Pre-versus post-formalin effects of intrathecal ketamine on spinal Fos-like immunoreactivity in rats

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Background & objectives: The spinal expression of the c-Fos immediate early gene in response to formalin pain of the hind paw of rat was used as a marker of neuronal activity. Ketamine, a N-methyl-D-aspartate (NMDA) receptor antagonist produces analgesic action due to the blockade of glutamate action at the NMDA receptor. Earlier study showed that ketamine acts differently depending on its route of administration. We undertook this study to compare a preemptive suppression of noxious stimulation induced spinal Fos-like immunoreactivity (FLI) after receiving intrathecal ketamine before or after formalin pain.

Methods: Male Sprague-Dawley rats received ketamine 1mg/kg or saline (control group) intrathecally either 5 min before (pre-treatment group) formalin or 5 min after (post-treatment group) formalin (5%, 50µl) injection. Animals were killed 2 h after the formalin injection, and the lumbar spinal cord was dissected, and processed by immunoperoxidase staining using an antibody against Fos protein.

Results: The FLI was significantly reduced in the pre-treatment group, only laminae I-II of the side ipsilateral to the formalin injection (P < 0.05 vs. control). In laminae V-VI, neither of the ketamine treatment groups showed a significant decrease than the control group.

Interpretation & conclusion: The results provide evidence that intrathecal ketamine does not have a preemptive blocking effect of FLI expression in whole spinal laminae area. FLI expression of laminae I-II only might not be a good predictor of the ability of agents to produce preemptive effect. The central patterns of activity generated during central sensitization differ regionally in the spinal dorsal horn.

Key words Formalin test - Fos - ketamine - preemptive - spinal cord

The formalin test is a widely used model of peripheral inflammation and central sensitization. Fos protein activates DNA transcription, and its noxious stimulus-induced expression has been linked to the increased expression of endogenous spinal opioids, such as dynorphin, which may play a role in spinal sensitization. The spinal expression of the c-Fos immediate early gene in response to formalin pain of the hind paw of rat was used as a marker of neuronal activity.
Preemptive analgesia is an analgesic strategy thought to be contingent on the prevention or suppression of spinal mechanisms of neuronal sensitization\(^3\). Ketamine is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist\(^4\), and its analgesic action is due to the blockade of glutamate action at the NMDA receptor. However, there is some controversy regarding preemptive analgesia induced by epidural ketamine in preventing postoperative pain\(^5-7\), whereas, intravenous ketamine alone or in combination with epidural morphine produces effective preemption\(^8-10\). Previous work in our laboratory showed that ketamine works differently according to the route of administration\(^11\). However, none of these investigations compared the effects of intrathecal ketamine on the formalin induced Fos-like immunoreactivity (FLI). The present study was undertaken to compare a preemptive blocking effect of formalin induced spinal FLI after receiving intrathecal ketamine.

**Material & Methods**

*Animal preparation:* The study protocol was approved by the Institutional Animal Care Committee of the Clinical Research Institute. Male Sprague-Dawley rats (250-275 g) were housed in plastic boxes in groups of four with food and water *ad libitum* under natural light. To habituate them to the formalin test environment, the rats were placed in the test chambers in groups of three for 15 min for four days, and then placed individually on the fifth day. The guidelines on the ethical standards for investigations of experimental pain in animals were followed throughout\(^12\).

During enflurane anaesthesia, chronic intrathecal PE-10 polyethylene tubing catheters were inserted through the atlantooccipital membrane to a position 9 cm caudal to the cistern. The catheter was externalized on the top of the skull and sealed with a piece of small bean sized silicon. The wound was closed with 3-0 silk sutures. Rats showing neurological deficits post-operatively were promptly killed by a barbiturate overdose. The day before the experiment, the location of the catheter was verified by injecting 10 µl of lidocaine 2 per cent.

Rats were allocated to three groups of 10 rats each: a 1 mg/kg ketamine (volume 10 µl) intrathecal pre-treatment group, a post-treatment group, and intrathecal saline control group. The intrathecal catheter was flushed with 10 µl saline following the drug administration. To evaluate the specific preemptive effect of the drugs studied, the pre-treatment group was compared with the post-treatment group. The intraplantar formalin first stimulates nociceptors, resulting in a barrage of primary afferent fiber activity (phase 1; 0-5 min after formalin) and 5-10 min quiescent period and ongoing stimulation of nociceptors increases primary afferent activity (phase 2; 10-60 min after formalin). In the pre-groups, rats received intrathecal ketamine 5 min before formalin injection so that the effect of ketamine could be maximal in the total phase 1 period, which means the period of acute pain. In the post-groups, ketamine was administered 5 min after the formalin injection to let the rats to be treated only during phase 2 (no preemption). Control animals were administered saline 20 µl intrathecally before formalin injection. Conscious rats (n=30) received subcutaneously 50 µl of 5 per cent formalin into the plantar surface of the right hind paw. Just after formalin injection, the rats were kept into a transparent Plexiglas and the number of flinches of formalin-injected paw was observed for 60 min by the blinded observer (data not shown).

Rats were killed 2 h after the formalin injection. After anaesthetizing rat with 75 mg/kg pentobarbital intraperitoneally, surgery proceeded with sternotomy, transcardiac aortic needle cannulation, and perfusion with 200 ml of phosphate buffered saline (PBS), followed by 500 ml of 4 per cent paraformaldehyde / PBS. The lumbar spinal cord was extracted, postfixed for 8 h in 4 per cent paraformaldehyde/PBS, then cryoprotected for 48 h in 30 per cent sucrose/PBS at 4°C. The spinal cords were frozen with dry ice and 50 µm transverse sections were cut using a refrigerated Leica cryostat (Houston, USA) and collected at intervals of 100 µm. These free-floating sections were collected and immunohistochemistry for Fos-like protein (polyclonal antibody 1:20,000 dilution) was performed by the avidin-biotin peroxidase method of
Fig. 1. Photomicrograph illustrating Fos-like immunoreactivity (FLI) in the lumbar spinal dorsal horn of control group. (a) - control rats (received intrathecal saline); (b) - pre-treatment group rats (received intrathecal ketamine before the formalin); and (c) - post-treatment group rats (received intrathecal ketamine after the formalin). There is increased FLI in laminae I-II (arrow) in the ipsilateral side to the formalin injection.
Hsu et al. After air drying for 24 h, sections were dehydrated in an ascending alcohol series and defatted in xylene. Negative control experiments were conducted with tissue sections from the formalin injected saline controls by omitting the primary antibody from the above protocol and adding 2 µg/ml of N-terminal Fos peptide to the primary antibody incubation solution. Neither of these controls resulted in any expression of FLI.

Quantification of FLI neurons was performed in the gray matter of both sides of the cord. Thus, tissue sections were first examined using light field microscopy to find the L4-5 segmental level. To study the laminar distribution of spinal dorsal horn three regions were defined: the superficial dorsal horn (laminae I-II), the nucleus proprius (laminae III-IV), and neck of the dorsal horn (laminae V-VI). The ventral border of the superficial dorsal horn (laminae I-II) and the reticular part of lamina V were readily distinguishable morphologically. Cells were considered positive only if they showed the appropriate size and shape, had dark brown to black nuclei, and were distinct from the background. For each rat, five randomly selected sections were counted. The gray matter was divided into regions: laminae I-II (superficial dorsal horn) and laminae V-VI (deep dorsal horn). The investigator responsible for plotting and counting the FLI neurons was unaware of the experimental procedures performed on the rats.

Data analysis: FLI results are expressed as mean counts per group. Two-way analysis of variance was done to compare the number of labeled cells between animals groups and laminar regions. \( P<0.05 \) was considered statistically significant.

Results

As previously described, hind paw injection of formalin induces a highly reproducible pattern of FLI in the lumbosacral spinal cord. The highest concentration of FLI neurons was in the ipsilateral superficial dorsal horn of the L4-5 lumbar segments (Fig.1). Counts of labeled neurons revealed the following lamina distribution in the L4-5 segment of control rats; 55 per cent in laminae I-II, 25 per cent in laminae V-VI, and rest in laminae III-IV (Fig.2).

The pre-treatment group showed a significant decrease in FLI only in laminae I-II comparing with the control group \( (P<0.05) \). The post-treatment group marginally suppressed FLI expression in lamina I-II. By contrast, in laminae V-VI, neither of the ketamine treatment groups showed a statistically significant decrease than the control group (Fig.2).

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**Fig. 2.** Histograms showing the distribution of Fos-like immunoreactivity (FLI) induced by the hind paw injection of formalin in the three groups of rats \( (n=10 \text{ in each group}) \). (a) ipsilateral side to the formalin injection, (b) contralateral side to the formalin injection. Results are expressed as a total mean number of FLI expression ± S.D. \( *P<0.05 \) compared to control group.
In rats that received intrathecal ketamine as pre-treatment, further decrease was recorded in the number of FLI neurons. In the superficial dorsal horn (lamina I-II) of L4-5, there was a significant decrease in the number of FLI neurons of ipsilateral side compared to the control group rats; but in the deep dorsal horn (laminae V-VI) of L4-5, the decrease in the number of FLI neurons was not significant. In intrathecal post-treatment group rats, although the number of FLI neurons of ipsilateral side decreased in the whole area of L4-5, there was no significant difference compared to control group.

Discussion

This study shows that intrathecal pre-treatment of ketamine does not have preemptive blocking spinal FLI expression of lumbar lamina V. There is an evidence that ketamine can only inhibit NMDA receptor activity when the receptor-controlled ion channel has been opened by a nociceptive barrage, which could explain why intrathecal pre-treatment of ketamine had no apparent preemptive effect of FLI expression in deep dorsal horn. But post-treatment of ketamine exerts a marginal (statistically non-significant) suppressive effect of FLI expression because the receptor channel had already been opened by formalin pain.

There were some positive results of preemption of intravenous ketamine. Why is intrathecal pre-treatment less effective than intravenous pre-treatment in preemption? There may be two possible explanations for this. First, the demonstration of preemptive analgesia with intravenous ketamine may suggest that the intravenous systemic ketamine predominates supraspinal effects and has a similar effect with opioids and masks any behavioral response. Second, the antinociceptive effects of intravenous ketamine involve an activation of the monoaminergic descending inhibitory system, and that spinal ketamine does not affect the responses to the acute noxious stimuli. Therefore, intrathecal ketamine pre-treated could not suppress the response of acute phase (phase 1) efficiently; this uninhibited phase 1 response triggered a tonic pain like phase 2 thereafter. This led us to assume that there was a different effect of intrathecal or intravenous ketamine on suppressing facilitation.

The failure of preemptive effect is not likely to be the result of ketamine’s action, duration, properties or dosage. Even though the concentration used (2.5-3%) produces analgesia rather than a NMDA blockade, many other studies on ketamine’s NMDA antagonistic action have used the same or similar concentrations. The fact that intrathecal ketamine had a subanaesthetic effect in intact rats is consistent with previous investigations which found that large doses of the drug (240 - 720 µg; 1-3 µM) are required to produce a detectable analgesic response in nonhyperalgesic animals. To study doses of ketamine, in preliminary experiments, we sought to determine the optimal drug dose (1mg/kg) that would produce spinal analgesic effect after receptor channel is opened by nociception.

The present study demonstrates that formalin induces the expression of FLI in superficial laminae (laminae I-II) and in the neck of the dorsal horn (laminae V-VI). Since the majority of small diameter myelinated and unmyelinated afferents terminate in the superficial dorsal horn, it is reasonable to hypothesize that many of the neurons in the superficial dorsal horn, which express FLI, are driven monosynaptically by small diameter, presumably nociceptive primary afferents from the inflamed paw. Moreover, since deeper dorsal horn neurons receive indirect nociceptive input from the superficial laminae, and do not express FLI when activated by innocuous stimuli, it seems highly probable that neurons in the neck of the dorsal horn, which express FLI, are also driven by nociceptive inputs from the inflamed paw. Though single unit studies have revealed the activity of lamina V neurons during both first and second phases of formalin test, an analysis of FLI expression suggested that lamina V neurons are predominantly driven during the second phase. Therefore, complete blocking of FLI in lamina V means a successful inhibition of second phase of formalin test and an accomplishment of preemption. In this study, we could not show a complete blocking of FLI expression in
pre-treatment group in lamina V. This shows that pre-treatment of intrathecal ketamine lacks preemptive effect.

Our previous study\textsuperscript{11}, has suggested that intrathecal pre-treatment of ketamine has no preemptive effects of behavioral study, we hypothesized that it could not decrease noxious stimulus-induced spinal FLI in whole laminae area. However, pre-treatment was observed to reduce FLI of lamina I-II (even if it was limited only in this laminae I-II area). Why is pre-treatment effective at suppressing the FLI of laminae I-II more than the control group, when no selective preemptive effect has been observed in previous behavioral studies (data not shown)? There are two possible explanations. First, this study on FLI reflects a 2 h period after formalin injection, whereas behavioral studies investigated only the first hour after formalin injection. Second, the first phases of formalin pain contribute equally to the expression of FLI in the superficial dorsal horn, but differently to the activity of deeper laminae. Thus, the reduction of FLI in laminae I-II was comparable after behavioral reduction in the first phase of the pre-treatment group. Therefore, nociceptive behaviors in the two phases are independent\textsuperscript{25}.

The longer duration of afferent firing during the second phase may produce sufficient temporal summation to drive neurons located in deeper laminae\textsuperscript{26}. If a blockade of the second phase could produce a greater suppression of FLI expression in deeper laminae, why was such suppression not observed in post-treatment group in the deeper dorsal horn when the second phase behavior of the formalin was markedly reduced in previous behavioral studies (data not shown)\textsuperscript{11}? Probably because nociceptive processing might continue one hour after formalin and residual expression could occur even in the absence of measurable pain behaviors. It was also appreciated that spinal cord FLI expression was not eliminated when the behavioral response of formalin was markedly reduced. In addition, not all neurons express the immediate early gene when activated, thresholds for FLI expression may differ between neurons\textsuperscript{27}. Thus, the absence of FLI expression may not indicate the absence of neural activity, and conversely the magnitude of FLI expression may not predict the magnitude of pain behavior.

In summary, the present results indicate that intrathecal pre-formalin ketamine has no preemptive effect, and FLI expression at laminae I-II only might not be a good indicator of the ability of an agent to produce a preemptive effect; the central patterns of activity generated by the formalin test differ regionally in the spinal dorsal horn. Since the intrathecal ketamine can only inhibit NMDA receptor activity when the receptor has been opened by a nociceptive barrage, pre-treatment has no apparent preemptive effect of FLI expression in deep dorsal horn.

\textbf{References}


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