NK$_1$ receptor and its interaction with NMDA receptor in spinal c-fos expression after lower urinary tract irritation

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The tachykinin neurokinin 1 (NK$_1$) receptor is widely distributed in both the central and peripheral nervous system. At the spinal cord level, NK$_1$ receptors are activated during the synaptic transmission, especially in response to noxious stimuli applied at the receptive field of primary afferent neurons (30). Neurons in lamina I of the spinal cord expressing NK$_1$ receptor are reported to encode for the intensity of noxious stimulation (13). There have been many studies that examined the role of substance P and NK$_1$ receptors in visceral pain because substance P is expressed by a much greater proportion of visceral than cutaneous or muscle afferents (29). Chemical irritation of the urinary bladder with formalin in rats induced c-fos expression in more than 80% of substance P receptor-like immunoreactive neurons of the lumbosacral (L-S) spinal cord (20). NK$_1$ antagonists inhibited nociceptive responses to visceral stimulation (22, 28). Moreover, NK$_1$ knockout mice showed profound deficits in behavioral responses to visceral chemical stimulation (intra-colonic capsaicin) and to cyclophosphamide-induced cystitis (17). Thus there is considerable evidence implicating a critical role of substance P and NK$_1$ receptor in visceral pain.

On the other hand, N-methyl-D-aspartic acid (NMDA) glutamatergic receptor is also involved in somatic as well as visceral nociception (7–9, 24, 32). Recent studies have suggested that the excitatory amino acid (EAA) glutamate interacts with substance P-containing sensory afferents to modulate nociceptive transmission. Substance P and glutamate have been found to coexist in primary afferents (12), and presynaptic NMDA autoreceptors were detected near the neurotransmitter release sites in primary afferents (19). Presynaptic NMDA receptors that were located on substance P-containing primary afferents facilitated nociception through a release of substance P (11, 18, 21). Coadministration of a low dose of NMDA receptor antagonist and NK$_1$ receptor antagonist markedly reduced NMDA receptor-mediated responses such as facilitated flexor reflex (36). Chapman and associates (6) showed that NK$_1$ and NMDA receptor interactions contributed to spinal c-fos expression after intraplantar injection of formalin in rats. Taken together, these findings suggest interaction between NK$_1$ and NMDA receptors in somatic nociception. However, there are few in vivo studies investigating the possible interaction of both receptors in visceral nociception, especially in bladder nociception.

Chemical irritation of the bladder has been used for producing noxious stimulation of the bladder. Intravesical instillation of acetic acid is known to increase spinal c-fos expression (1–4, 14, 15, 25). Previous studies have shown that NMDA receptors have a critical
role in spinal c-fos expression after lower urinary tract irritation (1, 16). However, neither the precise role of NK1 receptor nor the possible interaction between NK1 and NMDA receptors has been examined in spinal c-fos expression after lower urinary tract irritation.

In the present study, the selective NK1 receptor antagonist CP-99,994 (23, 33) was used to examine the role of NK1 receptor in spinal c-fos expression after lower urinary tract irritation. Then the effects of coadministration of a low dose of CP-99,994 and the NMDA receptor antagonist MK-801 (Sigma, St. Louis, MO) (1 mg/kg, n = 3, 16) were examined in the same model to evaluate the interaction between the two receptors in spinal nociceptive processing.

A preliminary report of these investigations was presented in abstract form (27).

METHODS

Adult female Wistar rats weighing between 160 and 220 g were used in the present study. All experimental procedures were performed in accordance with the Guideline for the Care and Use of Laboratory Animals by the Animal Research Committee of Hokkaido University Graduate School of Medicine. The work presented here fully conforms with the American Physiological Society’s “Guiding Principles for Research Involving Animals and Human Beings.”

In urethane-anesthetized (1.2 g/kg ip injection) rats, tracheal intubation was performed to facilitate respiration. A cannula (PE-50) was inserted in the right external jugular vein for intravenous drug administration. The urinary bladder was exposed through a lower midline abdominal incision, and 1% acetic acid in saline was infused (0.12 ml/min) for 2 h via a needle (23 gauge), which was inserted into the bladder dome just before the start of acetic acid infusion. Two-hour infusion time was adopted in this study according to the time course of c-fos expression after bladder irritation (2). Because the urethra remained open, fluid that was infused into the bladder could be expelled or leak out during the continuous infusion. To eliminate the possibility of irritation of the perineal skin and vaginal mucosa, mineral oil was applied to the area around the urethral meatus.

The effects of intravenous administration of CP-99,994 (Pfizer, Tokyo, Japan) (1, 3, and 10 mg/kg, n = 4 in each), CP-100,263 (Pfizer, Tokyo, Japan) [10 mg/kg, n = 4; the enantiomer of CP-99,994 with a very low affinity for NK1 receptor (33)], or a low dose of NMDA receptor antagonist MK-801 (Sigma, St. Louis, MO) (1 mg/kg, n = 4) on spinal c-fos expression were investigated. To examine nonspecific antinociceptive effects of CP-99,994 in rats, a high dose (10 mg/kg) of CP-100,263 was used. All drugs were dissolved in saline. For control animals without drug administration, the same amount of saline was administered intravenously as a vehicle. Effect of coadministration of a low dose of CP-99,994 (1 mg/kg) and MK-801 (1 mg/kg) was also examined (n = 4). CP-99,994 and MK-801 were administered 15 min before the start of lower urinary tract irritation.

Two hours after infusion of acetic acid, the animals were killed via intracardiac perfusion of 0.1 M phosphate buffer (PB), pH 7.4, followed by 4% paraformaldehyde fixative in PB (0.1 M, pH 7.4). The spinal cord was then removed and postfixed for 24 h in the same fixative at 4°C before cryoprotection in 0.1 M (pH 7.4) phosphate-buffered 30% sucrose solution overnight. With the use of avidin-biotin complex (ABC) method, alternate sections (30 μm) of the spinal cord were processed for immunoreactivity to c-fos protein (Fos), using nickel intensification (1–3, 16). Sections were incubated in Fos antiserum diluted for 2% rabbit serum in Tris-buffered saline (0.05 M, pH 7.6) containing 0.5% Triton X-100 (1:500; Chemicon) for 80 h at 4°C, and then in biotinylated secondary antibody (1:1,000, Chemicon) and ABC reagent (Chemicon), each for 2 h at room temperature. Tissue sections were then mounted on gelatin-coated slides, dehydrated in graded ethanol, cleared in xylene, and placed under a coverslip with Permount. All sections were examined with bright-field microscopy.

Because previous studies analyzed the distribution of Fos-positive cells within the spinal cord (2, 10), analysis in the present study was restricted to the L1 and L6 spinal segment, where the majority of the hypogastric and pelvic nerve afferent terminals project, respectively. Cells exhibiting Fos immunoreactivity were counted in three different spinal cord regions: dorsal horn (DH), dorsal commissure (DCM), and intermediolateral gray matter (ILG) (Fig. 1A and B). In the L1 spinal segment, because the number of Fos-positive cells in the DCM and ILG regions was significantly smaller than in the DH, the number of Fos-positive cells in the DCM and ILG was counted together (Fig. 1A). Counts of Fos-positive cells were performed on 20 sections. All values in the text are expressed as means ± SE. To evaluate changes in the number of Fos-positive cells in each group, analysis of variance followed by the Mann-Whitney U-test was used for examining differences in the distribution of Fos-positive cells at specific areas of the spinal cord, and P < 0.05 was considered significant.
RESULTS

Effects of NK1 receptor antagonist on c-fos expression. The total number of Fos-positive cells after lower urinary tract irritation was 53 ± 7 and 108 ± 6 cells/section at the L1 and L6 spinal cord, respectively (Figs. 2A (L6) and 3A (L1)). The largest number of Fos-positive cells at the L6 spinal cord was located at the DCM area (44 ± 3 cells/section), with smaller numbers at the ILG (34 ± 3 cells/section) and DH (30 ± 3 cells/section). At the L1 spinal cord, the number of Fos-positive cells was much greater at the DH (44 ± 4 cells/section) than at DCM and ILG (8 ± 2 cells/section).

CP-99,994 dose dependently reduced the total number of Fos-positive cells at both levels of the spinal cord (Table 1, Figs. 2B and 3B). However, CP-100.263 (10 mg/kg iv), the enantiomer of CP-99,994, did not affect the total number and regional difference of Fos-positive cells compared with control (Table 1).

At the L1 spinal cord, the number of Fos-positive cells after 1, 3, and 10 mg/kg of CP-99,994 was 96 ± 3, 90 ± 3 (P < 0.05), and 56 ± 2% (P < 0.01) of control, respectively, while at the L6 spinal cord the number of Fos-positive cells after 1, 3, and 10 mg/kg of CP-99,994 was 96 ± 4, 83 ± 4 (P < 0.03), and 35 ± 6% (P < 0.01) of control, respectively (Table 1, Fig. 4). Regional differences were observed in the effects of CP-99,994. At the L1 spinal cord, CP-99,994 did not have a significant effect on the number of Fos-positive cells at the DCM + ILG regions, whereas CP-99,994 decreased the number of Fos-positive cells in a dose-dependent manner at the DH region of the L1 spinal cord (Table 1, Fig. 5A). At the DH region of the L1 spinal cord, the number of Fos-positive cells after 1, 3, and 10 mg/kg of CP-99,994 was 97 ± 3, 90 ± 4 (P < 0.03), and 50 ± 2% (P < 0.01) of the control, respectively (Table 1). At the L6 spinal cord, CP-99,994 reduced the number of Fos-positive cells in a dose-dependent manner at all three regions. The number of Fos-positive cells after 1, 3, and 10 mg/kg of CP-99,994 was 96 ± 4, 78 ± 3 (P < 0.03), and 27 ± 7% (P < 0.01) of the control at the DCM; 99 ± 6, 91 ± 5 (P < 0.05), and 37 ± 4% (P < 0.01) of the control at the ILG; and 96 ± 4, 80 ± 7 (P < 0.03), and 44 ± 7% (P < 0.01) of the control at the DH of the L6 spinal cord, respectively (Table 1, Fig. 5B).

Effects of coadministration of NK1 receptor antagonist and NMDA receptor antagonist on spinal c-fos expression. Intravenous administration of a low dose of either CP-99,994 (1 mg/kg) or MK-801 (1 mg/kg) alone did not significantly alter the number of Fos-positive cells (Table 1, Figs. 6 and 7, A and B). At the L1 spinal cord, the number of Fos-positive cells as percentage of the control after 1 mg/kg of MK-801 was 96 ± 3% in total, 92 ± 1% at the DCM + ILG, and 95 ± 4% at the DH, while at the L6 spinal cord it was 94 ± 2% in total, 96 ± 4% at the DCM, 94 ± 5% at the ILG, and 91 ± 5% at the DH (Table 1). However, coadministration of a low dose of CP-99,994 (1 mg/kg) and MK-801 (1 mg/kg) significantly reduced the total number of Fos-positive cells at both levels of the spinal cord: L1, 65 ± 2% (P < 0.01) of control; L6, 47 ± 5% (P < 0.01) of control (Table 1, Figs. 2C, 3C, and 6). Regional differences were again observed in the effects of coadministration of a low dose.
of CP-99,994 and MK-801. After coadministration of a low dose of CP-99,994 and MK-801, the number of Fos-positive cells at the L1 spinal cord decreased significantly only at the DH region (59 ± 3% of the control) (Table 1, Fig. 7A), whereas at the L6 spinal cord the number of Fos-positive cells decreased significantly at all three regions (40 ± 6, 47 ± 6, and 57 ± 3% of the control at the DCM, ILG, and DH, respectively) (Table 1, Fig. 7B).

**DISCUSSION**

In the present study, c-fos expression in spinal neurons was used to examine the role of NK1 receptor and the possible interaction between NK1 and NMDA receptors in spinal nociceptive pathways activated by afferent input from the lower urinary tract. Because CP-99,994, which was used as a selective NK1 receptor antagonist in the present study, is known to have nonspecific antinociceptive effects in rats (33), we also examined its less active enantiomer, CP-10,263. CP-100,263 has the same nonspecific antinociceptive effect but has a very low affinity for NK1 receptor (33). CP-100,263 showed no significant effect on c-fos expression after lower urinary tract irritation. Therefore, the results indicate that NK1 receptor is involved in spinal c-fos expression after lower urinary tract irritation. A previous study on c-fos expression has shown that glutamatergic receptors (NMDA and non-NMDA receptors) play a synergistic role in bladder nociceptive processing (16). The results in the present study fur-

**Table 1. Effects of drugs on the number of Fos-positive cells after lower urinary tract irritation with acetic acid infusion**

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<tr>
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<th>L1</th>
<th>L6</th>
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<tr>
<td></td>
<td>Total DCM ILG DH</td>
<td>Total DCM ILG DH</td>
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<tr>
<td>CP-100,263 (10 mg/kg)</td>
<td>100 ± 1 98 ± 3 100 ± 1</td>
<td>100 ± 3 100 ± 3 100 ± 2 101 ± 4</td>
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<tr>
<td>CP-99,994</td>
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<tr>
<td>1 mg/kg</td>
<td>96 ± 3</td>
<td>96 ± 4</td>
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<tr>
<td>3 mg/kg</td>
<td>90 ± 3†</td>
<td>83 ± 4†</td>
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<tr>
<td>10 mg/kg</td>
<td>56 ± 2‡</td>
<td>35 ± 6‡</td>
</tr>
<tr>
<td>MK-801 (1 mg/kg)</td>
<td>96 ± 3</td>
<td>94 ± 2</td>
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<tr>
<td>CP-99,994 + MK-801 (1 mg/kg each)</td>
<td>65 ± 2‡</td>
<td>47 ± 5‡</td>
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Values are percent of control (means ± SE). DCM, dorsal commissure; DH, dorsal horn; ILG, intermediolateral gray matter. *P < 0.05, †P < 0.03, ‡P < 0.01.
ther indicate that NK₁ and NMDA receptors have a
synergistic interaction in the spinal processing of blad-
der nociception.

**Role of NK₁ receptor in spinal c-fos expression after lower urinary tract irritation.** Systemic administration of CP-99,994, which has central actions after intrave-
nous administration (33), reduced dose dependently the total number of Fos-positive cells at the L₁ and L₆ spinal cord after lower urinary tract irritation. However, the effects of CP-99,994 on c-fos expression at specific regions within the spinal cord were different between the L₁ and L₆ spinal cord. At the L₆ spinal cord, the reduction of Fos-positive cells by CP-99,994 was noted at all three regions within the spinal cord. In contrast, the reduction of Fos-positive cells at the L₁ spinal cord was restricted to the DH region. Thus it seems that spinal neurons located at the DCM and ILG of the L₁ spinal cord that are involved in c-fos expression are insensitive to NK₁ receptor antagonist. The number of Fos-positive cells at the L₁ spinal cord was significantly smaller at the DCM and ILG regions (8 ± 2 cells/section) than at the DH (44 ± 4 cells/section). This finding is consistent with a previous study that revealed that an increase in c-fos expression at the Th₁₂-L₂ spinal cord after lower urinary tract irritation was mainly noted in lamina I (10). Thus, at the L₁

![Graph](image1)

**Fig. 4.** Effects of graded doses of CP-99,994 (CP) on the total number of Fos-positive cells at the L₁ and L₆ spinal cord after lower urinary tract irritation (n = 4 in each dose). Percent changes in the number of Fos-positive cells per section are indicated. *P < 0.05, **P < 0.03, ***P < 0.01 compared with control (vehicle).

![Graph](image2)

**Fig. 6.** Effects of coadministration of a low dose (1 mg/kg) of CP-99,994 and MK-801 on the total number of Fos-positive cells at the L₁ and L₆ spinal cord after lower urinary tract irritation (n = 4 in each dose). Percent changes in the number of Fos-positive cells per section are indicated for pretreatment with a low dose of either CP-99,994 or MK-801 alone, or a combination of both (CP + MK) before lower urinary irritation. *P < 0.01 compared with control (vehicle).

![Graph](image3)

**Fig. 7.** Regional differences in the effect of coadministration of a low dose (1 mg/kg) of CP-99,994 and MK-801 on the number of Fos-positive cells at the L₁ and L₆ spinal cord after lower urinary tract irritation (n = 4 in each dose). Percent changes in the number of Fos-positive cells per section are indicated for pretreatment with a low dose of either CP-99,994 or MK-801 alone, or a combination of both before lower urinary irritation. A: L₁ spinal cord. B: L₆ spinal cord. *P < 0.01 compared with control (vehicle).
spinal cord, where the hypogastric nerve afferent terminals project, the effects of CP-99,994 was only noted at the DH region, which contains numerous nociception-specific neurons.

In accordance with the present study, Fos-positive neurons after chemical stimulation of the bladder with formalin were distributed in the DCM, ILG, and DH of the L6–S1 spinal cord where the spinal micturition center of the rat exists (20). These Fos-positive neurons in the L6–S1 spinal cord appear to be distributed in the areas of distribution of nociceptive spinal neurons that have been identified electrophysiologically (26). Interestingly, the distribution of these Fos-positive neurons was quite similar to that of substance P receptor-like immunoreactive neurons, and more than 80% of substance P receptor-like immunoreactive neurons in the DCM, ILG, and DH exhibited Fos immunoreactivity after nociceptive bladder stimulation (20). Taken together, it may be speculated that activation of NK1 receptors in the spinal neurons by nociceptive bladder stimulation is involved in inducing c-fos expression. Thus NK1 receptor antagonism by CP-99,994 significantly reduced c-fos expression in the pretest study, suggesting that NK1 receptor is involved in spinal processing of nociceptive input from the lower urinary tract. These results are consistent with previous findings that NK1 receptor antagonists inhibited nociceptive reflex responses to chemical stimulation of the gallbladder and to jejunal distension (28, 33). An essential role of NK1 receptor mediating central responses to nociceptive visceral stimulation (chemical stimulation of colon and cyclophosphamide-induced cystitis) has been well demonstrated in mice with a disruption of the NK1 receptor gene (17). However, because a large dose of NK1 receptor antagonist used in the present study did not block completely c-fos expression in spinal neurons, substance P may have a neuro-modulatory, as opposed to absolute neurotransmitter, role in spinal processing of nociception (6).

Interaction between NK1 and NMDA receptors in spinal c-fos expression after lower urinary tract irritation. It has been shown that NMDA glutamatergic receptor in spinal neurons is involved in visceral nociception. Preemptive intrathecal administration of NMDA receptor antagonist prevents hyperreflexia in a model of persistent visceral pain (32). Visceral hyperalgesia induced by colonic inflammation is mediated by the activation of spinal NMDA receptor (9). Thus many studies implicate the critical role of NMDA receptor in visceral pain (5). The present study, as the second part of experiments, focused on the possible interaction between NK1 and NMDA receptors in spinal processing of visceral nociception, instead of the well-documented role of NMDA receptor in visceral nociception.

Previous studies have shown that intravenous administration of a high dose (3.5 mg/kg) of MK-801 but not a low dose (0.8–1 mg/kg) significantly reduced spinal c-fos expression after chemical irritation of the lower urinary tract (1, 3, 16). The mean reduction of Fos-positive cells in the L6 spinal cord after intravenous administration of 3.5 mg/kg of MK-801 was 53, 55, and 54% at the DCM, ILG, and DH (1). In the present study, intravenous administration of a low dose (1 mg/kg) of either CP-99,994 or MK-801 alone did not significantly alter the number of Fos-positive cells. We selected only a dose of 1 mg/kg of MK-801 to confirm the ineffectiveness of a low dose of MK-801 in suppressing spinal c-fos expression after lower urinary tract irritation. However, combined administration of a low dose of CP-99,994 and MK-801 significantly decreased the number of Fos-positive cells at the L1 (35% reduction) and L6 spinal cord (53% reduction) to the almost similar extent as that observed after administration of the highest dose (10 mg/kg) of CP-99,994. In the L6 spinal cord, the mean reduction of Fos-positive cells after coadministration of a low dose of CP-99,994 and MK-801 was 60, 53, and 43% at the DCM, ILG, and DH, respectively. Compared with the results of a previous study (1), this inhibitory effect of coadministration of a low dose of CP-99,994 and MK-801 on spinal c-fos expression was equivalent to that of a high dose of MK-801. From these results, it seems that the effect of coadministration of a low dose of CP-99,994 and MK-801 is not simply additive, but there must be synergism between the NK1 and NMDA receptor antagonists. Because the present study did not focus on the dose-response characteristics of MK-801, we did not examine the effect of a medial (e.g., 2 mg/kg) or high dose of MK-801. Despite such limitations in the present study, we believe that NK1 and NMDA receptors have a synergistic interaction in the spinal processing of nociceptive input from the lower urinary tract.

A variety of studies has demonstrated mechanisms underlying the interaction between NK1 and NMDA receptor-mediated events. Synergistic activation of NK1 and NMDA receptors is anatomically possible because both receptors have been reported to coexist on single dorsal horn neurons (31, 35). The release of substance P is controlled by facilitatory NMDA receptors (11, 18, 21) and then the released substance P can also enhance the basal release of EAAs (34). Systemic coadministration of NK1 receptor antagonist and NMDA receptor antagonist significantly reduced the number of Fos-positive cells after intraplantar formalin injection, and the attenuating effect of the coadministration was significantly greater than the effect of either the NK1 or NMDA receptor antagonist alone (6). These results are consistent with the present study. Thus cooperativity between NK1 and NMDA receptors within the spinal cord, which has been shown in somatic nociceptive pathways as stated above, was also demonstrated in visceral nociceptive pathways by the present study.

We are grateful to Pfizer Pharmaceutical for a gift of CP-99,994 and CP-100,263.

REFERENCES


