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Activation of Spinal N-methyl-D-aspartate *Receptors Stimulates a Nitric Oxide/Cyclic Guanosine 3′,5′-monophosphate/Glutamate Release Cascade in Nociceptive Signaling*

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Background: **Increasing evidence has suggested the possibility that the activation of *N*-methyl-D-aspartate (NMDA) receptors modulates spinal nociceptive transmission *via* a nitric oxide (NO)/cyclic guanosine 3′,5′-monophosphate (cGMP) pathway. However, the existence and the role of an NO/cGMP pathway in the modulation of spinal nociceptive transmission has been unclear. The authors hypothesized that the activation of NMDA receptors stimulates an NO/cGMP pathway, and this pathway evokes glutamate release within the spinal cord, modulating spinal nociceptive transmission.**

Methods: **The authors have examined the effects of an NO synthase inhibitor and a soluble guanylate cyclase inhibitor on the concentrations of NO metabolites (NO_2^-/NO_3^-) and glutamate in the cerebrospinal fluid after intrathecal perfusion of NMDA, concomitantly observing pain-related behavior (scratching, biting, and vocalization) in unanesthetized, free-moving rats using an intrathecal microdialysis method. The contents of cGMP in the dorsal horn were also measured using enzyme immunoassay method.**

Results: **Intrathecal perfusion of NMDA produced pain-related behavior and increased glutamate and NO_2^-/NO_3^- concentrations in a dose-dependent manner. A competitive NMDA receptor antagonist, D,L-2-amino-5-phosphonovaleric acid, completely blocked the NMDA-induced responses. An NO synthase inhibitor, N^G-monomethyl-L-arginine acetate, at a dose that completely blocked the increase in NO_2^-/NO_3^-, inhibited both the NMDA-induced pain-related behavior and the increase in glutamate concentration. In addition, a soluble guanylate cyclase inhibitor, 1H-[1,2,4]oxadiazole[4,3-a]quinoxaline-1-one, also inhibited significantly NMDA-induced pain-related behavior and the increase in glutamate concentration. NMDA induced an increase in cGMP in the dorsal half of the spinal cord, which was blocked by N^G-monomethyl-L-arginine acetate.**

Conclusions: **The results of this study support the hypothesis that the activation of NMDA receptors modulated pain-related behavior *via* an NO/cGMP/glutamate release cascade within the spinal cord. (Key words: Neuron; nociception; pain.)**

SEVERAL lines of evidence support the hypothesis that the activation of spinal *N*-methyl-D-aspartate (NMDA) receptors plays a critical role in spinal mechanisms of pathologic pain states, such as hyperalgesia and allodynia.

The activation of NMDA receptors in the central nervous system initiates an influx of Ca^{2+}, inducing Ca^{2+}-dependent intracellular processes.[1] Nitric oxide synthase (NOS) is a Ca^{2+}/calmodulin-dependent enzyme that is stimulated by the activation of NMDA receptors and synthesizes nitric oxide (NO) from L-arginine.[2,3] NO increases cyclic guanosine 3′,5′-monophosphate (cGMP) through the activation of soluble guanylate cyclase (sGC).[3,4] Immunohistochemical studies demonstrated that high densities of neuronal NOS were found in neuronal fibers and interneurons in superficial layers of the dorsal spinal cord.[5,6] Behavioral studies have shown that the inhibition of either intrathecal NOS or sGC blocks hyperalgesia induced by inflammation and nerve injury but does not alter physiologic nociceptive responses.[7,8] Intrathecal administration of either an NO donor or a membrane-permeable cGMP analog enhances neuropathic pain-related behavior[9,10] and produces hyperalgesia.[11,12] Furthermore, electrophysiologic studies have shown that either NO or cGMP are involved in the sensitization of spinal secondary neuron after peripheral inflammation.[13] This evidence suggests a facilitatory role for NO and cGMP in spinal nociceptive transmission. Accordingly, it has been assumed that the activation of NMDA receptors stimulates a possible NO/cGMP pathway in spinal nociceptive transmission. However, it has

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been unclear whether the activation of NMDA receptors increases NO and NO-dependent cGMP in the spinal cord. Even if the activation of NMDA receptors increases NO and NO-dependent cGMP, the role of an NO/cGMP pathway in the modulation of spinal nociceptive transmission is also unclear.

Nociceptive afferents and interneurons release an excitatory amino acid, L-glutamate.[14,15] After peripheral inflammation, increased amounts of excitatory amino acids are released within the spinal cord.[16-18] There is an increase in the level of excitatory amino acids in the spinal cord dorsal horn ipsilateral to a peripheral nerve injury that produces neuropathic pain.[19] These increases are blocked by pretreatment with an NMDA antagonist.[18,19] In addition, it has been shown that NO or cGMP enhance neurotransmitter release in hippocampal neurons.[20,21] Therefore, we hypothesize that the activation of NMDA receptors stimulates an NO/cGMP pathway, and this pathway evokes glutamate release within the spinal cord, modulating spinal nociceptive transmission. To confirm this hypothesis, we measured the concentration of NO metabolites and glutamate in the cerebrospinal fluid (CSF) and the content of cGMP in the dorsal horn after the activation of spinal NMDA receptors, concomitantly observing pain-related behavior. We also have examined the effects of a NOS inhibitor and a sGC inhibitor on NMDA-induced increase in glutamate and pain-related behavior.

Materials and Methods

The protocol for this study was approved by Sapporo Medical University Animal Care and Use Committee. Experiments were conducted in male Sprague Dawley rats weighing 250-300 g (Japan SLC, Hamamatsu, Japan), which were housed individually in a temperature-controlled room (21 ± 1°C) with a 12-h light-dark cycle and given free access to food and water.

Construction of Microdialysis Probe and Animal Preparation

The dialysis probe was constructed according to our modification of the method described by Marsala *et al.*[22] A 6-cm cupraphan hollow fiber with an inner diameter of 200 μm, an outer diameter of 220 μm, and 50-kDa molecular weight cutoff (DM-22; Eicom Co., Kyoto, Japan) was coated with a thin layer of epoxy glue (Devcon Co., Danvers, MA) along the whole length, except for a 2-cm region in the middle. To make the fiber firm enough for implantation, a Nichrome-Formvar wire with a 78 μm inner diameter (A-M systems, Inc., Everret, WA) was passed through the fiber. Each end of the fiber was attached to 2-cm polyethylene catheters (PE-10, Clay Adams, NJ), and the other end of each polyethylene catheter was then attached to an 8-cm Teflon tube (JT-10; Eicom) with an inner diameter of 100 μm and an outer diameter of 400 μm. A U-shaped loop was formed by gently bending the whole fiber in the middle, and the two pieces were bound together at the fiber-polyethylene catheter connections using epoxy glue. The dead space volume of the dialysis probe was 7 μl.

During general anesthesia (isoflurane 3% in oxygen), a dialysis probe was inserted 30 mm cephalad into the lumbar subarachnoid space at the L4-5 intervertebral space, positioning the dialysis area of the probe over the lumbar enlargement of the spinal cord. The two distal ends of the probe were tunneled subcutaneously and externalized through the skin in the neck region. In some rats, an additional intrathecal polyethylene catheter (PE-10) that had 3 μl of dead space was inserted to administer a drug directly into the lumbar intrathecal space rather than by diffusion from the dialysis probe. The experiments were performed 5 days after the implantation of the dialysis probe. In the experiments, we used only animals that showed normal behavior and motor function. Each rat was used for only one experiment. After each experiment, rats were killed with an overdose of pentobarbital, and the positions of dialysis probe and additional intrathecal catheter were confirmed by gross examination of the spinal cord.

Drugs

The drugs and chemicals used were as follows: D,L-2-amino-5-phosphonovaleric acid (AP-5; Sigma Chemical Co., St Louis, MO), NMDA (Sigma), 1H-[1,2,4]oxadiazole[4,3-a]quinoxaline-1-one (ODQ; Sigma), N^G-monomethyl-L-arginine acetate (L-NMMA; Research Biochemicals Inc., Natrick, MA). AP-5, L-NMMA, and NMDA were dissolved in artificial CSF (ACSF). ODQ was first dissolved in dimethyl sulfoxide (DMSO) and then diluted in ACSF to 10% DMSO. The *p*H of all solutions was adjusted to 7.4.

Microdialysis Study

The animals freely moved in a plastic cage with dimensions of 30 × 30 × 35 cm during the dialysis experiments. A liquid switch (SI-50; Eicom) was placed between the syringe pump and the dialysis probe to enable different drugs to be administered locally *via* the probe.

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The dialysis probe was perfused with ACSF (140 mM NaCl, 4.0 mM KCl, 1.26 mM $CaCl_2$, 1.15 mM $MgCl_2$, 2.0 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, and *p*H 7.4) at a constant flow rate of 3 μl/min. The samples were collected as 10-min fractions. For each 10-min sample (30 μl), 15 μl was used for glutamate analysis and 10 μl for NO metabolites (total amount of NO_2^- and NO_3^-, NO_2^-/NO_3^-) analysis. The samples for glutamate were frozen at −80°C until analysis, and the samples for NO_2^-/NO_3^- were immediately analyzed.

Three consecutive samples were collected for determination of basal levels 60 min after starting perfusion of ACSF, and then the drugs were administered. To examine the dose-response interaction of NMDA, 0.1, 1.0, or 5.0 mM NMDA was infused for 10 min into the lumbar intrathecal space through a dialysis probe. The other series of experiments were designed to examine the effects of an NMDA receptor antagonist, NOS inhibitor, and sGC inhibitor on NMDA-induced responses. A competitive NMDA receptor antagonist, AP-5 (10 mM), or a NOS inhibitor, L-NMMA (10 mM), was perfused through the dialysis probe for 90 min. Thirty minutes after starting administration of AP-5 or L-NMMA, 1.0 mM NMDA was coperfused with AP-5 or L-NMMA for 10 min. The sGC inhibitor ODQ was dissolved in DMSO and then diluted in ACSF because of poor water solubility. ODQ was administered *via* an additional intrathecal catheter. Our preliminary study showed that, although intrathecal perfusion with 10% DMSO through the dialysis probe changed the glutamate and NO_2^-/NO_3^- concentrations progressively, direct intrathecal administration did not affect their concentrations. ODQ (35 nmol) could be dissolved in 10 μl of 10% DMSO in the present study. Based on the results of a previous behavioral study, the peak antihyperalgesic effect of intrathecal ODQ was observed 30 min after administration[8]; therefore, in this study, 35 nmol ODQ was directly administered 30 min before starting administration of 1.0 mM of NMDA.

Analysis of Glutamate

Glutamate in the dialysate was analyzed using high-performance liquid chromatography with electrochemical detection (Degasser; DG-100, liquid chromatograph; EP-100, electrochemical detector; ECD-100; all from Eicom) after derivatization with *o*-phthaldialdehyde (Sigma). The *o*-phthaldialdehyde derivatizing reagent was prepared by dissolving *o*-phthaldialdehyde (54 mg) in absolute methanol (1 ml) and adding 2-mercaptoethanol (40 μl) and sodium carbonate (0.1 M; *p*H 9.5; 99 ml). Ten microliters of this derivatizing reagent was mixed with 15 μl of the dialysate and allowed to react for 2.5 min. Fifteen microliters of this solution was analyzed. The chromatographic conditions were as follows: column = Eicompak (MA-5ODS 4.6 × 150 mm; Eicom); mobile phase = 0.1 M sodium dihydrogenphosphate and 0.1 M disodium hydrogenphosphate (*p*H 9.5) containing 30.0% methanol, and 0.1 mM disodium EDTA; working electrode = glassy carbon (WE-3G, Eicom); flow rate = 1 ml/min. Detector voltage was set at 0.6 V. Detector temperature was set at 30°C. Retention time for glutamate was 6.0 min. In analysis of dialysates containing AP-5, a mobile phase with 10.0% methanol was applied because of the overlap of the peaks of glutamate and AP-5 on the chromatogram. The retention time for glutamate in the samples containing AP-5 was 25.5 min.

To determine the *in vitro* recovery of glutamate across the dialysis probe used in the present study, a dialysis probe was put into aliquots containing 10 μM concentration of glutamate and perfused with ACSF at a constant flow rate of 3 μl/min at room temperature. The *in vitro* recovery was estimated based on the levels of glutamate in the 10-min dialysis sample.

Analysis of NO_2^-/NO_3^-

NO_2^-/NO_3^- in the dialysate was analyzed using an automated NO detector–high-performance liquid chromatography system (ENO-10; Eicom). NO_2^-/NO_3^- in the dialysate were separated by a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6 × 50 mm; Eicom), and NO_3^- was reduced to NO_2^- in a reduction column packed with copper-plated cadmium filling (NO-RED; Eicom). NO_2^- was mixed with a griess reagent to form a purple azo dye in a reaction coil. The separation and reduction columns and the reaction coil were placed in a column oven that was set at 35°C. The absorbance of the color of the product dye at 540 nm was measured by a flow-through spectrophotometer (NOD-10; Eicom). The mobile phase, which was delivered by a pump at a rate of 0.33 ml/min, was 10% methanol containing 0.15 M NaCl/NH_4Cl and 0.5 g/l 4Na-EDTA. The Griess reagent, which was 1.25% HCl containing 5 g/l sulfanilamide with 0.25 g/l *N*-naphthylethylenediamine, was delivered at a rate of 0.1 ml/min. The contamination of NO_2^-/NO_3^- in ACSF and the reliability of the reduction column were examined in each experiment.

To determine the *in vitro* recovery of NO_2^-/NO_3^- across the dialysis probe used in the present study, a dialysis probe was put into aliquots containing 10 μM concentration of $NaNO_2$ and $NaNO_3$ and perfused with

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ACSF at a constant flow rate of 3 μl/min at room temperature. The *in vitro* recovery was estimated based on the levels of NO_2^-/NO_3^- in the 10-min dialysis sample.

Behavioral Assessment

During the perfusion of NMDA *via* the microdialysis probe, the rats showed pain-related behavior (scratching, biting, and vocalization). The intensity of the pain-related behavior was assessed at 2-min intervals using a rating scale of 0 to 2 (0 = normal behavior, no detectable effect; 1 = occasional scratching or biting of hindlimbs or flank; 2 = frequent bouts of scratching or biting of hindlimbs or flank, or occasional vocalization) throughout the experiments. The intensity of the pain-related behavior was calculated by adding the individual scores over five observations during 10-min intervals (minimum = 0; maximum = 10). In addition, the duration of pain-related behavior was recorded at 10-min intervals throughout the experiments.

cGMP Analysis

The contents of cGMP in the dorsal horn were measured using an enzyme immunoassay method. After a washout period of 60 min, the dialysis probe was used to perfuse the lumbar intrathecal space of the rats with only ACSF for 40 min as control, 1.0 mM NMDA for 10 min after ACSF for 30 min, 10 mM L-NMMA for 40 min, 10 mM L-NMMA for 40 min with coperfusion of 1.0 mM NMDA for the last 10 min. The animals were killed by decapitation after each treatment. Upon decapitation, the lumbar spinal cord was quickly removed, and then the dorsal half of the lumbar enlargement of the cord was dissected and immediately frozen by immersion in liquid nitrogen. Tissue was homogenized in 1 ml ice-cold 6% trichloroacetic acid (Power Homogenizer S-203, Ikeda Scientific, Tokyo, Japan). The homogenate was centrifuged at 2,000g for 15 min at 4°C. The supernatant was washed with water-saturated diethyl ether and then assayed in duplicate for cGMP using a commercially available radioimmunoassay kit (Biotrak cGMP enzyme immunoassay system; Amersham, Buckinghamshire, United Kingdom). The resulting pellets were solubilized and used to measure protein concentrations by the Lowry method.

Data Analysis

The basal levels of the glutamate and NO_2^-/NO_3^- concentrations were defined by averaging the concentrations of the three consecutive samples 60 min after starting perfusion of ACSF. The changes of the glutamate and NO_2^-/NO_3^- concentrations are presented as mean ± SD of percentage of basal levels. The cGMP concentration and the duration of pain-related behavior also are presented as mean ± SD. The intensity of pain-related behavior is expressed as median and 25th–75th percentiles. With regard to the glutamate, NO_2^-/NO_3^- concentrations and the duration of pain-related behavior, statistical significance was determined by a two-way analysis of variance followed by multiple between-group comparisons using the Bonferroni correction. The contents of cGMP and the glutamate concentrations among NMDA-, L-NMMA-, and ODQ-treated rats were analyzed by a one-way analysis of variance followed by multiple between-group comparisons using the Bonferroni correction. The intensity of pain-related behavior was analyzed by the Kruskal-Wallis test followed by Dunn's test for multiple comparisons. A P value < 0.05 was considered to be statistically significant.

Results

General Behavior

All rats showed normal behavior and motor function after the implantation of the dialysis probe. In addition, none of the rats showed any behavioral changes during intrathecal perfusion with ACSF.

Basal Levels of Glutamate and NO_2^-/NO_3^- in dialysates

A preliminary study showed that dialysis equilibrium was obtained 60 min after starting of ACSF perfusion at a constant flow rate of 3 μl/min, and basal levels were stable for at least 180 min (data not shown). The absolute basal levels of glutamate and NO_2^-/NO_3^- in dialysates were 26.9 ± 4.76 and 60.93 ± 5.97 pmol/10 μl, respectively. The contamination of NO_2^-/NO_3^- in ACSF was < 0.5 pmol/10 μl. The *in vitro* recovery of glutamate and NO_2^-/NO_3^- was estimated to be 32.49 ± 0.01% and 68.97 ± 0.02%, respectively (n = 6), across the dialysis probe. Ten percent DMSO did not change the *in vitro* recovery.

Effects of NMDA Perfusion on Pain-related Behavior and Glutamate and NO_2^-/NO_3^- Concentrations

Figure 1 shows that NMDA perfusion evoked dose- and time-dependent increases in the scores of the intensity ($P < 0.01$) and duration of pain-related behavior ($P < 0.01$). Pain-related behavior was still

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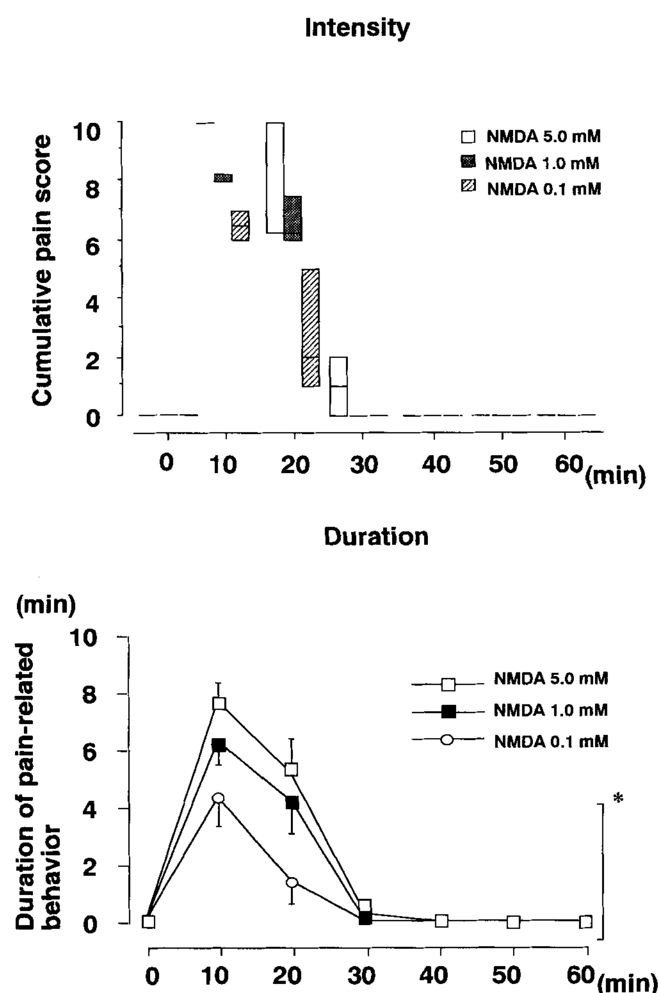

Fig. 1. Time courses of the intensity (*top*) and duration (*bottom*) of *N*-methyl-D-aspartate (NMDA)-induced pain-related behavior. NMDA was infused into the lumbar intrathecal space through a dialysis probe during 0–10 min. Data for the intensity and the duration of pain-related behavior are presented as median and 25th–75th percentiles (n = 6–9) and mean ± SD (n = 6–9), respectively. *Statistical significance among NMDA 0.1 mM, 1.0 mM, and 5.0 mM ($P < 0.01$). Statistical analyses also indicated the significant differences among NMDA 0.1 mM, 1.0 mM, and 5.0 mM ($P < 0.01$) in the intensity of the pain-related behavior.

present during the 20-30-min period after 5.0 mM NMDA perfusion was completed; 0.1 and 1.0 mM NMDA did not persist for long after the perfusion was completed. At 5.0 mM NMDA, five of six rats showed muscle tremor and hindlimb extensor-type rigidity; however, the rats treated with 0.1 or 1.0 mM NMDA had slight or no tremor and rigidity. Consistent with behavioral consequences, NMDA also evoked dose- and time-dependent increases in glutamate ($P < 0.05$) and NO_2^-/NO_3^- concentrations ($P < 0.01$; fig. 2). Although the peak concentrations in glutamate and NO_2^-/NO_3^- were observed at 0-10- and 10-20-min periods at 0.1 and 1.0 mM NMDA, respectively, the peak NO_2^-/NO_3^- concentration was observed during the 20-30-min period at 5.0 mM NMDA.

Effects of AP-5 on NMDA-induced Responses

D,L-2-amino-5-phosphonovaleric acid, which alone did not affect any general behavior, completely blocked 1.0 mM NMDA-induced pain-related behavior (data not shown). NMDA (1 mM)-induced increases in glutamate and NO_2^-/NO_3^- concentrations were also completely inhibited by 10 mM AP-5 (fig. 3).

Effects of L-NMMA and ODQ on NMDA-induced Pain-related Behavior

Although L-NMMA and ODQ alone did not affect general behavior, L-NMMA and ODQ significantly decreased both the intensity and duration of NMDA-induced pain-related behavior throughout the observation periods ($P < 0.001$; fig. 4). Ten percent DMSO, in which ODQ was dissolved, did not produce any changes of general behavior and NMDA-induced pain related behavior (data not shown).

Effects of L-NMMA and ODQ on NMDA-induced Increase in Glutamate and NO_2^-/NO_3^- Concentrations

N^G-monomethyl-L-arginine acetate and ODQ alone did not affect basal levels of glutamate 30 min after administration (fig. 5). L-NMMA blocked NMDA-induced increase in glutamate concentration (fig. 5). In the rats treated with ODQ, NMDA significantly increased glutamate concentration ($P < 0.01$), but the magnitude of increase in glutamate concentration was significantly smaller compared with that of NMDA alone ($P < 0.001$; fig. 5). ODQ alone had no effect on basal values of NO_2^-/NO_3^- release and no effect on the increase evoked by NMDA (data not shown). L-NMMA significantly decreased NO_2^-/NO_3^- concentrations. NO_2^-/NO_3^- concentrations were stable after the perfusion of L-NMMA for 30 min (fig. 6). L-NMMA completely blocked NMDA-induced increase in NO_2^-/NO_3^- concentrations (fig. 6).

cGMP Analysis

When the intrathecal space was perfused with ACSF as control, the content of cGMP in the dorsal half of the lumbar spinal cord was 99.12 ± 31.3 fmol/mg protein. NMDA perfusion significantly increased the content of

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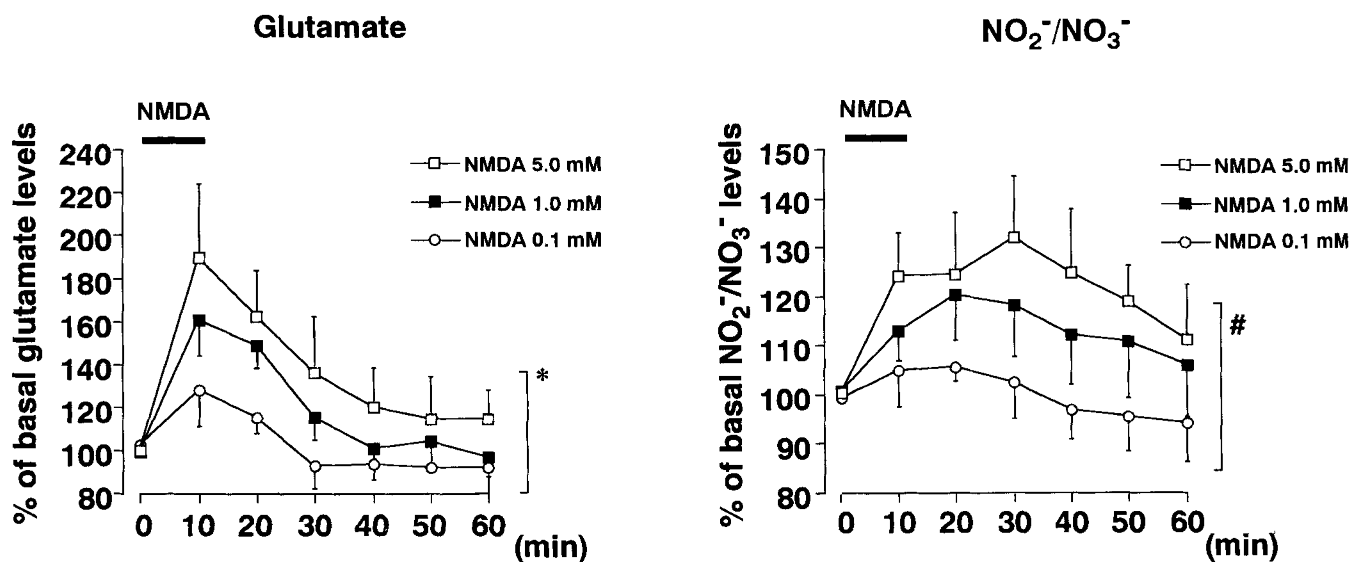

Fig. 2. Time courses of *N*-methyl-D-aspartate (NMDA)-induced spinal release of glutamate (*left*) and NO_2^-/NO_3^- (*right*). Dialysates were collected as 10-min fractions. NMDA was perfused into the lumbar intrathecal space through a dialysis probe during 0–10 min (as shown by the bar). Basal levels were measured by averaging three consecutive samples immediately before infusion of NMDA. Data are presented as mean ± SD (n = 6–9) of percentage values of basal levels. *#Statistical significance among NMDA 0.1 mM, 1.0 mM, and 5.0 mM ($P < 0.05$ and 0.01, respectively) in changes of glutamate and NO_2^-/NO_3^- concentrations, respectively.

cGMP compared with control ($P < 0.01$; fig. 7). L-NMMA itself did not change the content of cGMP compared with control and blocked NMDA-induced increase in cGMP (fig. 7).

Discussion

The present study has demonstrated that intrathecal perfusion of NMDA produced pain-related behavior and

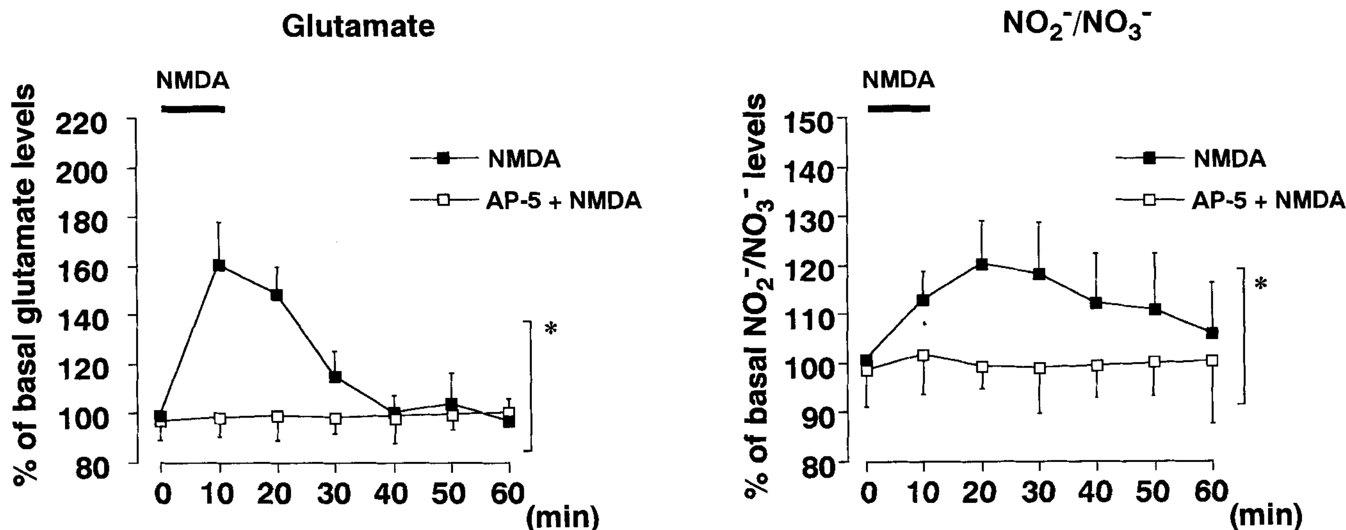

Fig. 3. Effects of D,L-2-amino-5-phosphonovaleric acid (AP-5) on *N*-methyl-D-aspartate (NMDA)-induced spinal release of glutamate (*left*) and NO_2^-/NO_3^- (*right*). Dialysates were collected as 10-min fractions. NMDA (1.0 mM) was perfused into the lumbar intrathecal space through a dialysis probe during 0–10 min (as shown by the bar). AP-5 (10 mM) was perfused into lumbar intrathecal space for 90 min. Thirty minutes after starting administration of AP-5, NMDA was coperfused with AP-5 for 10 min. Basal levels were determined by averaging three consecutive samples immediately before infusion of AP-5. Data are presented as mean ± SD (n = 8) of percentage values of basal levels. *Statistical significance between NMDA and AP-5 plus NMDA ($P < 0.001$ and 0.001, respectively) in changes of glutamate and NO_2^-/NO_3^- concentrations, respectively.

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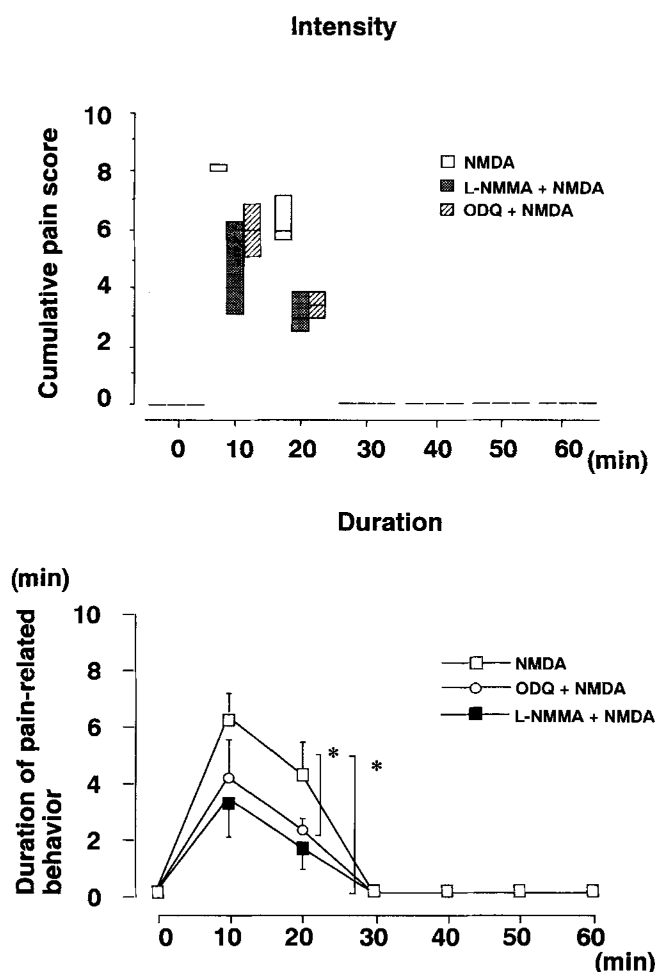

Fig. 4. Effects of N^G-monomethyl-L-arginine acetate (L-NMMA) and 1H-[1,2,4]oxadiazole[4,3-a]quinoxaline-1-one (ODQ) on the intensity (*top*) and duration (*bottom*) of *N*-methyl-D-aspartate (NMDA)-induced pain-related behavior. NMDA (1.0 mM) was infused into the lumbar intrathecal space through a dialysis probe during 0–10 min. Data for the intensity and the duration of pain-related behavior are presented as median and 25th–75th percentile (n = 8) and mean ± SD (n = 8), respectively. *Statistical significance between NMDA and L-NMMA plus NMDA ($P < 0.01$) or NMDA and ODQ plus NMDA ($P < 0.01$) in the duration of the pain-related behavior. Statistical analyses also indicated the significant differences between NMDA and L-NMMA plus NMDA ($P < 0.01$) or NMDA and ODQ plus NMDA ($P < 0.01$) in the intensity of the pain-related behavior.

increased glutamate and NO_2^-/NO_3^- concentrations in lumbar CSF in a dose-dependent manner. A NOS inhibitor, L-NMMA, at the dose that completely blocked the NMDA-induced increase in NO_2^-/NO_3^- concentration, significantly inhibited NMDA-induced pain-related behavior and increase in glutamate concentration. In addition, an sGC inhibitor, ODQ, significantly inhibited NMDA-induced pain-related behavior and increase in glutamate concentration. The analysis of cGMP showed that NMDA induced an increase in cGMP content in the dorsal half of the spinal cord and that L-NMMA blocked the increase. These results indicate that the activation of NMDA receptors evokes pain-related behavior *via* an NO/cGMP/glutamate release cascade within the spinal cord.

Methodologic Aspects

An intrathecal microdialysis method was used to measure the releases of amino acids and other chemicals into the CSF from the spinal cord concomitant with the observation of behavioral responses in awake, free-moving rats.[22,23] This method permitted the experiments to be performed on awake rats up to several days after implantation and, therefore, minimized interference of acute surgical stress and anesthetics.[22] In microdialysis study, although pharmacologic stimulation of neuronal pathways can induce an increase in extracellular glutamate that is related to neurotransmission, it is possible that this increase originates from both neuronal and nonneuronal pools.[24] In addition, the increased concentration of glutamate in the CSF may reflect not only the synaptic transmission but also the neurotransmission, which is characterized by long-distance signal diffusion

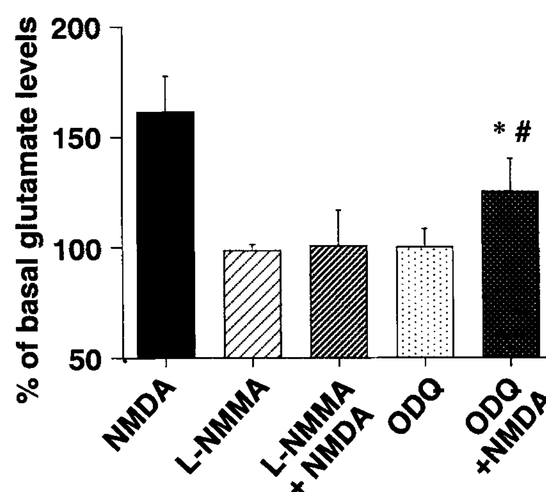

Fig. 5. Effects of N^G-monomethyl-L-arginine acetate (L-NMMA) and 1H-[1,2,4]oxadiazole[4,3-a]quinoxaline-1-one (ODQ) on *N*-methyl-D-aspartate (NMDA)-induced spinal release of glutamate. The bars of NMDA, L-NMMA plus NMDA, and ODQ plus NMDA show the concentrations of glutamate 10 min after NMDA perfusion. The bars of L-NMMA and ODQ show the concentrations of glutamate 30 min after administration of L-NMMA and ODQ alone, respectively. Data are presented as mean ± SD (n = 8) of percentage value of basal levels. *$P < 0.001$ *vs.* NMDA alone. #$P < 0.01$ *vs.* ODQ alone.

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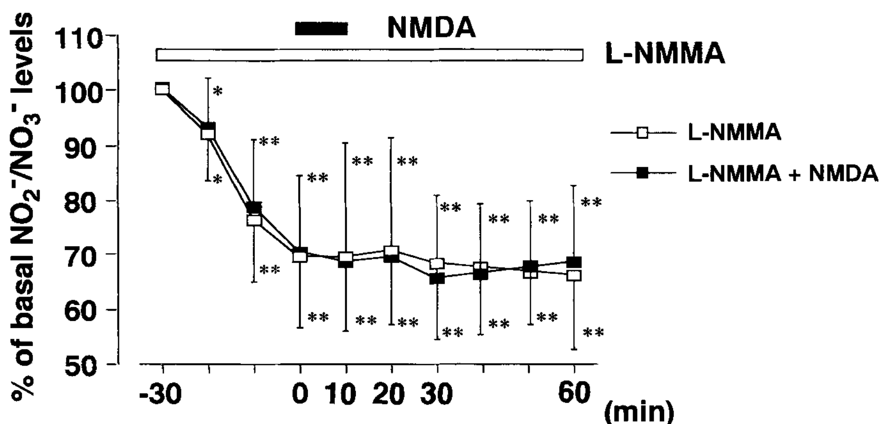

Fig. 6. Effect of N^G-monomethyl-L-arginine acetate (L-NMMA) on basal and *N*-methyl-D-aspartate (NMDA)-induced spinal release of NO_2^-/NO_3^-. Dialysates were collected as 10-min fractions. NMDA (1.0 mM) was perfused into the lumbar intrathecal space through a dialysis probe for 10 min (*solid bar*). L-NMMA (10 mM) was perfused into lumbar intrathecal space for 90 min (*open bar*). Thirty minutes after starting administration of L-NMMA, NMDA was coperfused with L-NMMA for 10 min. Basal levels were determined by averaging three consecutive samples immediately before infusion of L-NMMA. Data are presented as mean ± SD (n = 8) of percentage values of basal levels. *$P < 0.01$, **$P < 0.001$ *vs.* basal levels.

in a three-dimensional fashion through the extracellular fluid, including CSF.[25]

We examined the production and roles of NO *in vivo* by measuring NO_2^-/NO_3^- concentrations in the dialysate. NO_2^-/NO_3^- concentrations have been determined both *in vitro*[26] and *in vivo*[27,28] as a measure of NO production. Additionally, NO_2^-/NO_3^- concentration correlates closely with the activity of NOS in various brain regions.[29]

Activation of Spinal NMDA Receptors and NO/cGMP/Glutamate Release Cascade

Several lines of evidence have suggested that the activation of NMDA receptors evoked NO release from spinal neurons. In anesthetized rats prepared with a microdialysis probe transversing the dorsal spinal cord, capsaicin administered in peripheral tissue increased L-NAME–sensitive NO_2^- in perfusates,[30] and intrathecal administration of NMDA evoked L-NAME–sensitive spinal release of citrulline, a coproduction of NO synthesis.[31] Three isoforms of NOS—neuronal, endothelial and inducible NOSs—have been identified, and each of them has been cloned.[32] We did not determine which isoform of NOS was responsible for NMDA-induced NO release because L-NMMA is a nonselective inhibitor of NOS. Immunohistochemical studies demonstrated that high densities of neuronal NOS, which played an important role in nociceptive processing, were found in neuronal fibers and interneurons in superficial layers of the dorsal spinal cord.[5,6] In our results, although intrathecal perfusion of L-NMMA reduced NO_2^-/NO_3^- concentrations up to approximately 70% of the basal levels, AP-5 did not have any effects on basal NO_2^-/NO_3^- concentration, consistent with a previous report.[33] These imply that the tonic release of NO is mediated through a NMDA receptor–independent mechanism within the spinal cord. However, the role of the tonically released NO in spinal signal transmission is unclear.

Increasing evidence has supported the idea that NMDA-induced release of NO is involved in neuronal signal transmission. However, in the spinal cord, the role of NMDA-induced NO in the transmission of nociceptive signals has not been clear. Our results demonstrated that NMDA-induced NO produced cGMP *via* the activation of sGC, resulting in glutamate release. Because ODQ is a potent and selective sGC inhibitor and does not inhibit NOS activity or inactivate NO, unlike other sGC inhibitors such as methylene blue and LY83583,[34] our results

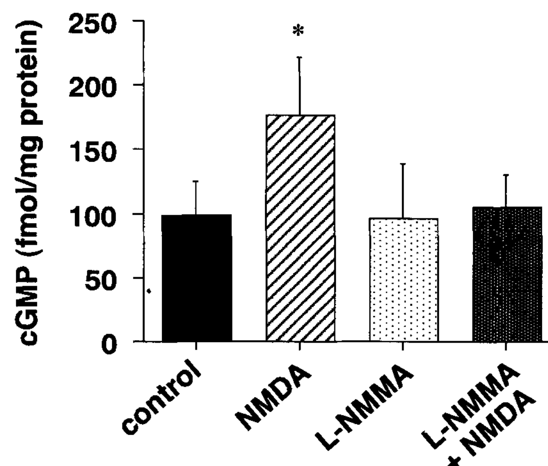

Fig. 7. Content of cyclic guanosine 3′,5′-monophosphate (cGMP) in the dorsal half of the lumbar spinal cord. Control = perfusion of artificial cerebrospinal fluid alone; NMDA = perfusion of *N*-methyl-D-aspartate (1.0 mM); L-NMMA = perfusion of N^G-monomethyl-L-arginine acetate (10 mM) for 40 min; L-NMMA + NMDA = L-NMMA (10 mM) for 40 min with coperfusion of NMDA (1.0 mM) for the last 10 min. Data are presented as mean ± SD (n = 6-8). *$P < 0.01$ *vs.* control.

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strongly indicate that the increase in cGMP caused glutamate release. In the present study, ODQ did not completely suppress the NMDA-induced increase in glutamate concentration because the solubility of ODQ limited the dose that could be administered. A higher dose of ODQ may show a greater inhibition of NMDA-induced glutamate release.

The mechanism underlying NO/cGMP-induced glutamate release in the spinal cord is unknown. An immunohistochemical study showed high densities of cGMP-dependent protein kinase I in laminae I and II of the spinal cord.[5] In addition, a recent study has demonstrated that spinal infusion of the cGMP-dependent protein kinase inhibitor blocks spinal release of glutamate after intradermal capsaicin administration.[35] Thus, it is possible that NO-induced cGMP evokes glutamate release *via* the activation of cGMP-dependent protein kinase in the spinal cord. These events are observed in the hippocampus.[20,21] In addition, recent evidence indicates that NO-induced cGMP directly activates a cyclic-nucleotide-gated channel, resulting in glutamate release in the retina.[36]

In the present study, the increase in glutamate and pain-related behavior preceded the increase in NO. Because NO_2^-/NO_3^- is a stable NO metabolite, it is possible that it would be cumulative in extracellular space. Therefore, we speculate that the cumulation of NO_2^-/NO_3^- in CSF would cause the delayed peak of increase in the concentration.

NO/cGMP/Glutamate Release Cascade in Spinal Nociceptive Signal Transmission

The present study showed that an NO/cGMP/glutamate release cascade is involved in NMDA-induced pain-related behavior. A NOS inhibitor completely suppressed the NMDA-induced increase in glutamate and NO releases, but this inhibitor did not completely block pain-related behavior. This residual pain-related behavior in the NOS or sGC inhibitor–treated rats may be caused by direct activation of not only spinothalamic tract neurons but also presynaptic NMDA receptors. Recent study has indicated that activation of presynaptic NMDA receptors located on terminals of small-diameter primary afferent fibers could evoke the internalization of the substance P receptor in the dorsal horn neurons, suggesting the release of substance P.[37] Previous studies have indicated that activation of the spinal NMDA receptors, NO and cGMP are involved in hyperalgesia observed in a rat model of neuropathic or inflammatory pain. In addition, it has been suggested that increased amounts of glutamate, which is blocked by pretreatment with an NMDA antagonist, are released within the dorsal horn of the spinal cord after peripheral inflammation[16-18] and peripheral nerve injury.[19] Thus, we suggest the possibility that the NO/cGMP/glutamate release cascade we demonstrated in this study plays a facilitated role in spinal nociceptive transmission in the pathologic pain states that the spinal NMDA receptors have activated.

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