Fos) has been extensively used as a marker of neuronal excitability after different noxious stimuli (Hunt et al., 1987; Harris, 1998). In the spinal cord, c-Fos expression has been reported to be increased in several animal models of nociception, including paw inflammation, neuropathic injury, and cancer pain (Coggeshall, 2005). c-Fos expression has also been reported to be involved in the signal transduction cascade that triggers long-term intracellular adaptation of spinal nociceptive input (Dubner and Ruda, 1992).

Both, the immediate-early gene *c-fos* and the late-response gene prodynorphin contain the CREB binding site cAMP-responsive element within their promoter regions (Lonze and Ginty, 2002). It has been described that activated extracellular signal-regulated kinases 1 and 2 (ERK1/2) contribute to CREB activation (Kawasaki et al., 2004). Therefore, in addition to immediate-early genes, the ERK1/2 pathway could regulate the expression of genes responsible for long-lasting synaptic plasticity, such as prodynorphin, and contribute to persistent pain hypersensitivity (Ji et al., 2002). ERK1/2, together with p38 MAPK and c-jun N-terminal kinase, are members of the serine-threonine MAPK family, which play important roles in the transmission and processing of physiological and pathological pain (Ji et al., 2009). Several studies have demonstrated that ERK1/2 is activated in the dorsal horn of the spinal cord in response to noxious stimulation, nerve or inflammatory injury, and after morphine administration (Ji et al., 2002; Crown et al., 2006; Komatsu et al., 2007).

The aim of the present study was to assess the role of spinal prodynorphin/dynorphin in the initiation and maintenance of postoperative hyperalgesia when remifentanyl is used as the main anesthetic and its possible association with the expression of the protein c-Fos and/or activated ERK1/2.

Materials and Methods

Animals. Swiss CD1 male mice weighing 25 to 30 g were obtained from Charles River (CRIFFA, Charles River, Lyon, France) were used in all experiments. Animals were housed under 12-h light/dark conditions in a room with controlled temperature ($21 \pm 1^\circ\text{C}$) and relative humidity ($55 \pm 10\%$). Animals had free access to food and

water except during behavioral evaluation. All procedures and animal handling met the guidelines of the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and the European Communities directive 86/609/EEC regulating animal research. The protocol was approved by the institutional review board (Ethical Committee of Animal Experimentation–Parc Recerca Biomèdica de Barcelona, Barcelona, Spain).

Surgery. We used the incisional postoperative pain model recently validated in our laboratory (Célérier et al., 2006; Cabañero et al., 2009b), that was adapted from Brennan et al. (1996). Animals were anesthetized with sevoflurane delivered for 30 min via a nose mask [induction, 3.5% (v/v); surgery, 3.0% (v/v)] in a sterile operating room. A 0.7-cm longitudinal incision was made with a number 20 blade through the skin and fascia of the plantar surface of the right hind paw, starting 0.3 cm from the proximal edge of the heel extending toward the toes. The underlying plantaris muscle was exposed and incised longitudinally, keeping the muscle origin and insertion intact. After homeostasis with slight pressure, the skin was closed with two 6-0 silk sutures, and the wound was covered with povidone-iodine antiseptic ointment. After surgery, the animals were allowed to recover under a heat source in cages with sterile bedding.

Drug Administration. Remifentanyl (Ultiva; GlaxoSmithKline, Madrid, Spain) and sevoflurane (Sevorane; Abbot Laboratories S.A., Madrid, Spain) were supplied by the Department of Anesthesiology of the Hospital del Mar (Barcelona, Spain). Remifentanyl (80 $\mu\text{g}/\text{kg}$) was dissolved in saline (NaCl 0.9%) and infused subcutaneously over a period of 30 min (rate, 0.8 ml/h) using a KD Scientific pump (KD Scientific Inc., Holliston, MA). Control animals (noninjured mice) underwent a sham procedure that consisted of the administration of sevoflurane plus the same volume of saline in identical conditions.

Behavioral Testing. Hyperalgesia to noxious thermal stimulus and to punctate mechanical stimulus (referred to as “mechanical allodynia” throughout the text) served as measures of nociception. Before the experiments, animals were habituated to the equipment (without nociceptive stimulation) for 3 days. All behavioral experiments were performed between 9:00 AM and 6:00 PM.

Thermal hyperalgesia was evaluated as described previously by Hargreaves et al. (1988). Paw withdrawal latency in response to radiant heat was measured using the Hargreaves equipment (Ugo Basile, Varese, Italy). In brief, mice were placed in methacrylate cylinders (30 cm high, 9 cm in diameter; acquired from Servei Estació, Barcelona, Spain) positioned on a glass surface. Animals were habituated to the environment for 2 h before testing. The heat source was then positioned under the plantar surface of the hind paw and activated with a light beam intensity that, in preliminary studies, gave baseline latencies of 9 to 11 s in control mice. A cutoff time of 20 s was used to prevent tissue damage in the absence of response. The mean paw withdrawal latencies for both hind paws were obtained from the average values of three separate measurements taken at 5-min intervals to prevent nonspecific responses.

Mechanical allodynia was measured by the hind paw withdrawal response to von Frey filament stimulation. Animals were placed in methacrylate cylinders with a wire grid bottom through which the von Frey filaments were applied (bending force range from 0.008 to 2 g; North Coast Medical, Inc., San Jose, CA). Animals were allowed to habituate for 2 h before testing to achieve immobility. The filament force was increased or decreased according to the response. The upper limit value (2 g) was assigned when there was no withdrawal response, and the threshold of response was calculated using the up-down method (Chaplan et al., 1994). Clear paw withdrawal, shaking, or licking was considered a nociceptive-like response. Both hind paws were alternately tested.

Prodynorphin mRNA Quantification by Real-Time Reverse Transcriptase-Polymerase Chain Reaction. Animals were sacrificed at different time points after manipulation. The lumbar spinal cord (L4–L6) was removed and immediately frozen in liquid nitrogen. Tissue was then homogenized in ice-cold buffer (homogenizer Ultra-Turf, T8; Ika Werke, Staufen, Germany), and the total

RNA was isolated with TRIzol (Invitrogen, Renfrewshire, England). In all experiments, 2 μ g of total RNA was transcribed into cDNA using SuperScript II RNase H reverse transcriptase (Invitrogen, Renfrewshire, England).

The expression of prodynorphin was determined by relative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) using a premade TaqMan gene expression assay (Applied Biosystems, Foster City, CA) for the prodynorphin gene (Mm00457572_m1). A probe against phosphoglycerate kinase 1 gene (Mm00435617_m1) was used as endogenous control. Polymerase chain reactions were set up in 384-well plates containing the corresponding cDNA, 2 \times universal master mix (Applied Biosystems), the forward and reverse primers, and the TaqMan probe. The assays were performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

Dynorphin Immunoassay. The lumbar spinal cord (L4–L6) was removed after sacrifice and frozen in liquid nitrogen. To perform the assay, tissue samples were placed in 1 N acetic acid, disrupted with a homogenizer, and incubated for 30 min at 95°C. After centrifugation at 12,000 rpm for 20 min (4°C), the supernatant was lyophilized and stored at –80°C. Protein concentrations were determined using the bicinchoninic acid method with bovine serum albumin as standard. Immunoassay was performed with a commercial enzyme immunoassay system using a specific antibody for dynorphin A (1–17) (Peninsula Laboratories, Belmont, CA). Each experiment was repeated three or four times. Standard curves were constructed, and the dynorphin content was determined with the Prism program (GraphPad Software Inc., San Diego, CA).

Immunohistochemistry. Mice were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused intracardially with phosphate-buffered saline (PBS; 0.1 M, pH 7.4) followed by a fixative containing 4% paraformaldehyde with PBS. The spinal cord from the L4–L6 segments was removed and cryoprotected in 20% sucrose in 0.1 M PBS at 4°C. After 48 h, the lumbar spinal cord was embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Zoeterwoude, The Netherlands), and transverse sections (12 μ m thick) were obtained with a cryostat (Leica, Madrid, Spain) maintained at –25°C. Sections of spinal cord were serially cut, placed on gelatinized slides, and processed for immunohistochemistry. After blocking with 2% normal goat serum (Vector Laboratories, Burlingame, CA), tissue sections were incubated in primary polyclonal anti-c-Fos antibody (diluted 1:3000; Santa Cruz Biotechnology, Santa Cruz, CA) for 36 h at 4°C. Then, the bound primary antibody was localized by a biotinylated secondary anti-rabbit IgG (dilution, 1:200) incubated for 1 h in normal goat serum in PBS (containing 2% goat serum and 0.5% Triton-X-100; Vector Laboratories) and subsequently with the avidin-biotin complex (ABC kits; Vector Laboratories) at room temperature for 1 h. Visualization of the antigen-antibody complex was performed using 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO). The same immunohistochemical procedure was followed to determine pERK1/2. In this occasion, a mouse monoclonal anti-pERK1/2 antibody was used as primary antibody (diluted 1:500 in normal goat serum in PBS, incubated at 4°C overnight; Santa Cruz Biotechnology) and a horse anti-mouse IgG as secondary antibody

(diluted 1:400, incubated for 1 h at room temperature; Vector Laboratories). All sections were then cover-slipped with distyrene-plasticizer-xylene (Sigma-Aldrich). For double-label immunohistochemistry, c-Fos and pERK1/2 were revealed with 3,3'-diaminobenzidine intensified with nickel in the first and second position, respectively.

Quantification of c-Fos and pERK1/2 Immunoreactivity. The number of c-Fos or pERK1/2-immunopositive cells was determined using a video camera (DFC290; Leica, Madrid, Spain) adapted to a light microscope (DM400B; Leica, Madrid, Spain). Seven or eight adjacent sections from the L4–L6 lumbar spinal cord were randomly selected. The number of c-Fos- or/and pERK1/2-positive cells was determined under a 20 \times objective lens in the superficial dorsal horn (laminae I-II), in which most positive cells were observed after the different treatments. The values from seven or eight sections were averaged for each group of experiments.

Experiments Performed. All experimental groups received the same inhaled concentration of sevoflurane [3.0–3.5% (v/v)] for 30 min. Special care was taken to reduce interindividual variability and to use the smallest number of animals per group. Four groups of experiments were performed.

In the first group, we determined the effect of remifentanyl on thermal hyperalgesia and mechanical allodynia. After the habituation period, a baseline response was obtained in all animals, and the average of the measures of three consecutive days was registered. All mice were first tested for mechanical allodynia (von Frey) followed 2 to 3 h later by the assessment of thermal hyperalgesia (Hargreaves test). For each group of treatment, 8 to 10 animals were used. Animals were tested at 4 h and at 2, 7, 10, 14, and 21 days after one of the following treatments, administered over a period of 30 min: sevoflurane + subcutaneous infusion of saline, a treatment that does not alter nociceptive thresholds (C  lerier et al., 2006) (control group); sevoflurane + subcutaneous infusion of 80 μ g/kg remifentanyl (remifentanyl group); surgery performed under sevoflurane + subcutaneous infusion of saline (incision group); and surgery performed under sevoflurane + subcutaneous infusion of 80 μ g/kg remifentanyl (remifentanyl + incision group).

In the second group, we determined the expression of prodynorphin mRNA in the spinal cord after surgical incision and/or remifentanyl administration. To assess the expression of prodynorphin mRNA in the lumbar spinal cord, animals pertaining to the four study groups (control, remifentanyl, incision, and remifentanyl + incision) were sacrificed at 30 min, 4 h, and 2, 4, 7, 10, 14, and 21 days after manipulation. We used the entire lumbar spinal cord (two animals per sample) for the control and remifentanyl groups, whereas for the groups with an incision, the ipsilateral spinal cord was used (four animals per sample). Prodynorphin mRNA expression was determined in animals not exposed to nociceptive testing (nonstimulated). Each experiment was repeated at least four times.

In the third group, we determined the expression of dynorphin in the spinal cord after surgical incision and/or remifentanyl administration. To assess changes in dynorphin levels in the lumbar spinal cord, animals from the four study groups were sacrificed at 30 min, 4 h, and 2, 4, 7, 10, 14, and 21 days after manipulation. We pooled

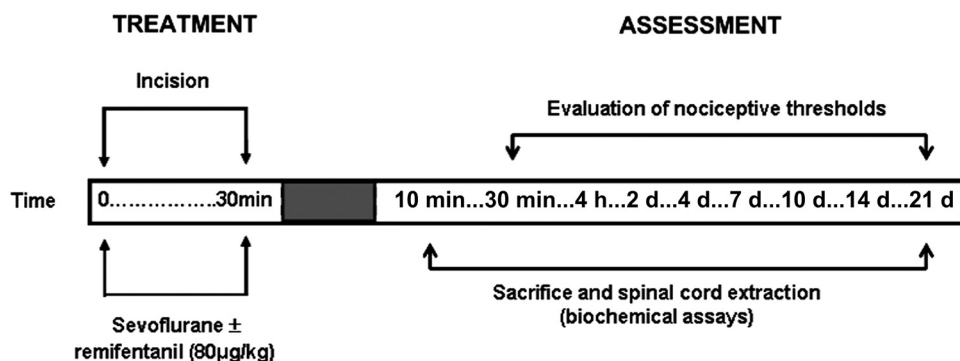


Fig. 1. Experimental protocol. Surgery (or sham) was performed under sevoflurane plus remifentanyl or saline anesthesia infused during a period of 30 min. For the biochemical assays, nonstimulated animals were sacrificed at different time points from 30 min to 21 days (d). Nociceptive thresholds were periodically assessed from 4 h up to 21 days (as described under *Materials and Methods*).

two or four animals per sample (nonstimulated mice) depending on the experimental condition. In these experiments, we also evaluated dynorphin levels in the contralateral spinal cord in the incision and remifentanil + incision groups. Each experiment was repeated at least four times.

Finally, in the fourth group, we determined the expression of c-Fos, pERK1/2, and c-Fos/pERK1/2 (double-labeled) after surgical incision and/or remifentanil administration. c-Fos and pERK1/2 were assessed in the four experimental conditions. To obtain tissue samples for immunohistochemical determination, nonstimulated mice were sacrificed 30 min, 4 h, and 2 and 4 days after treatment (four animals per group). pERK1/2 expression was also assessed in the remifentanil + incision group 10 min after manipulation. Figure 1 shows the experimental protocol used in the study.

Statistical Analysis. To estimate the pronociceptive effects induced by remifentanil, incision, and the combination of both, a general linear-mixed model was used. For each mouse and time point, the responses in seconds (Hargreaves test) or grams (von Frey) are expressed as the percentage of change \pm S.E.M. with respect to the baseline values; negative values indicate net pronociceptive effects and positive values, antinociception. The statistical analysis was performed using R software (The R Project for Statistical Computing, available at <http://www.r-project.org/>), using its library "nlme." This model allows multiple between-group comparisons to assess the effect of the treatment, the effect of time, and the animal hind paw (left and right) compared with baseline values. Areas above the curve (AACs) \pm S.E.M. were calculated to illustrate the overall pronociceptive effect in each experimental condition. The AACs (0–21 days) were obtained with the GraphPad Prism software, and the AAC for each treatment group was normalized (subtracted) to the AAC of the control group. Because the AAC data are reduced to a single

value per mouse, the comparison of the AAC was performed with the nonparametric Wilcoxon Mann-Whitney test for independent samples using SPSS version 13.0 (SPSS Inc., Chicago, IL).

Relative expression of prodynorphin in the spinal cord and statistical significance of real-time RT-PCR data were calculated using REST-384 software (Pfaffl et al., 2002). This software tests statistical significance using a pair-wise fixed reallocation randomization test. The software provides a mean relative value \pm S.E.M. for each time point. In the text, data are shown as the mean percentage of change \pm S.E.M. with respect to baseline.

Data obtained from the dynorphin A (1–17) immunoassay (expressed in picograms per milligram of total protein) was normalized with respect to the mean control values set to a value of 1. The results were plotted using the mean relative value \pm S.E.M. for each time point; in the text, data are shown as the mean percentage of change \pm S.E.M. with respect to baseline. For the statistical analysis, we used one-way analysis of variance followed by a post hoc Newman-Keuls test. The comparison of dynorphin levels between the ipsilateral and the contralateral spinal cord were analyzed by paired Student's *t* test.

The number of c-Fos and pERK1/2-immunoreactive cells is shown as mean values \pm S.E.M. Data were analyzed with a one-way analysis of variance followed by a post hoc Newman-Keuls test. The c-Fos, pERK1/2, and c-Fos/pERK1/2-positive neurons in the ipsilateral and contralateral dorsal horn were compared by paired Student's *t* test. A *p* value less than 0.05 was considered statistically significant.

Results

Behavioral Studies. Baseline thresholds in both paws to thermal and mechanical stimuli were similar in all groups,

TABLE 1

Thermal hyperalgesia and mechanical allodynia in the different experimental conditions

Nociceptive thresholds were evaluated at baseline conditions (before treatment), at 4 h, and at 2, 7, 10, 14, and 21 days after manipulation. In all groups, the maximal decrease in nociceptive thresholds was observed at 4 h and 2 d, and the values are expressed as the percentage change \pm S.E.M. with respect to the baseline. The values of the AAC \pm S.E.M. illustrate the overall effects of each treatment. Negative values indicate net pronociceptive effects (*n* = 8–10 per group).

Experimental Condition and Time of Evaluation	Hind Paw		Duration of Effect	AAC (0–21 days)
	Ipsilateral	Contralateral		
	<i>% change</i>		<i>days</i>	
Thermal hyperalgesia				
Control				
4 h	-1.7 \pm 1.9	-6.2 \pm 0.6	0	-25.5 \pm 65.2
2 days	-3.7 \pm 2.1	1.6 \pm 2.5		
Remifentanil				
4 h	-39.0 \pm 2.9**	-38.8 \pm 2.9**	7–10	-278.2 \pm 52.2*
2 days	-26.8 \pm 4.9*	-22.3 \pm 7.3*		
Incision				
4 h	-74.7 \pm 4.3***	-17.3 \pm 6.0	4–7	-362.4 \pm 110.0*
2 days	-60.7 \pm 7.7***	-24.8 \pm 4.5*		
Remifentanil + Incision				
4 h	-83.7 \pm 2.6***	-45.2 \pm 2.3***	14–21	-694.6 \pm 69.8**+,+,&
2 days	-64.2 \pm 5.9***	-31.3 \pm 4.8***		
Mechanical allodynia				
Control				
4 h	16.2 \pm 3.0	10.1 \pm 5.0	0	84.9 \pm 52.4
2 days	-4.4 \pm 3.0	-0.1 \pm 4.6		
Remifentanil				
4 h	-26.3 \pm 7.0*	-22.3 \pm 8.0*	7–10	-271.8 \pm 99.6*
2 days	-22.9 \pm 7.2*	-25.9 \pm 2.3*		
Incision				
4 h	-69.1 \pm 5.5***	-12.8 \pm 8.7	4–7	-335.7 \pm 115.1*
2 days	-39.8 \pm 2.3***	-12.6 \pm 6.2		
Remifentanil + Incision				
4 h	-59.1 \pm 5.0***	-49.1 \pm 4.8***	10–14	-696.9 \pm 101.7***+,+,&
2 days	-61.8 \pm 10.5***	-49.6 \pm 6.4***		

* *p* < 0.05 compared with the control group.

** *p* < 0.01 compared with the control group.

*** *p* < 0.001 compared with the control group.

+ *p* < 0.05 compared with the remifentanil group.

++ *p* < 0.01 compared with the remifentanil group.

& *p* < 0.05 compared with the incision group.

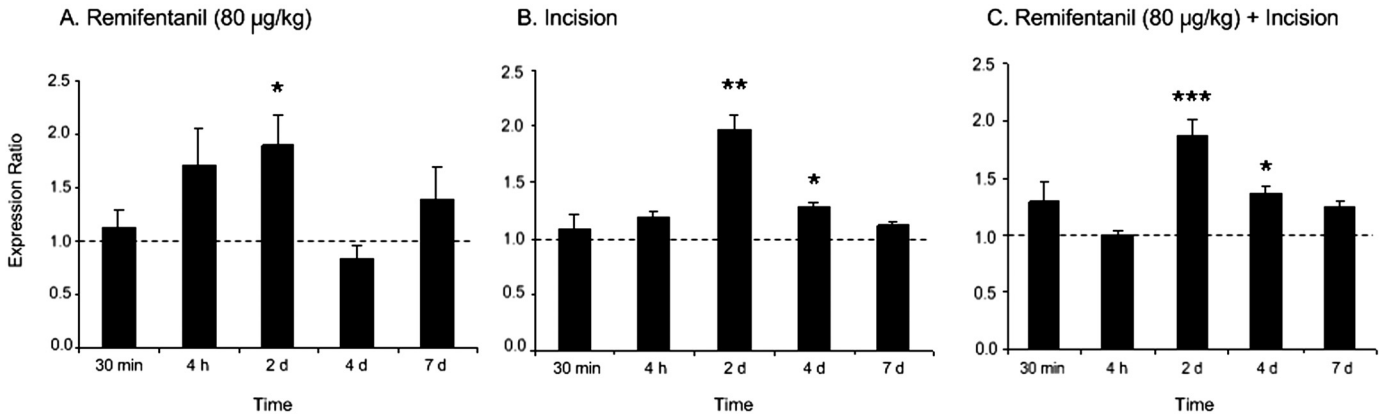


Fig. 2. Time course of prodynorphin mRNA expression in the lumbar spinal cord (L4–L6) in the different experimental conditions. Prodynorphin mRNA is expressed as mean relative values \pm S.E.M. compared with the control group (set to 1, horizontal broken lines). A, remifentanyl group; B, incision; C, remifentanyl + incision. For each time point, we performed at least four determinations using two to four animals per sample. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the control group.

with mean values of 10.8 ± 0.6 s (thermal) and 1.1 ± 0.08 g (mechanical). Saline administration (control group) did not induce significant changes in nociceptive thresholds. In all treatment groups (remifentanyl, incision, and remifentanyl + incision), the maximal pronociceptive effects were observed between 4 h and 2 days (Table 1).

Remifentanyl induced generalized hyperalgesia and allodynia in both paws, still present 7 days after treatment ($p < 0.05$ for thermal hyperalgesia and mechanical allodynia compared with the control group). No significant pronociceptive effects were observed on day 10 or later. The incision decreased nociceptive thresholds only in the operated paw (right), except on day 2, when heat hyperalgesia could be observed also in the contralateral paw. Pain sensitization lasted approximately 4 to 7 days ($p < 0.05$ in both tests). In the remifentanyl + incision group, an increase in the duration of mechanical allodynia in the operated paw, which was statistically significant until day 10 ($-28.5 \pm 5.8\%$; $p < 0.05$), was found. Thermal hyperalgesia was also prolonged up to

day 14 ($-15.8 \pm 1.6\%$; $p < 0.05$). In all groups, animals were completely recovered 21 days after manipulation.

When AACs were assessed in the different experimental conditions, we observed a global increase in hyperalgesia and allodynia after each treatment. No significant differences were observed between the remifentanyl and incision groups. Nevertheless, the increase was significantly greater when remifentanyl and incision were combined (Table 1).

Up-Regulation of Prodynorphin mRNA in the Spinal Cord after Remifentanyl Administration and/or Surgical Incision. Changes in the expression of prodynorphin mRNA in the different groups were determined with RT-PCR. Remifentanyl infusion significantly increased prodynorphin mRNA at 2 days ($90.2 \pm 28.1\%$ increase; $p < 0.05$ compared with controls), although a moderate, but not significant, increase was also observed at 4 h ($70.9 \pm 35.6\%$) (Fig. 2A). After incision, an up-regulation was observed on days 2 ($96.7 \pm 13.8\%$; $p < 0.01$) and 4 ($28.3 \pm 4.3\%$; $p < 0.05$) (Fig. 2B). In the remifentanyl + incision group, prodynorphin

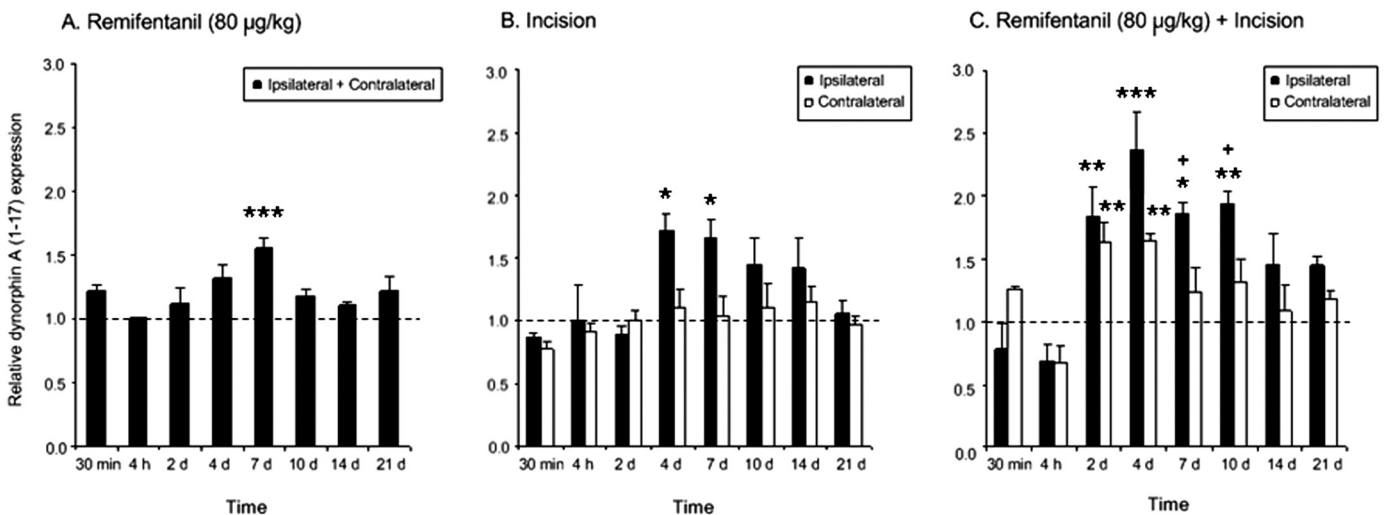


Fig. 3. Dynorphin levels in the lumbar spinal cord (L4–L6) of mice receiving remifentanyl (A), incision (B), or remifentanyl + incision (C). Dynorphin levels (peptide) are expressed as mean relative values \pm S.E.M. compared the control group (value set to 1, horizontal broken lines). A, dynorphin levels after remifentanyl administration in the whole spinal cord (ipsilateral + contralateral sides). B and C, filled and empty columns represent dynorphin levels in the ipsilateral and contralateral side, respectively. We performed at least four determinations for each time point with two to four animals per sample. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the control group. +, $p < 0.05$ compared with the contralateral side of the spinal cord.

mRNA expression was increased at 2 and 4 days ($87.2 \pm 13.7\%$, $p < 0.01$, and $35.7 \pm 7.2\%$, $p < 0.05$, respectively) (Fig. 2C). Prodynorphin mRNA levels were assessed up to 21 days after manipulation (results not shown) but in all groups returned to baseline values 7 days after treatment.

Increased Dynorphin Levels in the Spinal Cord after Remifentanyl Administration and/or Incision. In control mice, mean dynorphin levels in the spinal cord were 393.0 ± 21.6 pg/mg protein, without significant differences between the right and left sides (data not shown). Remifentanyl infusion significantly increased dynorphin levels on day 7 ($55.0 \pm 7.7\%$ compared with control; $p < 0.001$) (Fig. 3A). After incision, an increased dynorphin expression was observed in the ipsilateral spinal cord on days 4 and 7 (71.3 ± 14.0 and $66.7 \pm 13.3\%$, respectively; $p < 0.05$), without significant changes in the contralateral side (Fig. 3B). In the remifentanyl + incision group, we observed a sustained increase of dynorphin in the ipsilateral spinal cord (incision) that lasted from days 2 to 10 (Fig. 3C). The percentages of increase were 83.1 ± 23.8 ($p < 0.01$), 136.7 ± 29.8 ($p < 0.001$), 86.0 ± 8.6 ($p < 0.05$) and $92.7 \pm 11.3\%$ ($p < 0.01$) on days 2, 4, 7, and 10, respectively. In the contralateral spinal cord, significant increases were observed at days 2 and 4 after manipulation (62.1 ± 16.9 and $63.2 \pm 6.6\%$, respectively; $p < 0.01$ compared with controls). Dynorphin levels in the ipsilateral side were significantly higher than in the contralateral one on days 7 and 10 ($p < 0.05$). In all experimental conditions, dynorphin levels returned to baseline 14 days after manipulation.

c-Fos Expression in the Spinal Cord after Remifentanyl and/or Surgical Incision. In all of the experimental conditions, c-Fos was mainly expressed in laminae I–II of the spinal cord. In the control group, a small number of c-Fos-positive neurons were detected both in the ipsilateral and contralateral sides of the lumbar spinal cord (Fig. 4). In the remifentanyl and incision groups, a small but significant increase of the number of c-Fos-immunoreactive spinal cord neurons was observed ipsilaterally ($p < 0.05$) and contralaterally ($p < 0.05$) only at 30 min and 4 h, without significant differences between sides. Non-c-Fos expression could be detected on days 2 and 4 after manipulation (data not shown). In the remifentanyl + incision group, we observed a substantial increase in c-Fos expression in the ipsilateral side at 30 min (17.9 ± 0.3 ; $p < 0.001$) and 4 h (23.0 ± 0.2 ; $p < 0.001$). A significant increase but of a smaller magnitude was also present in the contralateral side at the same time points ($p < 0.001$ compared with the ipsilateral side). In the same group (remifentanyl + incision), c-Fos-immunoreactive neurons were also slightly increased only in the ipsilateral side of the spinal cord on days 2 (7.6 ± 0.7 ; $p < 0.05$) and 4 (5.0 ± 0.4 ; $p < 0.05$) after manipulation.

Activation of ERK1/2 in the Spinal Cord after Remifentanyl and/or Incision. pERK1/2-labeled cells were predominantly localized in laminae I–II of the spinal cord. In control mice, pERK1/2 was undetectable. In the remifentanyl or incision groups, nonsignificant levels were also obtained in both sides of the dorsal horn at all times of evaluation (30 min, 4 h,

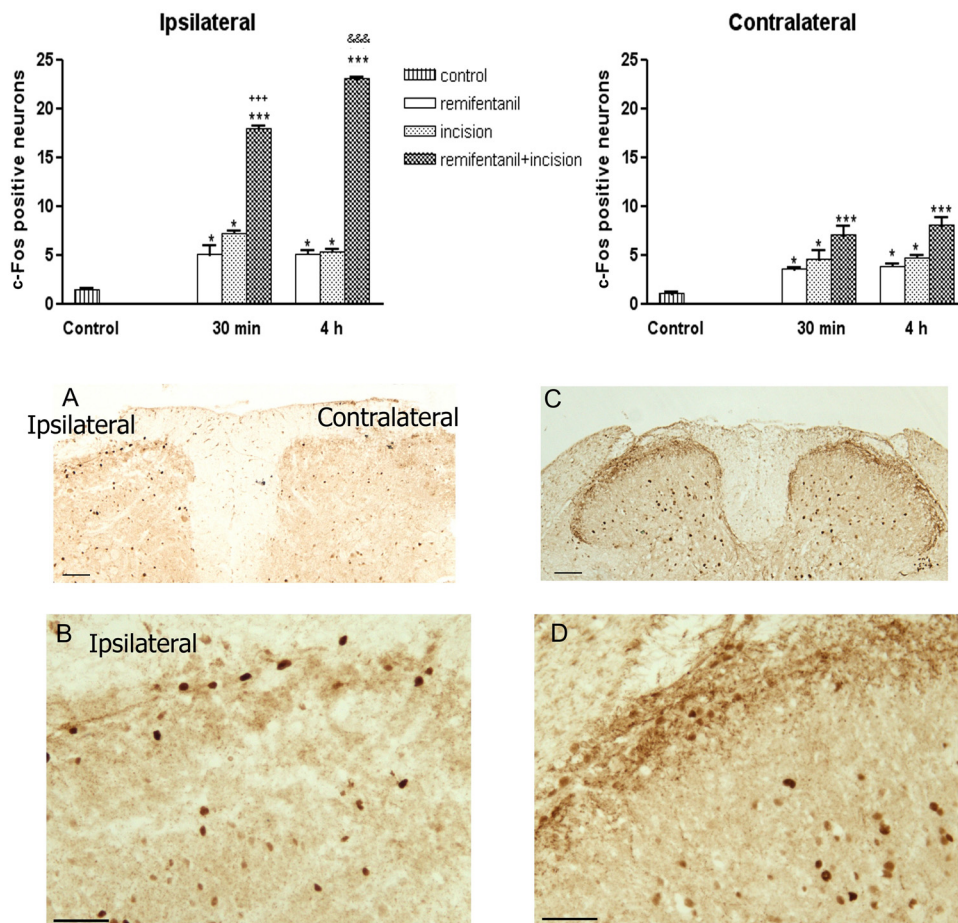


Fig. 4. c-Fos-immunoreactive neurons in the lumbar spinal cord (L4–L6) after remifentanyl and/or incision. Histograms show the number of c-Fos-positive neurons \pm S.E.M. in the ipsilateral and contralateral dorsal horn, assessed at 30 min and 4 h after manipulation. *, $p < 0.05$, ***, $p < 0.001$ compared with the control group. + + +, $p < 0.001$ compared with the remifentanyl or incision groups at 30 min. &&&, $p < 0.001$ compared with the remifentanyl + incision groups at 4 h. Bottom, photomicrographs of c-Fos expression at 4 h (A and B) and 30 min (C and D) in the remifentanyl + incision group ($n = 4$ per group). Scale bars, 100 μ m (A and C) and 50 μ m (B and D).

and 2 and 4 days). In the remifentanyl + incision group, ERK1/2 activation was assessed at 10 min, 30 min, 4 h, and 2 and 4 days. An increase of pERK1/2-positive cells was observed at 4 h ipsilaterally (38.2 ± 1.0 ; $p < 0.001$ compared with the other treatment groups) and contralaterally (12.3 ± 1.5 ; $p < 0.001$), as well as on day 2 (5.36 ± 0.2 ; $p < 0.05$), but only in the ipsilateral side (Fig. 5). Significant differences were also observed between both sides of the spinal cord at 4 h after manipulation ($p < 0.001$). No pERK1/2 expression was present at 10 or 30 min or 4 days after manipulation.

Coexpression of pERK1/2 and c-Fos in the Spinal Cord. In this set of experiments, we analyzed ERK1/2 activation in cells expressing c-Fos, in the spinal cord. Because the main increase in the number of pERK1/2-positive cells was observed in the remifentanyl + incision group at 4 h, we performed pERK1/2/c-Fos colocalization assays in these experimental conditions. As shown in Fig. 6A, c-Fos was localized in neurons expressing activated ERK1/2 in the dorsal horn (Fig. 6, B and B'). Quantitative analysis revealed a significant increase in the number of pERK1/2/c-Fos-positive neurons in the ipsilateral versus contralateral side ($p < 0.001$). The number of c-Fos-positive non-ERK1/2-activated cells is shown in Table 2. Although the proportion of double-labeled cells after remifentanyl + incision was high, absolute counts revealed that a similar number of c-Fos-positive neurons were also detected outside of pERK1/2-positive cells.

Discussion

In our study, surgery performed under remifentanyl anesthesia induced significant postoperative hyperalgesia that was maximal between 4 h and 2 days, lasting up to 10 to 14 days. In the same experimental conditions, we also show for the first time an earlier expression of c-Fos immunoreactivity

in the superficial laminae of the dorsal horn of the spinal cord (peak at 4 h), together with a major activation of ERK1/2 (4 h). A delayed increase in prodynorphin mRNA expression and dynorphin levels was also observed. The results suggest that activated ERK1/2 could induce c-Fos expression and trigger the transcription of prodynorphin in the spinal cord. This in turn would result in long-lasting increased levels of dynorphin (2–10 days) that roughly correlate with the duration of postoperative hyperalgesia in our model.

In the same model, we have reported previously that a subcutaneous infusion of remifentanyl (40 $\mu\text{g}/\text{kg}$) during surgery enhanced postoperative hyperalgesia and allodynia at least up to 7 days (C  lerier et al., 2006; Caba  ero et al., 2009b). In the present study, we used a higher dose of remifentanyl (80 $\mu\text{g}/\text{kg}$) because it induces a greater pronociceptive effect (Caba  ero et al., 2009a). The maximal decrease in nociceptive thresholds was observed between 4 h and 2 days after remifentanyl + incision, which lasted 10 to 14 days. Residual effects of anesthesia prevented behavioral nociceptive evaluation 10 or 30 min after surgery.

Although mechanisms underlying opioid-induced hyperalgesia and the enhancement of postoperative pain by perioperative opioid administration are not completely characterized, spinal dynorphin seems to play a critical role. In a model of postoperative pain in rats, Rivat et al. (2009) found a significant increase in spinal dynorphin 1 day after fentanyl administration or surgical incision, with a greater increase when both procedures were combined. In the present report, we were able to confirm and expand these results in mice, but using remifentanyl. It is likely that increased dynorphin also plays an important role in postoperative pain sensitization when other short-acting opioids such as alfentanil are used (C  lerier et al., 2006).

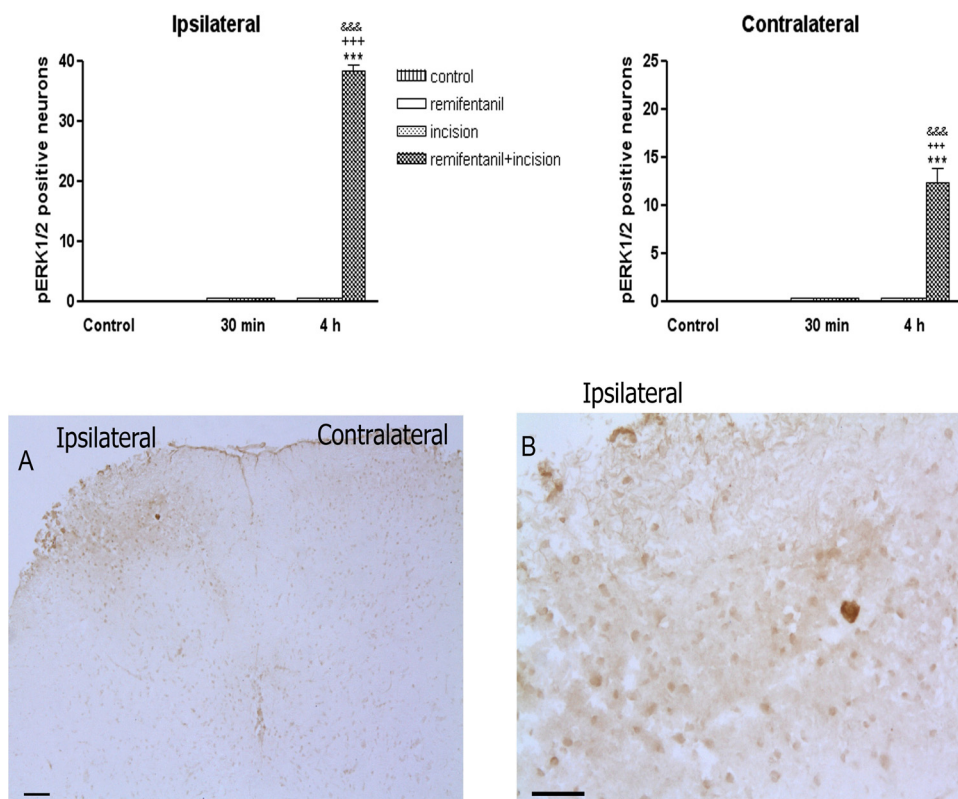


Fig. 5. pERK1/2-immunoreactive cells in the lumbar spinal cord (L4–L6) after remifentanyl and/or incision. Histograms show the number of pERK1/2-positive cells \pm S.E.M. in the ipsilateral and contralateral dorsal horn, assessed at 30 min and 4 h after manipulation. ***, $p < 0.001$ compared with the other experimental conditions and time points. Bottom, photomicrographs of pERK1/2 expression at 4 h in the remifentanyl + incision group ($n = 4$ per group). Scale bars, 100 μm (A) and 50 μm (B).

Our results show that prodynorphin gene transcription peaked at 2 days in all of the experimental conditions and disappeared after 4 days. Our results roughly agree with those of Zhu et al. (2006) who, in a model of incisional pain, reported an early and sustained increase in immunoreactive prodynorphin lasting up to 7 days. We observed that the increase in dynorphin levels parallels the reduction in prodynorphin mRNA over time, suggesting that the treatments enhance the processing and subsequent release of spinal dynorphin. A similar finding was reported after the administration of (D-Ala2-N-methyl-Phe-Gly-ol5)-enkephalin, which elicited a sequential increase of spinal prodynorphin and dynorphin expression (Vanderah et al., 2001).

The onset and duration of dynorphin expression was considerably different in the groups of study. In the remifentanyl + incision group the increased dynorphin levels was observed at an earlier time (2 days) and persisted up to 10 days compared with remifentanyl or incision alone; in this group (but not in the others), we also observed an increase in spinal dynorphin in the contralateral side. Accordingly, a parallelism between dynorphin levels and nociceptive behavior could be suggested. Maximal hyperalgesia was observed at 4 h and 2 days after manipulation. However, increased dynorphin levels were detected somewhat later, on days 4 to 7 in the remifentanyl and incision groups and on days 2 to 10 in the remifentanyl +

TABLE 2

Coexpression of pERK1/2 and c-Fos-positive cells in the dorsal horn of the spinal cord in the remifentanyl + incision group

Estimated number of pERK1/2-immunoreactive cells, c-Fos-positive neurons, and c-Fos-positive neurons plus pERK1/2-negative cells in the ipsilateral and contralateral sides of the spinal cord at 4 h after manipulation. Results are expressed as mean values \pm S.E.M. ($n = 4$ animals).

	pERK1/2 ⁺	c-Fos ⁺	c-Fos ⁺ / pERK1/2 ⁻
Ipsilateral	37.4 \pm 1.0***	23.3 \pm 0.9***	11.7 \pm 1.6***
Contralateral	12.3 \pm 1.5	10.3 \pm 1.0	4.6 \pm 0.9

*** $p < 0.001$ compared with contralateral spinal cord.

incision group. Onset and duration of dynorphin up-regulation could be relevant to explain postoperative sensitization. The delayed increase in dynorphin observed in our study (days 4–7) could explain the failure to reverse incisional pain after intrathecal administration of anti-dynorphin antiserum on the first day after surgery (Rivat et al., 2009). Moreover, intrathecal anti-dynorphin antiserum also failed to reduce pain hypersensitivity 2 days after sciatic nerve ligation in mice but was effective 10 days after surgery (Wang et al., 2001). Our data suggest that spinal dynorphin could be involved in the persistence of pain but not in the expression of first pain.

It has been postulated that after nociceptive stimuli,

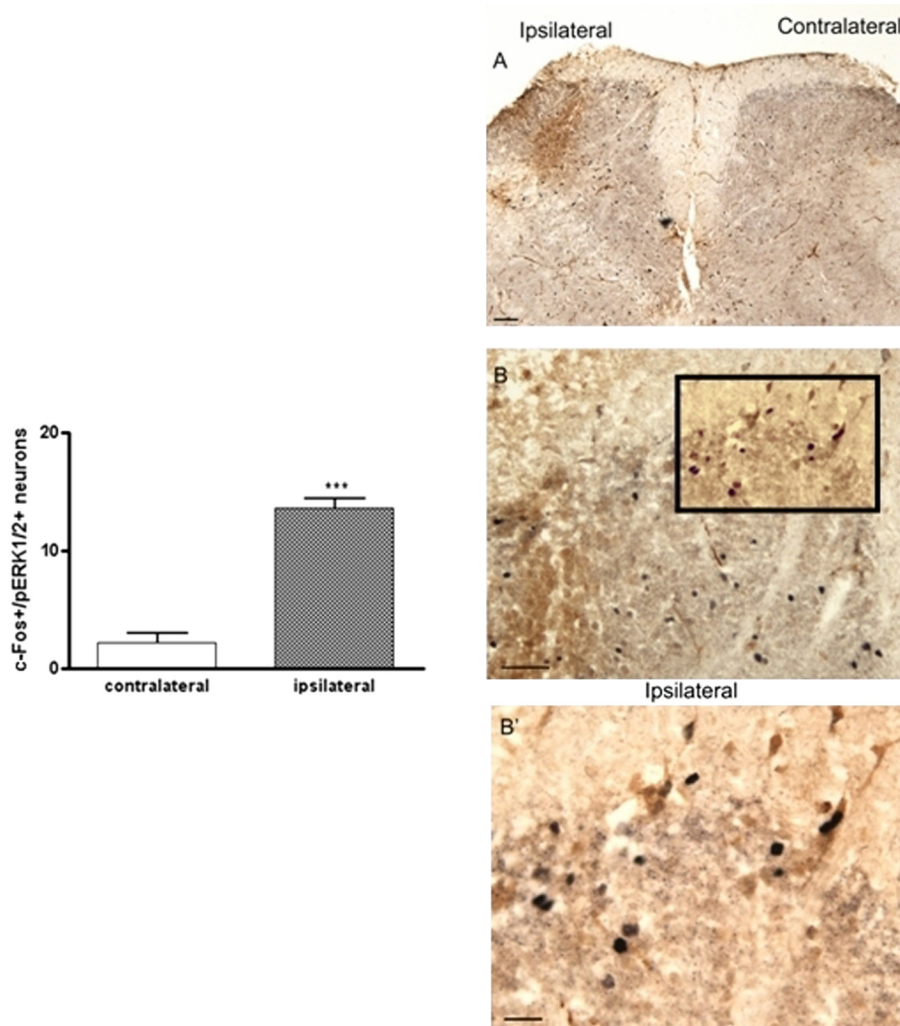


Fig. 6. Coexpression of c-Fos and pERK1/2 in the lumbar spinal cord (L4–L6) in the remifentanyl + incision group. Shown are the mean number of c-Fos/pERK1/2-positive neurons \pm S.E.M. 4 h after manipulation in the ipsilateral and contralateral dorsal horn. ***, $p < 0.001$ compared with the contralateral side. Photomicrographs show c-Fos-positive (black) and pERK1/2-positive (brown) cells. B and B', ipsilateral dorsal horn at a higher magnification. Scale bars, 100 μ m (A), 50 μ m (B), and 20 μ m (C). ($n = 4$ per group).

dynorphin gene expression could be promoted by c-Fos (Hunter et al., 1995). In our study, the temporal sequences of c-Fos and mRNA prodynorphin expression suggest that c-Fos regulates the expression of the prodynorphin gene as supported by others (Kawasaki et al., 2004). We identified an increase in c-Fos-positive neurons in the spinal cord at 30 min and 4 h in all treatment groups, although the greater increase was observed in the remifentanyl + incision group. At the same site, other studies have shown a rapid (within minutes) and transient expression of c-Fos after noxious stimuli, peaking at approximately 2 h and disappearing a few hours later (Hunt et al., 1987; Le Guen et al., 1998).

Analyzing each experimental condition individually, few c-Fos-immunoreactive cells were identified in the control group, in agreement with previous reports (Coggeshall, 2005). Remifentanyl or incision alone induced a slight increase in c-Fos immunoreactivity, both in the ipsilateral and contralateral sides of the spinal cord. Beitz et al. (2004) also observed increased spinal Fos expression 4 h after incision, which is consistent with our findings. However, little is known about changes in c-Fos associated with the pronociceptive effects of systemic opioids. Vera-Portocarrero et al. (2007) reported that sustained intrathecal administration of morphine induced pain hypersensitivity, increasing touch-evoked spinal Fos expression after repeated stimulation. In the remifentanyl + incision group, we demonstrated a robust increase in c-Fos immunoreactive neurons in the ipsilateral dorsal horn at 30 min and 4 h, followed by a lesser expression on days 2 and 4. Thus, the combination of both remifentanyl + incision greatly enhanced neuronal activity in our model.

pERK1/2 regulates prodynorphin expression through c-Fos or CREB pathways. In the study, we determined c-Fos and pERK1/2 because they are good markers of neuronal activation after noxious stimulation and tissue injury. Moreover, pERK1/2 is a good indicator of central sensitization closely correlating with pain behavior (Gao and Ji, 2009). Our results show a remarkable increase in pERK1/2-immunoreactive cells in the remifentanyl + incision group at 4 h in both sides of the spinal cord, although it was of a lesser magnitude contralaterally. We also observed a persistent up-regulation of prodynorphin mRNA 2 to 4 days after manipulation, suggesting that ERK1/2 could regulate prodynorphin gene transcription in this experimental condition. A persistent up-regulation of prodynorphin mRNA (<48 h) associated with ERK1/2 activation in a model of inflammatory pain in rats has been reported previously (Ji et al., 1999). In our study, pERK1/2-immunoreactive cells were undetectable when the incision or remifentanyl individually was used, probably related to the low intensity of the stimuli (Ji et al., 1999) that would be insufficient to activate ERK1/2. However, when remifentanyl + incision were combined, the intensity of the stimuli reached the required threshold to activate ERK1/2 in the spinal cord.

Different studies have shown pERK1/2 after opioid administration or tissue/nerve injury (Ji et al., 2009); however, changes in spinal ERK1/2 activation induced by remifentanyl during surgery have not been reported. It has been proposed that spinal ERK1/2 contributes to short-term pain hypersensitivity after intense and persistent noxious stimuli. In our study, the activation time course of ERK1/2 and its distribution (laminae I and II) suggest that this MAPK may contribute to pain sensitization after intraoperative remifentanyl.

The expression of pERK1/2 precisely coincides with the time of greater reduction in nociceptive thresholds. Moreover, the higher number of pERK1/2-immunoreactive cells in the ipsilateral side concurs with the lower nociceptive thresholds observed in the operated paw.

Because in the remifentanyl + incision group pERK1/2 was greater at 4 h, we investigated a possible coexpression with c-Fos at this time. We observed a partial colocalization (laminae I-II), suggesting that pERK1/2 could contribute to c-Fos expression in our in vivo model. However, a similar number of c-Fos-positive neurons did not coexpress pERK1/2, indicating that alternative pathways such as protein kinase A, protein kinase C, and p38 kinase could also regulate c-Fos expression (Benavides et al., 2005; Svensson et al., 2005; González-Cuello et al., 2007). Regarding the association between ERK1/2 and c-Fos, some in vivo studies show that in the spinal cord, ERK1/2 is required for c-Fos expression after noxious stimulation (Cruz et al., 2007; Zhang et al., 2009), whereas others illustrate c-Fos expression in the absence of ERK1/2 activation in different animal models (Ji and Rupp, 1997; Kominato et al., 2003).

We conclude that in the present mice model, surgery performed under remifentanyl anesthesia induced an early activation of c-Fos immunoreactivity in the superficial laminae of the dorsal horn of the spinal cord, which was partially associated with an increase in pERK1/2 immunoreactivity. These results suggest that the ERK1/2 pathway and c-Fos expression could regulate prodynorphin mRNA levels in the spinal cord, resulting in a long-lasting increase of dynorphin that roughly correlated with the time course of postoperative pain sensitization. Likely strategies to prevent postoperative hyperalgesia could include decreasing the dose of intraoperative opioids (Cabañero et al., 2009a), antagonism of the NMDA or bradykinin receptors, and/or blocking dynorphin with antiserum or MAPK activation in the spinal cord.

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