

Effects of MK-801 and Morphine on Spinal C-Fos Expression during the Development of Neuropathic Pain

Youn-Woo Lee¹, Kyung Ah Park², and Won Taek Lee²

Departments of ¹Anesthesiology and Pain Medicine, ²Anatomy, Yonsei University College of Medicine, Seoul, Korea.

The purpose of this study was to investigate the expression of c-fos in the spinal cord during the development of allodynia, induced by peripheral nerve injury. Following tight ligation of the left L5 and L6 spinal nerves of Sprague-Dawley rat, the lumbar spinal cord was postfixed following perfusion. Frontal frozen sections of 40 μ m were immunostained according to the peroxidase-antiperoxidase method. The allodynic threshold was checked with 8 calibrated von Frey filaments. MK-801 (0.3 mg/kg), morphine (3 mg/kg) and saline (as a placebo) were administered subcutaneously 30 min before, and 24 and 48 hrs after surgery. The tactile threshold decreased below 3 g since 2 days after surgery in the saline and morphine groups, but delayed a little in the MK-801 group. In the superficial layer the number of Fos-like immunoreactive neurones (Fos-LI) peaked at 2 hours and decreased thereafter, and reached normal levels 24 hrs following operation, for all groups. In the deep layer they were biphasic, - peaking at 2 and 24 hrs - in the saline group, but were suppressed in the morphine and MK-801 groups until 7 days following operation. The above discrepancy between the number of Fos-LI and the allodynic threshold showed that central sensitizations are not critically involved in the development of nerve injury induced tactile allodynia.

Key Words: Allodynia, central sensitization, spinal c-fos, MK-801, morphine

INTRODUCTION

Physiological stimulation of rat somato-sensory neurones results in the spinal expression of c-fos

immunoreactive protein in spinal dorsal horn neurons.¹ Studies using c-fos showed its appearance could be used to mark cells that had been stimulated and activated. C-fos activation can be used not only to map metabolic activity in cells but also to study the effectiveness of therapies suppressing the activity of cells. Spinal c-fos expression is not a specific marker of neurons activated by noxious inputs, and there are relatively few spinal Fos-like immunoreactive neurones (Fos-LI), expressed by non-noxious mechanical and proprioceptive stimulation compared to that produced by noxious stimulation of rat hind paw. The relative preferential noxious stimulation evoked expression of spinal c-fos was exemplified by a study demonstrating the electrical activation of A δ and C-fibers, but not A β fibers. Allodynia refers to pain or discomfort, brought on by a non-painful stimulus, which is transmitted by A β primary afferent fibers to the spinal cord. Therefore a few studies have reported that c-fos expression does not have to be fulfilled to light up the mechanism of allodynia.

Following peripheral nerve injury, neuropathic pain rats will show a tactile allodynia and hyperalgesia. Kim and Chung² reported that ligation of L5 and L6 spinal nerve produced tactile allodynia and hyperalgesia, which were relieved by surgical sympathectomy³ and intrathecal alpha-2 agonists,⁴ but not by intrathecal morphine.⁵ This indicated that this animal model simulates the sympathetically maintained pain (SMP) in humans.

In other studies, peripheral nerve injury induced a massive discharge in the injured axon and dorsal root ganglion,⁶ this massive discharge can trigger long-lasting changes in the central

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Reprint address: requests to Dr. Youn-Woo Lee, Department of Anesthesiology, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul 120-752, Korea. Tel: 82-2-361-5847, Fax: 82-2-312-7185, E-mail: ywoleepain@yumc.yonsei.ac.kr

nerve system excitability through N-methyl-D-aspartate (NMDA) receptor activation. Nerve injury induced allodynia was relieved by the intrathecal NMDA antagonist, dextrorphan⁷ and MK-801.⁸ These data showed that NMDA receptors are one of the keys in ongoing pain maintenance following peripheral nerve injury. In our preliminary studies perioperative MK-801 delayed the development of tactile allodynia a little more than morphine following spinal nerve ligation. The mechanism of NMDA receptors in the spinal cord for the development of allodynia after peripheral nerve injury is still obscure.

The purpose of the current study is to investigate the time course change of Fos-LI on the spinal dorsal horn, and to find the central mechanism during the development of allodynia following spinal nerve ligation. We examine the effects of perioperative MK-801 and morphine on Fos-LI expression in the spinal dorsal horn by comparing with behavioral responses to tactile stimuli following ligation of the spinal nerve.

MATERIALS AND METHODS

Surgical preparation

The Institutional Animal Care Subcommittee of the Yonsei University Medical Center approved the studies. Male Sprague Dawley rats, weighing 120-150 g at the beginning of the experimental procedure, were housed in an environmentally controlled room on a 12-h light/12-h dark cycle. Rats were anesthetized with halothane and the left L5 and L6 spinal nerves ligated tightly according to the method devised by Kim and Chung.² Briefly, under the halothane/oxygen anesthesia a dorsal midline incision was made and the exposed L6 transverse process and pedicle were partially resected. This was followed by careful teasing of the underlying fascia and dragging the separated left L5 and L6 spinal nerves, which were ligated tightly with 6-0 silk suture material at the distal to the dorsal root ganglion. The rats with inability to flex the left hind limb postoperatively, indicating damage to the L4 nerve, were discarded, since this precluded testing.

Drug treatment

The drugs employed were MK-801 (0.3 mg/kg: RBI, N=30), morphine sulphate (3 mg/kg, N=30) and saline (as a placebo, N=30). They were administered subcutaneously 30 min before skin incision, with the next doses being administered subcutaneously 24 and 48 hrs postoperatively. Drugs were dissolved in saline and prepared such that the total volume delivered was 5 ml/kg.

von Frey filament test

In a group of rats (N=5 at each group) other than those used for Fos-LI studies, pain related tests were performed before operation and every 24 hrs after operation. The rats were placed in a clear plastic wire mesh-bottomed cage, and placed into individual compartments of 12 × 14 × 22 cm, which permitted access to the paws for testing. We waited until animals accommodated to the test cage for approximately 15 min or until animals ceased cage exploration. The methods for assessing the mechanical threshold for paw withdrawal were followed the paradigm previously described by Chaplan et al.⁹ Briefly, thresholds were assessed by applying a von Frey filament (Stoelting Co., Wood Dale, IL, USA) to the plantar mid-hind-paw, avoiding the footpad. The filaments were designated by log and ranged from 0.4 to 15.1 g. Each filament was pressed perpendicularly against the paw with sufficient force to cause slight bending, and held for approximately 3 to 4 sec. A positive response was noted if the paw was sharply withdrawn. Absence of a response was a cause to present the next consecutive stronger stimulus; but with a positive response, the next weaker stimulus. Six successive stimuli were presented from descending to ascending or vice versa and if the minimum stimulus or maximum stimulus was reached the threshold was assigned an arbitrary minimum value of 0.25 g or maximum value of 15 g. The resulting pattern of responses was tabulated and the 50% response threshold computed from the formula: $\log(\text{threshold in milligrams} \times 10) = X_f + k\delta$, where X_f =value of the last von Frey filament applied; k =correction factor based on pattern of responses and δ =mean distance in log units

between stimuli.

Immunohistochemistry

In order to detect Fos-LI of the spinal dorsal horn, rats (N=25 at each group) were divided 5 time sequence - 2 hrs, 8 hrs, 24 hrs, 3 days, and 7 days after operation. At each time 5 animals at each group anesthetized deeply with diethylether and perfused intracardially with 200 ml of saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4). The lumbar spinal cord was then removed, postfixed for 4 hrs and cryo-protected overnight in 30% sucrose. Tissue sections from the L5 and L6 lumbar segment were used for analysis of Fos-LI. Frontal frozen sections of 40 μ m were cut and collected in phosphate buffer (PB) to be processed immunocytochemically as free floating sections. The serial sections were immunostained for c-fos like protein according to the peroxidase-antiperoxidase (PAP) method of Sternberger.¹⁰

Counting of Fos-labeled cells

Fos-LI was studied at the L5 spinal cord segment, in which the number of Fos-LI are the greatest in nerve ligation induced neuropathic pain rats. Tissue sections were first examined using dark-field microscopy to determine the segmental level and gray matter landmarks. The sections were then examined under light-field microscopy to localize Fos positive cells. For each rat the number of Fos-LI per specific laminar region of the spinal dorsal horn was counted. Two regions were defined: superficial layer (Rexed laminar I and II) and deep layer (Rexed laminar III - VI).

Data analysis

All results are expressed as the mean and mean standard error of the thresholds for tactile allodynia and the number of Fos-LI per section. Statistical analysis was made for comparison of the results of three groups, using one way analysis of variance (ANOVA) for each time point, and repeated measures ANOVA for the time course variances in each group. The Fisher's test

was used for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Tactile thresholds for allodynia

Before the operation of nerve ligation the mean threshold was 13.8g with no significant change in the threshold for the left hind paw withdrawal response to the touch of von Frey filaments in the sham operated rats, until 2 weeks following the operation. The tactile threshold for allodynia decreased below 3.5 g the first postoperative day after spinal nerve ligation in the saline and morphine groups, while the rats in the MK-801 group showed a slightly higher threshold until 5 days postoperatively. The decreased thresholds in all three groups persisted over 2 weeks. The tactile threshold in the MK-801 group was significantly higher than in the saline group until the third postoperative day, but the significant difference versus the morphine group only showed for the first postoperative day (Fig. 1).

These results demonstrated that MK-801, but not morphine, delayed the reduction of the tactile

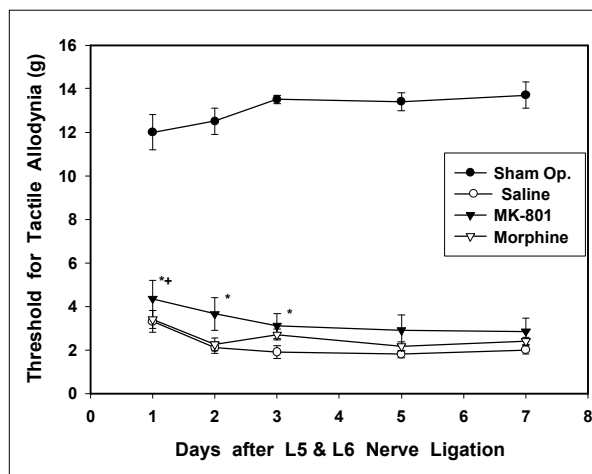


Fig. 1. Time course changes of thresholds for tactile allodynia on the left hind paw. There has been no significant change of the threshold in the sham operated rats. The tactile thresholds in MK-801 group were significantly higher than those in the saline group until the third postoperative day ($*p < 0.05$), but compared to the morphine group showed significant differences only on the first postoperative day ($†p < 0.05$).

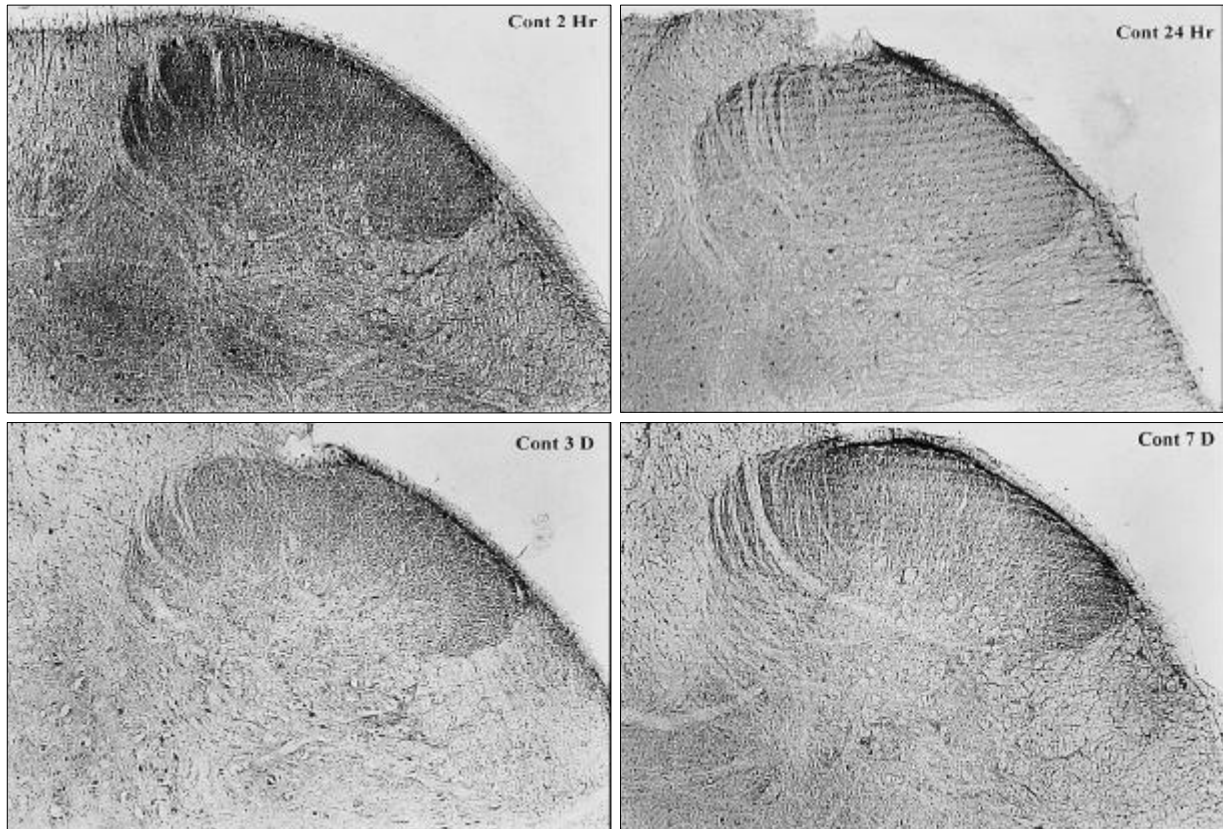


Fig. 2. Fos-like immunoreactive neurones in the dorsal horn of the spinal cord (L5). 2 hrs (Cont 2Hr), 24 hrs (Cont 24Hr), 3 days (Cont 3D) and 7 days (Cont 7D) after nerve ligation operations in placebo injected rats. Fos-LI presented as dark brown granules in the nuclei of the neurones. The number of Fos-like immunoreactive neurones are plotted on Fig. 3.

threshold at least three days following spinal nerve ligation.

Fos-LI

In the left superficial layer of the spinal dorsal horn, the number of Fos-LI peaked at 2 hrs, had markedly decreased at 8 hrs, and there after decreased gradually to normal levels by 7 days postoperatively in all groups. In the left deep layer of the spinal dorsal horn, the time course change of the number of Fos-LI was biphasic. It peaked at 2 and 24 hrs postoperatively, and after 3 days decreased gradually, reaching normal levels by day 7 in the saline group, but a lesser increase in the number of Fos-LI was maintained until 3 days postoperatively in the morphine and MK-801 groups ($p < 0.01$) (Fig. 2 and 3).

In the right superficial and deep layer of the spinal dorsal horn, the time course changes of the

numbers of Fos-LI showed the same pattern as that of the left side, although they were much less than that of the left spinal dorsal horn in each group.

DISCUSSION

Both acute pain and chronic inflammatory arthralgia, or hyperalgesia, are mediated by unmyelinated C-fibers, but tactile allodynia is mediated by large myelinated A β -fibers. In the studies by Kim & Chung² the neuropathic pain rat model dominantly represents the sympathetically maintained pain, and clinically simulates to both hyperalgesia and allodynia in humans. From experimental animal studies, three major types of modifications potentially inducing a pathologic activation of central nociceptive neurons responsible for the genesis of neuropathic pain have

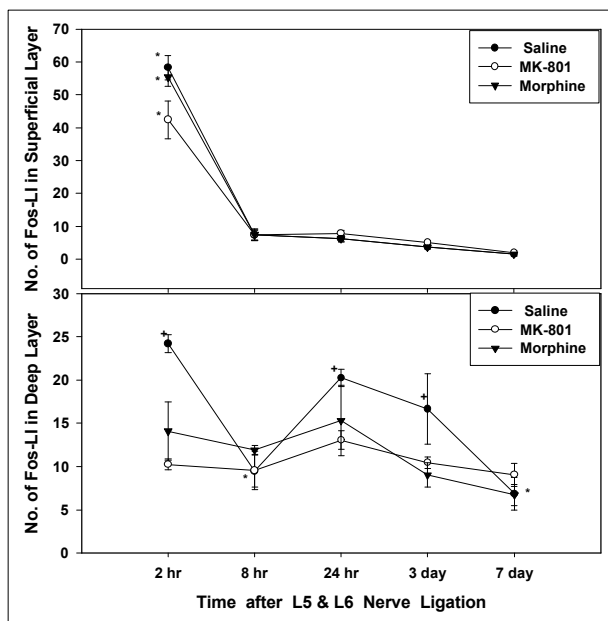


Fig. 3. Time course changes of the number of Fos-LI in the left superficial (top) and deep layer (bottom) of the spinal cord. There were no significant difference between the three groups in the superficial layer of the spinal dorsal horn. In the left deep layer of the spinal cord, Fos-LI expressions were suppressed significantly in the morphine and MK-801 groups. * $p < 0.01$ vs the number of Fos-LI at other times. † $p < 0.01$ vs other groups.

been proposed. These being: a modification of the modulatory controls of the transmission of nociceptive messages, anatomical reorganization of the central nociceptive neurons responsible for pathological activation, and central sensitization of central nociceptive neurons due to modification of their electrophysiologic properties.¹¹

Other pharmacological studies have described that systemic morphine but not intrathecal morphine⁵ or the NMDA antagonist,^{7,8} reduced nerve injury induced tactile allodynia in the Kim and Chung model. These results show that spinal NMDA receptor activation, by repetitive small primary afferent fiber stimulation, augments the pain response and modulation of the pain processing system in the spinal dorsal horn. Spinal NMDA receptor activation is one of the important mechanisms in the maintenance of allodynia and hyperalgesia following nerve injury. The purpose of our experiment was to investigate the mechanism for the development of allodynia and find key drugs to prevent nerve injury induced

allodynia.

Chi et al.¹² reported that c-fos was expressed when the sciatic nerve was totally transected. Huang and Simpson¹³ reported sciatic nerve constriction induced expression of c-fos protein in dorsal horn neurons of the spinal cord 90 min after ligation. They also found intrathecal administration of c-fos antisense 18 hrs prior to nerve ligation attenuated Fos immunoreactivity by 80%, but could not exclude the effect of noxious stimulation during the operation of nerve resection. Most of the previous reports showed that c-fos expression increased in the superficial layer following noxious stimulation, but this normalized in 12 hrs. The suggestion that neonatal capsaicin treatment attenuates spinal c-fos activation, and dynorphin gene expression, following noxious stimulation indicate that input from small diameter unmyelinated primary afferent C-fibers are important for the noxious stimulus induced increase in Fos-LI and preprodynorphin mRNA.¹⁴

Williams et al.¹⁵ reported a significant increase in c-fos expression in the superficial layer 2 hrs after nerve ligation, which normalized in 8 hrs, while in the deep layer the peak expression of c-fos was observed at 8 hrs which persisted until 24 hrs, even on the opposite side of the spinal cord. Our experimental results were concur with these findings, but the pretreatment with morphine and MK-801 could not prevent the expression of Fos-LI in the superficial layer of the spinal cord until 8 hrs postoperatively. Morphine had an enhanced potency on the C-fiber evoked and noxious natural stimuli evoked neuronal response of spinal nerve ligated rats.¹⁶ In contrast to morphine, the NMDA antagonist does not prevent the noxious input transmitted through the primary afferent nerve fiber. The reason for the increased Fos-LI in the superficial layer postoperatively was interpreted as being two folds. One, that the administration of morphine in a dosage of 3 mg/kg could not block the large scale of noxious stimuli completely resulting in irreversible nerve injury. This dosage could block most of the acute and chronic nociceptive pain transmission, resulting in the prevention of Fos-LI expression.¹⁷ Two, that morphine should activate the descending inhibitory pathway, and the secondary neurons and interneurons connected

with the inhibitory pathway in the spinal cord. These neurons are located in the superficial layer of the dorsal horn, and have numerous opioid receptors on their synaptic membrane.

However, in our experiment, both morphine and MK-801 pretreatment decreased the expression of Fos-LI in the deep layer of the spinal cord. This result revealed that both morphine and MK-801 blocked the transmission of noxious stimulation to the secondary neurons. Conversely, the threshold of tactile allodynia decreased in 24 hrs and persisted over 7 days. Catheline et al.¹⁸ reported the intravenous administration of 3 mg/kg morphine did not change the levels of spinal Fos-LI observed following light touch stimuli, but reduced the number of Fos-LI neurons induced by heat stimulation in neuropathic pain rat by 30%. With that rat, allodynia and hyperalgesia had already induced. C-fos expression in spinal cord neurons occurs in chronic pain diseases such as poly- or monoarthritis.^{19,20} In contrast to acute inflammation, which mainly induces c-fos expression in the superficial dorsal horn, polyarthritis induces c-fos expression in the deep laminae of the dorsal horn and ventral horn. The peak distribution of c-fos expression, in this study, being well matched to maximum hyperalgesia, that is 3 weeks after inoculation for the disease. Furthermore, pretreatment with morphine prevented the hyperalgesia, and corresponded with a marked decrease in Fos expression in this polyarthritis animal model. Formalin injection related hyperalgesia could be treated by both the NMDA antagonist and morphine.²¹ In our experiments, perioperative treatment with MK-801 prevented the decreasing threshold slightly more than morphine, but the decreased Fos-LI was almost the same as the morphine group. The above results indicate that morphine and MK-801 could prevent the development of hyperalgesia which is mediated by central sensitization, but not tactile allodynia which is also possibly mediated by central sensitization. NMDA antagonists have treatment effects on previous induced tactile allodynia^{7,8} and hyperalgesia,²² so it was inevitable that our results would show preventable effects in the development of tactile allodynia.

It has been shown that injury triggers central sprouting of large myelinated afferent fibers into

the upper laminae of the spinal dorsal horn,²² and A β -fibers appear to contribute to inflammatory hypersensitivity by switching their phenotype to one resembling pain fibers.²³ Nerve injury also triggers the sympathetic nerve terminals sprouting into dorsal root ganglia and forms basket like structures around large myelinated neurons.²⁴ Peripheral nerve injury induces spontaneous firing in neuronal cell bodies freshly isolated from dorsal root ganglion.^{6,25,26} Therefore, the development of tactile allodynia could be accomplished mainly by alteration of pain modulatory control systems and structures with functional modification in the dorsal root ganglion and spinal dorsal horn. Peripheral nerve ligation induced NMDA receptor activation, which results in central sensitization, was not critically involved in the development of tactile allodynia in our experiment. However Fos-LI inhibition patterns did not clearly correlate with pain reduction, providing further evidence that Fos-LI inhibition is not always predictive of behavioral analgesia.^{27,28}

Conclusion: C-fos expression in the spinal dorsal horn indicated the pain processing system after spinal nerve ligation injury. Morphine (3mg/kg) and MK-801 (0.3 mg/kg) blocked the expression Fos-LI neurons in the deep layer, but not in the superficial layer of the dorsal horn. They could not prevent the development of nerve injury induced tactile allodynia, although we did confirm the anti-allodynic effect of MK-801.^{7,8} The above discrepancy between the time course changes of Fos-LI and the allodynic threshold suggest that central sensitization is not critically involved in the development of nerve injury induced tactile allodynia.

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