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Comparison of the Effects of Treatment with Intrathecal Lidocaine Given before and after Formalin on Both Nociception and Fos Expression in the Spinal Cord Dorsal Horn

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Background: **It has been proposed that the measure of noxious stimulus-induced Fos (the protein product of the immediate early gene c-*fos*) expression in the spinal cord dorsal horn of laboratory animals may provide an estimate of the potential of specific treatments to produce preemptive analgesia. The present study examined this hypothesis by comparing the effects of intrathecal lidocaine given before and after hindpaw formalin injection on persistent nociceptive responses and Fos expression in spinal cord dorsal horn of rats.**

Methods: **Formalin-induced nociception and Fos expression in the spinal cord, in response to a 50-μl injection of 2.5% formalin into the hind paw, were assessed in rats given an intrathecal injection of 50 μl 2% lidocaine by lumbar puncture between the L5 and L6 vertebrae, either 3 min before (pretreatment) or 5 min after (post-treatment) formalin injection.**

Results: **Pain behaviors (hindpaw licking, elevation, and favoring) in the second phase of the formalin test were significantly reduced by pretreatment, but were unaffected by post-treatment. The number of immunocytochemically stained Fos-positive cells and the immunoprecipitation of the Fos antibodies were reduced by pretreatment, and were also reduced, to a lesser extent, by post-treatment.**

Conclusions: **The finding that persistent nociceptive behaviors and Fos expression were suppressed by intrathecal lidocaine pretreatment suggests that nociception in the second phase of the formalin test depends on increases in central hyperexcitability generated during the first phase. On the other hand, the finding that the intrathecal injection of lidocaine after formalin treatment reduced Fos expression but not nociceptive responses indicates an uncoupling of the behavioral and Fos protein responses to formalin and suggests that changes in Fos expression may not be a good predictor of the ability of agents to produce preemptive analgesia. (Key words: Anesthesia; analgesia; formalin test; pain; proto-oncogenes; preemptive analgesia.)**

THERE is a growing body of basic and clinical evidence demonstrating that noxious peripheral stimulation produces sustained changes in central nervous system excitability that may contribute to pathologic pain processes,[1] and that central nervous system hyperexcitability is more effectively attenuated by analgesic pretreatments than by post-treatments.[2] Preemptive analgesia refers to clinical situations in which analgesic or local anesthetic treatments are given before surgery to reduce postoperative pain by preventing or minimizing central nervous system hyperexcitability. The formalin test[3] has been used as an animal model of injury-induced central nervous system hyperexcitability[4,5] and to test the potential of treatments for preemptive analgesia.[6–11] Several investigations indicate that various treatments, including intrathecal[4,5] and systemic[6] lidocaine or opiates[7,12] or N-methyl-D-aspartate receptor[13–18] and neurokinin-1 receptor[19–22] antagonists, are often more effective at reducing nociceptive responses to formalin when given as pretreatments compared with when given as post-treatments. Together these findings suggest that central hyperexcitability that occurs early

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after formalin injection contributes to the persistent nociceptive responses to formalin, and that various treatments can preempt the development of hyperexcitability and reduce persistent nociceptive behaviors.

Sensory inputs,[23] particularly nociceptive inputs,[24] have been reported to produce increased expression of c-*fos* in the mammalian spinal dorsal horn. Investigators have studied the levels of the immediate early gene c-*fos* and its protein product Fos. Increases in c-*fos* mRNA, or Fos protein-like immunoreactivity, have been reported after high-intensity electrical stimulation,[24] noxious mechanical stimulation,[25] and either noxious heat[26] or cold[27] stimulation of the rat hind paw. Fos expression is also enhanced after chemical irritation of cutaneous tissue with formalin[28–32] and mustard oil,[33] and in response to chronic inflammation produced by hind paw injections of complete Freund's adjuvant[34] or carrageenan.[35] It has been suggested there may be a parallel between the ability of anesthetic and analgesic agents to preemptively inhibit nociceptive behaviors and to suppress Fos expression in the spinal cord dorsal horn.[36] The objective of the present study was to test this hypothesis by determining whether there is a parallel between the effects of intrathecal lidocaine pretreatment and post-treatment on nociceptive scores and Fos expression after formalin injection in rats.

Materials and Methods

The following experiments were done under protocols approved by the Institutional Animal Care Committee of the Clinical Research Institute of Montreal. The experiments were performed on Long Evans male rats (weight, 250–300 g) from Charles River, Quebec. Efforts were made to minimize animal suffering and reduce the numbers of animals used. Two approaches were taken to detect Fos-like immunoreactivity after the noxious stimulus was applied. The first approach was taken to measure Fos-like immunoreactivity quantitatively by immunoprecipitation. The second approach was taken by using immunocytochemical methods to demonstrate qualitative differences in Fos-like immunoreactivity among various groups.

Experimental Protocol

In all the studies, the peripheral noxious stimulus consisted of injecting 50 μl 2.5% formalin into the plantar surface of one hind paw of the rat. To study the effect of spinal anesthesia on nociceptive behavior in the formalin test, 50 μl 2% lidocaine was given intrathecally by lumbar puncture between the L5 and L6 vertebrae using a 27-gauge needle. Rats were briefly anesthetized with 3% halothane during the lumbar punctures, which normally took 2–3 min. Typically, rats recovered from the halothane anesthesia within 1 min after completion of the lumbar puncture. The spinal lidocaine produced a complete anesthetic blockade (as measured by the loss of all responses, including an orienting response, to hind paw pinch) between 1 and 7 min, with partial effects lasting until 20 min after treatment. We found that the same dose of intrathecal lidocaine used in the present study produces a complete block of a pressor response to hindpaw injection of 10% formalin, without affecting increases in mean arterial pressure in response to forepaw injection of 10% formalin. For behavioral studies, each of 13 rats in each group received either lidocaine 3 min before formalin (lidocaine + formalin), lidocaine 5 min after formalin (formalin + lidocaine), saline 3 min before formalin (saline + formalin), or saline 5 min after formalin (formalin + saline). For Fos-like immunoreactivity and Fos immunoprecipitation (Fos-IP), three or four animals in each group received either no lidocaine and no formalin (control), formalin but no lidocaine (formalin), lidocaine 3 min before formalin (lidocaine + formalin), or lidocaine 5 min after formalin (formalin + lidocaine), and were compared with a group that received only lidocaine. In the lidocaine pretreatment group, when rats received intrathecal lidocaine 3 min before formalin, all rats had fully recovered from the halothane anesthesia when the formalin was given. However, at this time the intrathecal lidocaine was fully active, so that rats had a sensory and motor block of the hindlimbs and did not respond in any way to the formalin injection. In the lidocaine post-treatment group, when rats received lidocaine 5 min after formalin, rats were completely unanesthetized at the time of formalin injection, but they were briefly anesthetized with halothane when the lumbar puncture was performed to inject lidocaine 5 min after formalin. All rats, in the pretreatment and post-treatment groups, had fully recovered from the spinal anesthesia when behavioral assessment of formalin nociception began 30 min after formalin injection.

Behavioral Studies

In behavioral studies, the rats were assessed for their nociceptive scores in the formalin test. These observations were taken 30 to 60 min after the formalin injection. A nociceptive score was determined for each

5-min block by measuring the amount of time spent in each of the four behavioral categories: 0, the injected paw is not favored; 1, the injected paw has little or no weight on it; 2, the injected paw is elevated and is not in contact with any surface; and 3, the injected paw is licked, bitten, or shaken. A weighted average nociceptive score ranging from 0 to 3 was calculated according to the method of Dubuisson and Dennis[3] by multiplying the time spent in each category by the category weight, summing these products, and then dividing by the total time for each 5-min period.

Immunoprecipitation

It is well established that Fos-like immunoreactivity in the spinal cord peaks approximately 2 h after formalin injection.[30] Thus 2 h after the formalin injection, the rats were anesthetized with sodium pentobarbital (75 mg/kg), decapitated, and the spinal cords removed rapidly by pressure ejection using cold phosphate-buffered saline (PBS; 0.15 M, *p*H 7.4). The lumbar segment was separated from the spinal cord and cut into approximately 1-mm-thick slices. The dorsal and ventral horns were isolated under the microscope, weighed, and homogenized in PBS. After homogenization, 100 μl tissue homogenate containing 10 μCi ^{35}S-methionine was incubated at 37°C for 45 min. The reaction was stopped using 100 μl PBS-thiamine disulfide on ice for 15 min. The homogenate was again centrifuged for 45 min at 11,200 rpm in a cold room. The supernatant was removed and reacted with Fos monoclonal primary antibody (Ab-1, 1:3,000 dilution; Oncogene Science, Manhassett, NY) for 48 h at 4°C. Protein A agarose (100 μl) was added to precipitate the antigen-antibody complex. The amino precipitate was constructed in 100 μl 1:1 nitric acid:perchloric acid solution, vortex mixed, and counted in a liquid scintillation counter (Beckman B Scintillation spectrometer, Fullerton, CA) using a scintiverse II cocktail. The protein concentration was measured using Bradford's[37] method. Quantification of Fos immunoreactivity was based on this completely objective immunoprecipitation technique.

Immunocytochemical Analysis

Although Fos-like immunoreactivity was quantified using immunoprecipitation, spinal cord sections were also taken from some rats for immunocytochemical analysis. This allowed us to determine the laminar distribution of Fos-like immunoreactivity and to compare our findings with those of previous investigations that used immunocytochemical analysis. Two hours after the formalin injection, rats were killed with sodium pentobarbitol (60 mg/kg given intraperitoneally) and given an intracardiac perfusion of heparin-prepared saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer with 0.01% thimersol. The spinal cord was rapidly removed by pressure ejection using cold PBS, and the lumbar segment of the spinal cord was postfixed in the paraformaldehyde solution for 12 h and then cryoprotected by soaking in 30% sucrose in 0.1 M phosphate buffer for 48 h. The spinal cords were frozen with dry ice and 30-μm transverse sections were cut using a refrigerated Leica cryostat. These slides were stored in 0.1 M PBS until the sections were immunocytochemically stained for Fos protein. For immunostaining, we used the methods of Dragunow and Robertson,[38] as described in the guidelines produced by Oncogene Science (Uniondale, NY). This method incorporates the avidin-biotin-peroxidase method of Hsu *et al.*,[39,40] using a commercial kit (Vector Laboratories, Burlingame, CA). Briefly, staining consisted of incubating sections in a solution of 1% horse serum, 0.01 M PBS, and 0.2% Triton X-100 for 20 min followed by washing with 0.01 M PBS. Sections were incubated in 1.0 μg/ml Fos primary antibody (polyclonal primary antibody, Ab-2, 1:100 dilution; Oncogene Science) for 24 h at 4°C. Negative control sections were not incubated in the primary antibody. All sections were then rinsed in 0.01 M PBS and incubated in biotinylated secondary antibody (rabbit IgG, PK-4001; Vector Laboratories) for 60 min at room temperature. After rinsing with 0.01 M PBS, sections were incubated in the Vectastin avidin-biotin-peroxidase mixture from these kits for 60 min at room temperature. A 0.03% 3,3 diamino-benzidine tetrahydrochloric/0.0015% hydrogen peroxide solution was placed on the sections for 1–3 min. Sections were rinsed in distilled water and mounted on gelatin-coated slides. After mounting, sections were dehydrated in 70% and 90% alcohol and 100% 2-propanol for 1 min each. Sections were soaked in AmeriClear (Baxter Scientific) and mounted with coverslips using Permount (Fisher Scientific). These slides were visually scanned and photographed using a light microscope (Vanox; Olympus Corp., Tokyo, Japan).

Statistical Analyses

Data are presented in text and figures as means ± SD. Statistics are based on Newman-Keuls *post hoc* comparisons after one-way analysis of variance (across groups) for the behavioral study and two-way analysis of vari-

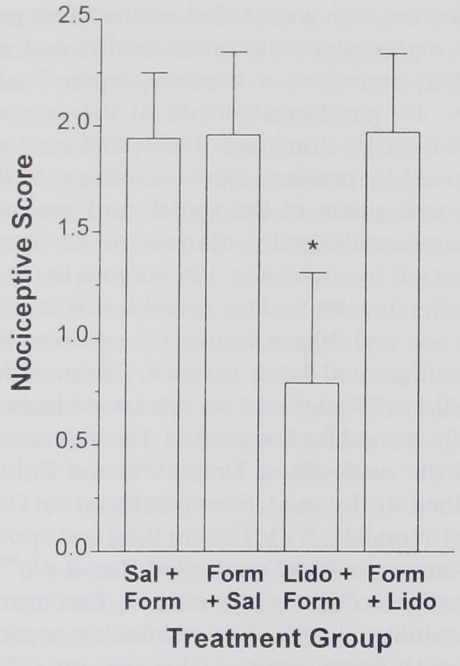

Fig. 1. Histograms representing the formalin pain scores (mean ± SD) averaged over the 30-min test period for rats treated with lidocaine either before (lidocaine + formalin) or after (formalin + lidocaine) formalin injection. Saline (saline + formalin or formalin + saline) replaced the lidocaine in the two control groups. Lidocaine given before formalin ($P < 0.01$, Newman-Keuls test), but not after formalin ($P > 0.05$, Newman-Keuls test), produced a significant reduction in nociceptive scores compared with the two saline groups. No difference in nociceptive scores was found between saline given before (saline + formalin) or after (formalin + saline) the formalin injection ($P > 0.05$, Newman-Keuls test).

ance (across groups and spinal cord sites) for the immunoprecipitation study.

Results

As shown in figure 1, nociceptive scores were significantly reduced compared with the saline/formalin control group (saline + formalin), when rats were given intrathecal lidocaine 3 min before injection of formalin (lidocaine + formalin; $P < 0.01$). However, when the same treatment was given 5 min after the formalin injection (formalin + lidocaine), or when saline was given 5 min after formalin (formalin + saline), the pain scores remained unaffected ($P > 0.05$).

Fos-like immunoreactivity in response to formalin injection was restricted to the spinal cord ipsilateral to the injected hind paw. Fos-like immunoreactivity was maximal in the lumbar (L2–L6) spinal cord, predominantly in the superficial (lamina I) and deeper layers (lamina V-VI). Rats given formalin but no lidocaine (fig. 2A) had greater Fos-like immunoreactivity in the superficial spinal cord dorsal horn than control rats, which received neither formalin nor lidocaine (fig. 2B). The number of Fos-positive cells was also dramatically reduced in rats that received lidocaine before formalin (fig. 2C), and reduced to a lesser degree in rats that received lidocaine after formalin (fig. 2D).

The amount of Fos in the spinal cord was measured using immunoprecipitation of the monoclonal antibody (Ab-1). Kinetic studies on formalin-induced Fos expression (data not shown) exhibited a maximal induction at 2 h, with a greater enhancement in the dorsal horn compared with the ventral horn. The current results, shown in figure 3, indicate that significantly higher counts of immunoprecipitated Fos were found in the group of animals injected with formalin compared with control animals ($P < 0.05$). Furthermore, Fos expression was significantly suppressed in rats that received intrathecal lidocaine before the formalin injection (lidocaine + formalin; $P < 0.05$) and suppressed to a lesser extent in rats that received lidocaine after the formalin injection (formalin + lidocaine; $P < 0.05$). The suppression of formalin-induced Fos expression by pretreatment or post-treatment with lidocaine may be underestimated because we also found that intrathecal lidocaine treatment alone produced a significant increase in Fos expression over untreated controls (counts of 1,253 ± 8.1 and 677 ± 8.2 for the dorsal and ventral horns, respectively). Regardless of the treatment condition, Fos expression was always greater in the dorsal horn compared with the ventral horn ($P < 0.05$).

Discussion

The present results replicate our previous findings[4,5] that intrathecal administration of lidocaine to the lumbar spinal cord depresses the second-phase nociceptive response to hind paw formalin injection, only when it is given before the formalin and not shortly after formalin. They are also consistent with a previous report that second-phase nociceptive responses to formalin are reduced by systemic pretreatment but not by early post-treatment with local anesthetics.[6] Furthermore, they support previous data demonstrating that subcutaneous injection of formalin also produces an enhanced expression of Fos-like immunoreactivity in the spinal cord dorsal horn.[28–32] The present study also provides novel

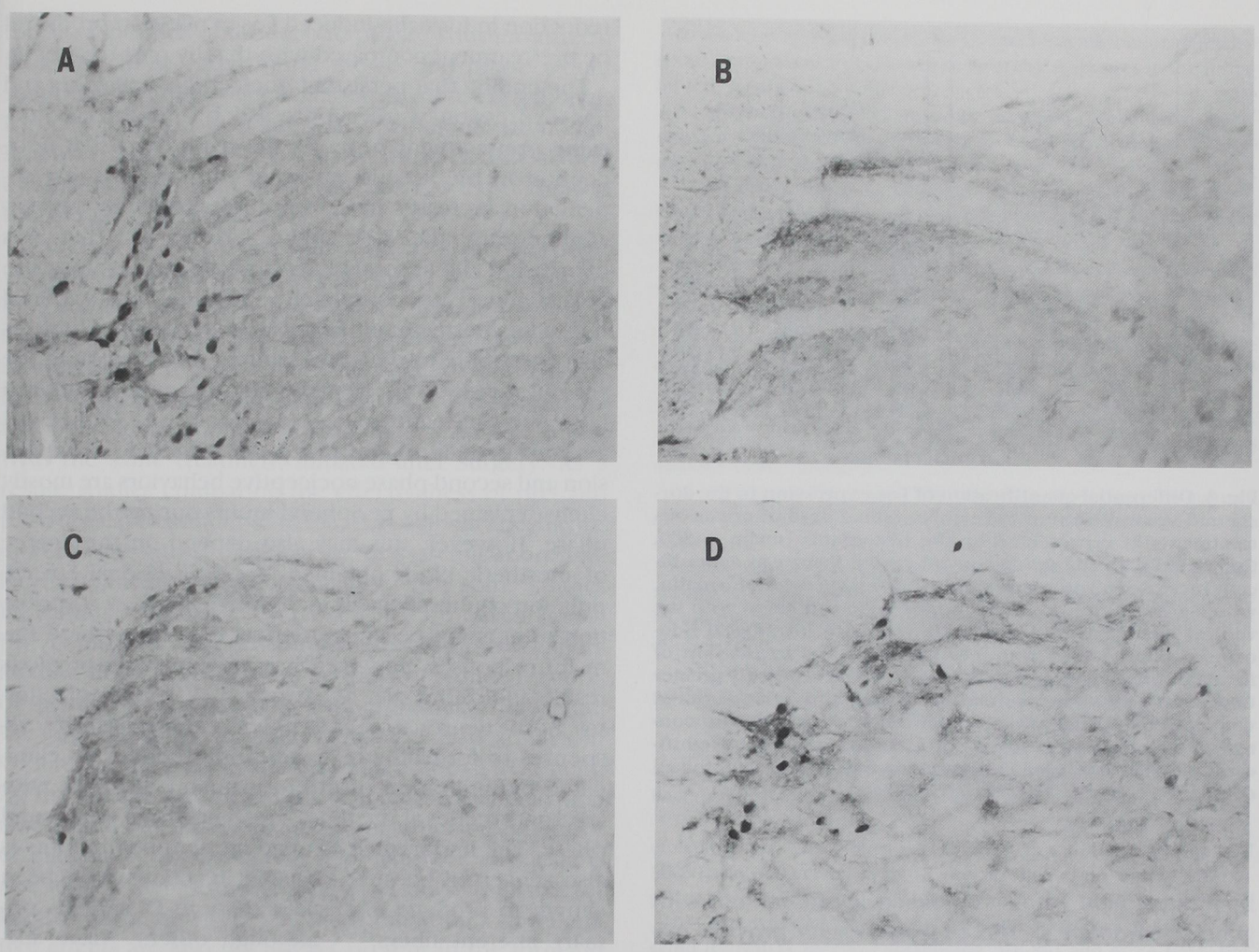

Fig. 2. The photomicrographs of the lumbar cord illustrating the differential distribution of immunoreactive *Fos* neurons in the dorsal horn with (*A*) injection of formalin alone, (*B*) control group (no formalin and no lidocaine), (*C*) lidocaine given before formalin, and (*D*) lidocaine given after formalin injection. The most prominent changes in Fos expression were observed in laminae I and II of the dorsal horn. The image is magnified ×250 its original size, and images are oriented with the superficial layer of the spinal dorsal horn to the left of each photomicrograph.

evidence for a formalin-induced enhancement of Fos expression in the spinal cord as measured by immunoprecipitation. This method allows for an easily quantifiable measure of changes in Fos expression that compliments the accurate localization, but less-objective quantitative assessment, provided by immunocytochemical analysis.

Recently investigators[36] have suggested that studies using Fos expression in the spinal cord may provide a rational basis for designing preemptive anesthetic regimens. This conclusion was based on the findings of Sun *et al.*,[31] who examined the effects of inhalation anesthetics and fentanyl on nociceptive responses and Fos expression in spinal cord after hind paw injection of formalin. They observed that when inhalation anesthetics (2% halothane, 75% nitrous oxide, or both) are used to block nociceptive responses in the first phase of the formalin test, they produce very little effect on either nociceptive responses in the second phase of the formalin test or Fos expression in the spinal cord dorsal horn. However, when fentanyl (100 μg/kg given intraperitoneally) is used to reduce first-phase responses, second-phase nociceptive responses and spinal cord Fos expression to formalin are suppressed. Thus the persistent nociceptive responses and spinal cord Fos expression after formalin injection can be preempted

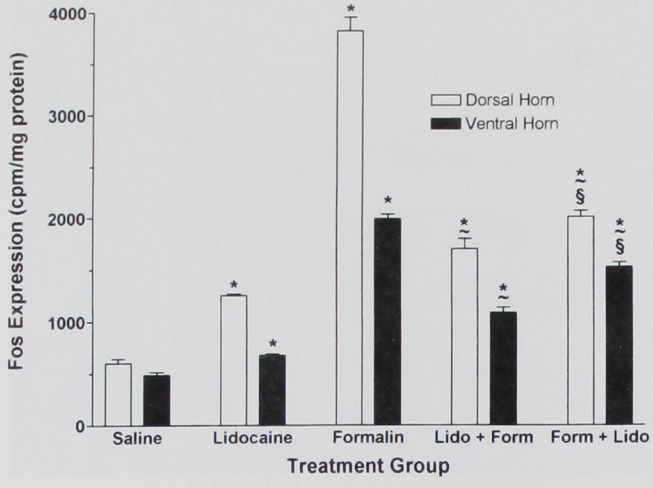

Fig. 3. Differential quantification of Fos expression in the dorsal and ventral horn of the lumbar spinal cord after various treatments as represented by the histograms (mean ± SD). Compared with the saline control group, there was a significant increase in Fos expression in rats injected with formalin, with a greater increase in the dorsal horn compared with the ventral horn. Pre- and post-treatment with intrathecal lidocaine produced significant reductions in Fos expression of formalin-injected rats. The reduction was significantly greater in the rats given pretreatment compared with post-treatment with intrathecal lidocaine. *Significantly > than saline controls; ~significantly < than formalin-injected rats; §significantly > than lidocaine pretreatment group ($P < 0.05$ for all comparisons by the Newman-Keuls test).

by pretreatment with an opioid analgesic, but not with general anesthetics. The present results provide evidence that spinal anesthetics can also produce preemptive effects by demonstrating that pretreatment with intrathecal lidocaine suppresses second-phase nociceptive responses and Fos expression induced by formalin.

Although in the current study intrathecal lidocaine pretreatment reduced formalin-induced nociception and Fos expression, post-treatment with intrathecal lidocaine reduced Fos expression but not nociceptive responses to formalin. The present results parallel those of Tokunaga *et al.*,[32] who found that post-treatment with 3% prilocaine (given subcutaneously at the ankle) produced a similar suppression of formalin-induced Fos expression in the dorsal horn as did pretreatment with 4% lidocaine. Remarkably, despite differences in the formalin concentration (2.5% *vs.* 5%), the location of the anesthetic blockade (spinal cord *vs.* ankle) and the assessment method (immunoprecipitation *vs.* immunocytochemical analysis), our own study and that of Tokunaga *et al.*[32] found only a marginally greater (8% *vs.* 6%) reduction in formalin-induced Fos expression produced by pretreatment compared with that by post-treatment.

The finding that persistent nociceptive behaviors and Fos expression were suppressed by intrathecal lidocaine pretreatment supports the hypothesis that nociception in the second phase of the formalin test depends on increases in central hyperexcitability generated during the first phase. However, that Fos expression and second-phase nociceptive responses to formalin were not completely eliminated by intrathecal lidocaine pretreatment suggests that peripheral inputs also contribute to the expression of nociception in the second phase of the formalin test. Because intrathecal lidocaine pretreatment would substantially reduce spinal activation during the first phase, residual Fos expression and second-phase nociceptive behaviors are mostly likely explained by peripheral inputs during the second phase. However, this may also depend on the degree of anesthetic block obtained, because residual Fos expression could reflect an incomplete block. A contribution of ongoing peripheral inputs to second-phase formalin responses, however, is consistent with previous results indicating that behavioral,[4,41,42] spinal cord dorsal horn neuronal,[12] and reflex cardiovascular[42] responses to formalin are significantly reduced or eliminated by infiltration of the formalin-injected site with local anesthetics at the time of testing during the second phase. The importance of ongoing peripheral inputs is also suggested because formalin injections have been shown to produce biphasic activity in C- and Aδ- primary afferent fibers,[43,44] although others[45] have suggested that primary afferent activity in the second phase is considerably less than in the first phase.

The finding that the intrathecal lidocaine post-treatment reduced Fos expression but not nociceptive responses was unexpected and reveals an uncoupling of the behavioral and Fos protein responses to formalin. It is perhaps not surprising that post-treatment with intrathecal lidocaine would *not* reduce nociceptive behaviors, because by acting only for 5–10 min during the quiet intermediate phase of the formalin test, the lidocaine treatment would block neither the development of central hyperexcitability during the first phase nor ongoing inputs during the second phase. It is, however, unexpected that this same treatment would reduce overall Fos expression. It is possible that because Fos expression was measured only at a single time point after prolonged (2 h) exposure to the stimulus, the net Fos expression is more greatly influenced by activation that would normally occur in the earlier, rather than

the later, periods of the second phase. Thus intrathecal lidocaine given early during the second phase would significantly reduce overall Fos expression, even though its effects on behavior are minor. It should be noted, however, that others[46] have also reported a dissociation of analgesics on pain behaviors and Fos expression in the spinal cord dorsal horn.

Although the Fos expression data do not correspond with the behavioral data in this study, the Fos results bare some resemblance to clinical observations. Thus, although clinical studies comparing preemptive *versus* no treatment are overwhelmingly suggestive of a beneficial effect in pretreated patients, the value of preemptive treatment becomes less obvious when compared with the same treatment initiated after surgery. As a result, studies comparing the effectiveness of pre- and postsurgical treatment with local or regional anesthesia, epidural anesthesia or analgesia, or systemic morphine have produced conflicting results, with some studies indicating a limited[47–50] or even no[51–54] advantage of pre- compared with postsurgical treatment. By demonstrating that Fos expression is similarly reduced by pre- or postformalin treatment with intrathecal lidocaine, the present study resembles those clinical studies in which there was no advantage to preemptive treatment. By the same token, it could be argued that behavioral assessments in the formalin test may not be reflective of clinical situations in which preemptive analgesia is not always demonstrated. However, as discussed at length in our previous article on this topic,[55] preemptive effects are also not always present in the formalin test and may depend on formalin concentration. Thus higher concentrations of formalin, which produce significant long-lasting peripheral inflammation (similar to that observed after many clinical surgeries), are more resistant to the preemptive effects of intrathecal lidocaine.[55]

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