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RESEARCH ARTICLE

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Effects of *N*-methyl-D-aspartate (NMDA) and non-NMDA receptor antagonists on excitation of the tooth-pulp-evoked C1 spinal neurons in the rat

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Abstract To examine whether there is a difference between the effects of iontophoretically applied *N*-methyl-D-aspartate (NMDA) and non-NMDA receptor antagonists on the activity of C1 spinal neurons with input from the tooth pulp, extracellular single recordings were performed in pentobarbital-anesthetized rats. The activity of 16 C1 spinal neurons and the amplitude of the digastric electromyogram increased proportionally by 1.0–3.5 times the threshold for jaw-opening reflex. After iontophoretic application (10, 30 and 50 nA, 5 min) of NMDA receptor blocker (5R,10S)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cycloheptene-5,10-imine hydrogen maleate or non-NMDA receptor blocker (6-cyano-7-nitroquinoxaline-2,3-dione), the mean number of spikes responding to the tooth pulp stimulation at ×3.5 threshold for jaw-opening reflex was significantly decreased. Under these conditions, there were no significant differences between the amplitudes of the dEMG before and after applications of both NMDA and non-NMDA receptor antagonists. These results suggest that the release of endogenous excitatory neurotransmitters is necessary for activation of C1 spinal neurons that are associated with the transmission of nociceptive information, and that both NMDA and non-NMDA receptors contribute to the mechanism of excitation of tooth-pulp-evoked C1 spinal neurons.

Key words C1 spinal neuron · Excitatory amino acid · NMDA · MK-801 · Non-NMDA · CNQX · Tooth pulp

Introduction

The trigeminal nerves transmit sensory information from the head and orofacial regions including the tooth pulp (TP). Previous studies have shown that horseradish peroxidase (HRP) applied to the rat trigeminal ganglion results in transport to both trigeminal nuclear complex and cervical dorsal horn (Jacquin et al. 1982; Pfaller and Arvindsson 1988; Ruggiero et al.1981). Using the expression of proto-oncogene c-*fos* which is triggered by external stimuli (Bullitt 1990; Dragunow and Faull 1989; Morgan and Curran 1989), after both noxious and non-noxious stimulation, c-*fos*-positive cells are found in the trigeminal spinal nucleus and upper cervical cord (Bereiter and Bereiter 1996; Coimbra and Coimbra 1994; Strassman and Vos 1993; Sugimoto et al. 1994). Notably, noxious thermal or mechanical stimulation of the dental pulp causes c-*fos* expression in the dorsomedial and ventrolateral regions of the C1–C2 segments (Coimbra and Coimbra 1994). However, no studies have evaluated the neurotransmitter responsible for the nociceptive transmission from the TP to C1 spinal neurons.

Excitatory amino acids such as glutamate and aspartate contribute to the processing of somatosensory information including nociception at the spinal level (Wilcox 1991). Iontophoretic application of glutamate receptor antagonist to subnucleus caudalis neurons reduces the response to intense mechanical, but not thermal, stimuli of facial skin (Salt and Hill 1983). Clements et al. (1991) have demonstrated that the primary afferent pathways projecting from the TP to the trigeminal nucleus caudalis are glutaminergic. Indeed, the highest densities of N-methyl-D-aspartate (NMDA) and non-NMDA receptors are found in the trigeminal spinal nucleus (Tallaksen-Greene et al. 1992). Bereiter and Bereiter (1996) have shown that in the acute trigeminal pain model produced by mustard oil application to the cornea in rats, pretreatment with either NMDA or non-NMDA receptor blockers induces a decrease in the number of c-*fos*-positive cells in the subnucleus caudalis/upper cervical cord transition. From these observations, it is possible that neurotransmission from the TP afferents to the C1 spinal neurons correlates with the activation of glutamate receptors, but there are no studies that have examined this possibility.

Since the jaw-opening reflex (JOR) induced by electrical stimulation of the TP is a valid index for reflex re-

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sponses to noxious stimuli (Mahan and Anderson 1970; Mason et al. 1985; Takeda et al. 1998b), the JOR threshold has been used as an indicator of the intensity of stimulus applied to TP. In the present study, therefore, the implication of glutamate receptor subtypes on the C1 spinal neurons responding to electrical stimulation of the TP was tested by iontophoretic applications of NMDA and non-NMDA receptor antagonists in anesthetized rats.

Materials and methods

Animal preparation

The experiments were performed on seven male Wistar rats (340–390 g). All experimental protocols used in this study were approved by the Animal Use and Care Committee of Nippon Dental University. Each animal was initially anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and anesthesia was maintained with additional doses of 2–3 mg/kg/h through a cannula in the jugular vein, as required. The trachea was cannulated. The rectal temperature was maintained at 37±0.5°C with a radiant heater. Arterial blood pressure was measured by means of a pressure transducer through a cannula inserted into the femoral artery. Adequacy of the anesthesia was determined by the lack of a response to pinching the paw.

Tooth pulp stimulation

Bipolar stimulating electrodes made from stainless steel wire (diameter 150 µm, enamel insulated except for the tip) were inserted into the pulp of the upper incisors and insulated from the surrounding tissue with dental cement to limit current spread, as described in a previous study (Takeda et al. 1998b).

Recording of single-unit and dEMG activities

The rats were then placed in a stereotaxic apparatus, and a laminectomy was performed to expose the C1 spinal region of the spinal cord. The single neuronal activity was recorded extracellularly from the C1 spinal neuron, by means of a four-barreled glass micropipette filled with 2% pontamine sky blue and 0.5 M sodium acetate. The neuronal activity was amplified (WPI, DAM 80), filtered (0.3–10 kHz) and monitored with an oscilloscope (Nihon Kohden, VC-10). By means of stainless steel electrodes (interpolar distance 2 mm, insulated except for tip), the digastric electromyogram (dEMG) was recorded from the ipsilateral anterior belly of the digastric muscle to record the JOR. Since this study was concerned with the effects of NMDA and non-NMDA receptor antagonists on the activity of TP-stimulated C1 spinal neurons (at nociceptive intensity), our attention was focused on the neurons responding to TP stimulation that show a proportional increase in the firing rate to the stimulation intensity (1.0–3.5 times threshold for dEMG).

Iontophoretic application of drugs

Of the three lateral barrels of the micropipette, one containing 160 mM NaCl was used for autonomic current balancing to prevent the occurrence of tip polarization artifacts. The remaining barrels contained the following solutions: (5R,10S)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclo-heptene-5,10-imine hydrogen maleate (MK-801; 15 mM in 160 mM NaCl, pH 8.0; RBI, USA) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 5 mM in 160 mM NaCl, pH 8.0, Tocris Cookson, UK). Ejection, retention and balancing currents were provided by a constant current unit (Dia Medical, DPI-25, Japan). All drugs were ejected with anionic currents of 10–50 nA. Retaining currents of 10–25 nA were used.

Experimental protocols and data analysis

Recordings of the C1 unit and the dEMG activity responding to TP stimulation and their data analysis were carried out in the following steps. The threshold for JOR was determined from the dEMG, that is, pulse duration was set at 0.1 ms and pulse intensity (at a stimulation frequency of 1 Hz) was increased until three of five consecutive dEMG responses to TP stimulation were obtained. The peak-to-peak amplitudes in five stimulus trials were averaged. Poststimulus histograms of C1 unit activity induced by TP stimulation (intensity of tooth pulp stimulation was set at 3.5 times the dEMG threshold) were constructed (16–32 sweeps, bin width 1 ms). After the control responses were obtained, NMDA and non-NMDA receptor antagonists were iontophoretically administered and the neuronal and dEMG activities responding to the TP stimulation were observed after 5–10 min. To determine ejection-current-related effects of NMDA and non-NMDA receptor antagonists, we tested TP-evoked unit activity after both MK-801 and CNQX applications at three current ejections (10, 30 and 50 nA, 5–10 min). Respective ejection current applications were made at intervals of 15–20 min. If the change from control activity was more than 20%, a given stimulus was considered effective. Concerning the effects of drug applications on the TP-evoked C1 spinal unit activity, the neuronal discharges were quantified by subtracting background discharges from the evoked activities. The conduction velocity for each neuron was calculated by dividing the distance between the site of TP and the C1 region by the latency between the stimulus artifact and evoked spikes. Latency values were corrected for a 0.5-ms synaptic delay. Latency was determined from the peak of the poststimulus histogram. The data were stored on magnetic tape for off-line analysis. Statistical significance was calculated by Duncan's new multiple-range test. A probability less than 0.01 was considered significant. Values were expressed as means±SEM.

Histology

At the end of recording sessions, rats were deeply anesthetized. Then cathodal DC currents (30 µA,·5 min) were passed through a recording micropipette. The animals were transcardially perfused with saline and 10% formalin. Frozen coronal sections were cut into 30-µm sections and stained with hematoxylin-eosin. Recording sites were identified from the blue spots, and construction of the electrode tracks was done by means of a combination with micromanipulator readings.

Results

Extracellular single-unit activity was recorded from 16 C1 spinal neurons responding to TP stimulation. NMDA receptor antagonist (MK-801) was applied iontophoretically to 11 neurons and non-NMDA receptor antagonist (CNQX) to 9 neurons. Iontophoresis of vehicle (160 mM, NaCl, pH 4.5) had no significant effect on the C1 spinal neuronal activity evoked by TP stimulation to increasing current intensities (10, 30 and 50 nA, 10 min, n=4).

dEMG and C1 spinal neuronal activities

Electrical stimulation of the TP induced reflex responses of digastric muscle at a latency of 5.87±0.55 ms (n=7). The mean threshold intensity was 0.87±0.17 mA (n=7). As shown in Fig. 1, these neurons were located in laminae I/II (n=7) and III/IV (n=9). Four out of 16 neurons

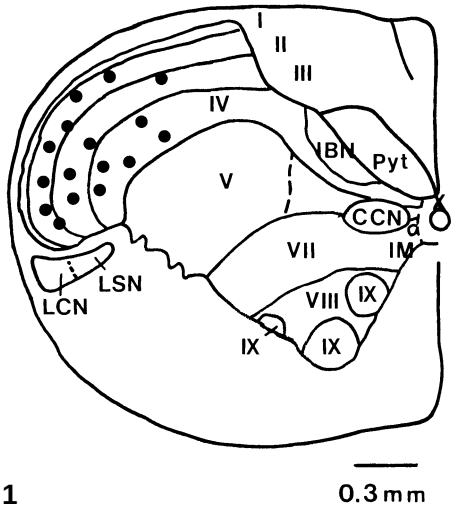

Fig. 1 Locations of recorded C1 spinal neurons (•) responding to ipsilateral TP stimulation (*I–X* laminae, *IBN* internal basilar nucleus, *Pyt* pyramidal tract, *CCN* central cervical nucleus, *IM* intermediolateral nucleus, *LSN* lateral spinal nucleus, *LCN* lateral cervical nucleus)

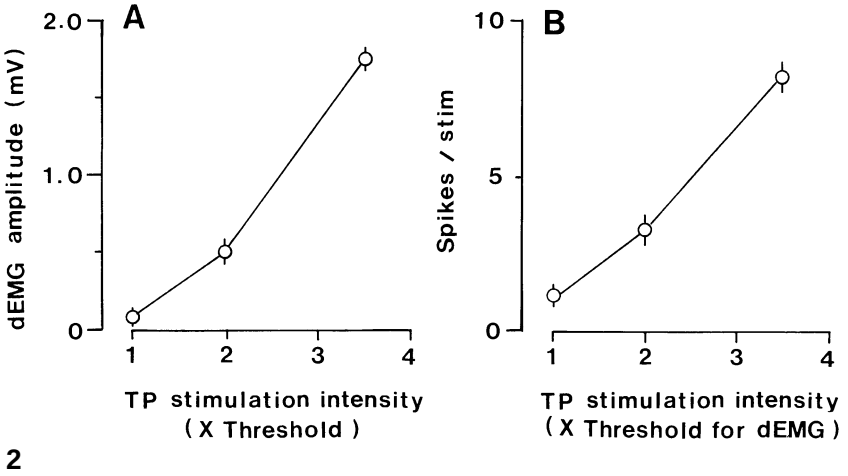

Fig. 2 Threshold-related responses of dEMG **(A)** and C1-spinal neurons **(B)** to TP stimulation. *Vertical bars* are means±SEM (**A** *n*=7, **B** *n*=16)

(25%) showed spontaneous activities (5–19 Hz). The mean latency of C1 spinal neurons (*n*=16) during TP stimulation was 10.9±0.79 ms. The mean threshold in 16 units was 0.55±0.03 mA and the value was lower than that of dEMG. Since the distance from the site of TP stimulation to the ipsilateral C1 segment was approximately 46 mm, the average conduction velocity was 4.56±0.33 m/s. The summarized results of the responses of dEMG and C1 spinal neuron activities to ipsilateral TP stimulation at 1.0–3.5 times the threshold for JOR are shown in Fig. 2. The activity of 16 C1 spinal neurons and the amplitude of dEMG increased proportionally during 1.0–3.5 times the threshold for JOR.

Effects of MK-801 iontophoretic application on the TP-evoked C1 spinal neuronal discharge

Typical examples of effects of NMDA receptor blocker administration are shown in Fig. 3A,B. After iontophoret-

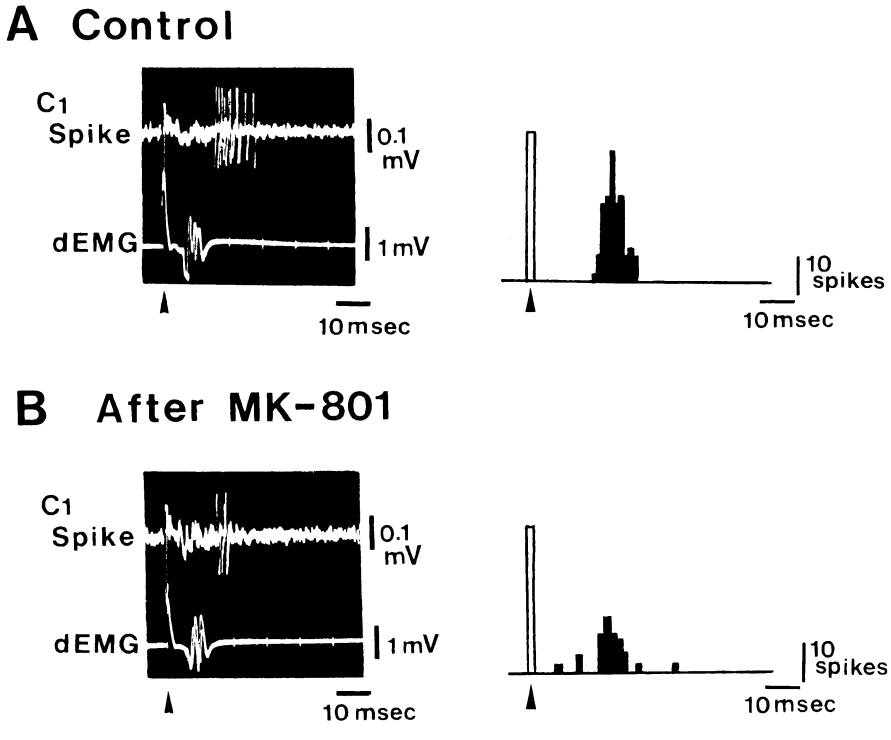

Fig. 3 Examples of effect before **(A)** and after **(B)** the iontophoretic application of NMDA antagonist (MK-801, 30 nA·5 min) on the C1 spinal neuronal and dEMG responses to TP stimulation. **A, B** Left side traces, unit response evoked by electrical stimulation of TP (stimulus intensity was ×3.5 threshold for evoking the dEMG, three superimposed traces). Poststimulus histogram for the same cell elicited by electrical stimulation (1-ms bin width, 16 sweeps). Timing of the TP stimulation is indicated by *filled triangles* (*open column* stimulus artifact, *solid column* recorded responses)

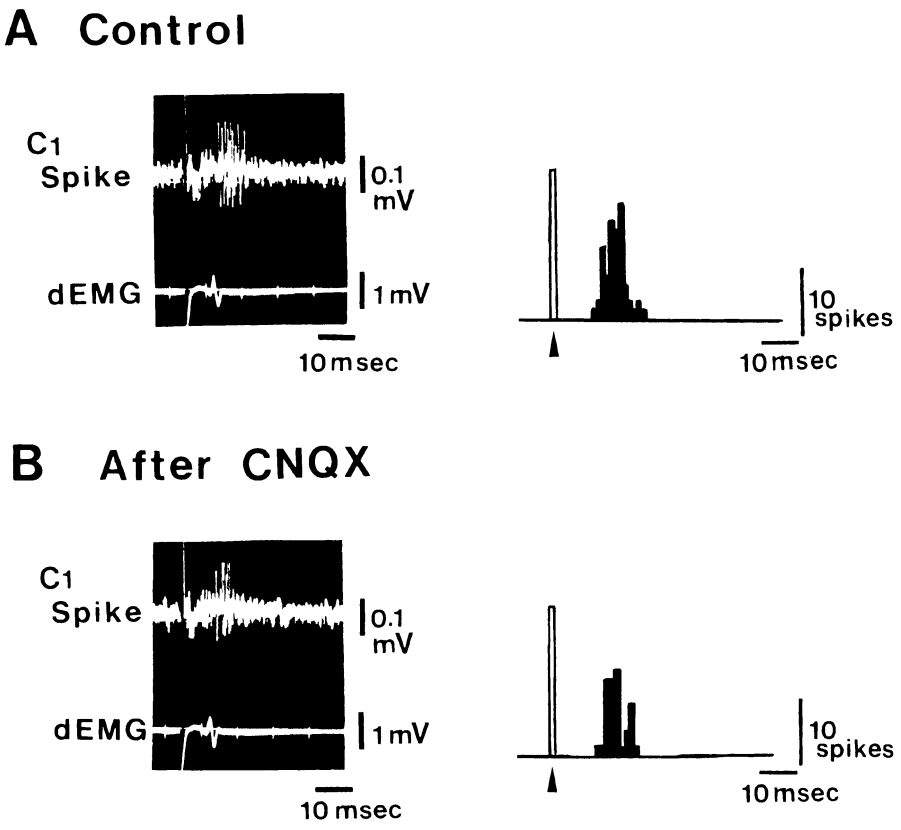

Fig. 4 Examples of effects before **(A)** and after **(B)** iontophoretic application of non-NMDA receptor antagonist (CNQX) on the C1 spinal neuronal and dEMG responses to TP stimulation. **A,B** Left side traces, unit responses evoked by electrical stimulation of TP (stimulus intensity was ×3.5 threshold for evoking the dEMG, three superimposed traces). Poststimulus histogram for the same cell elicited by electrical stimulation (1-ms bin width, 16 sweeps). Timing of TP stimulation is indicated by *filled triangles* (*open column* stimulus artifact, *solid column* recorded responses)

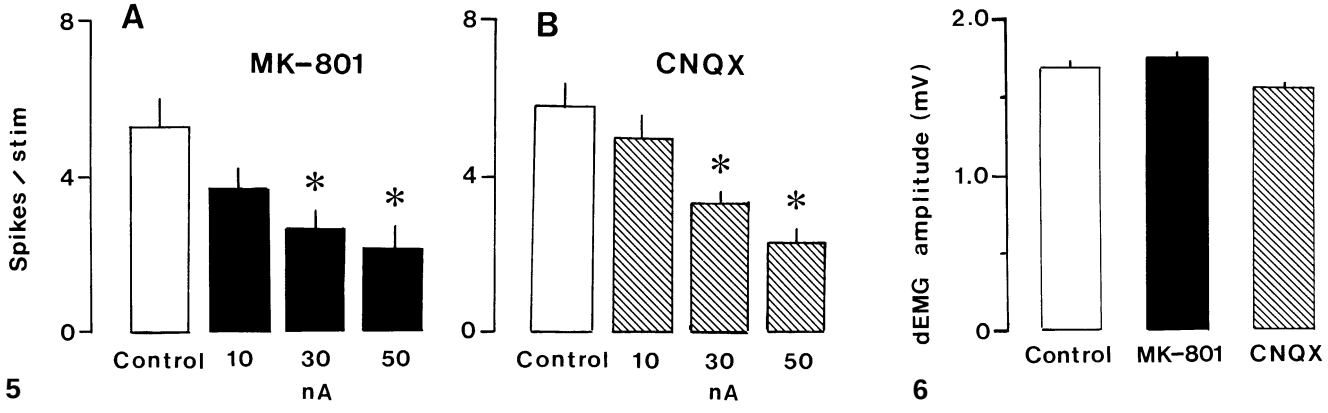

Fig. 5 Effects of iontophoretic application of NMDA (MK-801) **(A)** and non-NMDA (CNQX) **(B)** receptor antagonists on the mean spike numbers of C1 spinal neuron responding to TP stimulation. *Each column* represents the mean±SEM (n=5). *P<0.01

Fig. 6 Effects of iontophoretic application of MK-801 (30 nA·5 min) and CNQX (30 nA·5 min) on the amplitude of dEMG evoked by the TP stimulation. *Each column* represents the mean±SEM (control n=11, MK-801 n=11, CNQX n=9)

ic application of NMDA receptor blocker (30 nA·5 min), C1 spinal neurons decreased their discharge frequency evoked by TP stimulation (at 3.5 times the threshold for JOR). The summarized results of MK-801 effect are shown in Fig. 5A. Iontophoretic application of MK-801 induced a suppressive effect on the TP-evoked C1 spinal activities in a current-dependent manner (10–50 nA). After MK-801 application (30 and 50 nA), the mean TP-evoked neuronal activity was significantly decreased compared to that before application of MK-801 (P<0.01).

Effects of CNQX iontophoretic application on the TP-evoked C1 spinal neuronal discharge

Examples of effects of the non-NMDA receptor blocker (CNQX) application are shown in Fig. 4A,B. After application of CNQX (30 nA·5 min), C1 spinal neurons decreased their activity evoked by TP stimulation (at 3.5 times the threshold for dEMG). The results of CNQX effect are summarized in Fig. 5B. Pretreatment with CNQX induced suppressive effects in a current-dependent manner (10–50 nA). After CNQX application (30 and 50 nA),

firing discharges were significantly decreased compared to those before application of CNQX ($P<0.01$).

Effects of MK-801 and CNQX iontophoretic application on the amplitude of dEMG

As shown in Fig. 6, there were no significant differences in dEMG amplitude after iontophoretic applications of MK-801 and CNQX.

Discussion

The present series of experiments showed that release of endogenous excitatory neurotransmitters is necessary for activation of C1 spinal neuronal activity that is associated with the transmission of nociception, and that both NMDA and non-NMDA receptors contribute to excitation of the C1 spinal neuron activity during TP stimulation.

Validation of the microiontophoresis method

The iontophoretic application method has some disadvantages, since the drugs applied have little dose dependency. In the present study, however, we obtained evidence that application of NMDA or non-NMDA receptor antagonists decreased the firing rates of C1 spinal neuronal activity evoked by TP stimulation in a current-dependent manner (10–50 nA). We also found no significant differences in the dEMG amplitude before and after the iontophoretic applications of NMDA and non-NMDA antagonists. Thus, the results support the validity of the iontophoretic application method.

TP stimulation and nociception

Recently, Dong et al. (1993) have reported that non-noxious mechanical stimulation of the TP activated intrapulpal Aβ fibers. Low-threshold mechanoreceptors are known to be innervated by myelinated Aβ fibers in the cutaneous tissues which convey non-noxious peripheral information to the central nervous system (Freeman and Johanson 1982a, 1982b). These observations raise the possibility that electrical stimulation of the TP at a lower threshold activates the intrapulpal Aβ fibers conveying non-pain sensation to the central nervous system. The TP-evoked JOR has been considered to be a valid model of pain if it is evoked by adequate TP stimulation (e.g., with 3–5 times the threshold of the JOR, as this threshold is very close to the sensory threshold in humans) (Mason et al. 1985). The simultaneous recordings with dEMG were useful to assess the responses of C1 spinal neuron activity to a given sensory threshold of TP stimulation. In the present study, we found that: (1) the number of spikes of these neurons increased proportionally as the stimulus intensity above threshold of JOR was increased and (2), based on the calculated afferent conduction velocity, most C1 neurons received inputs from Aδ- and C-fibers. The results of this study are in agreement with the observations that dorsal horn neurons in the C1 segment of the rat spinal cord play a significant role in nociceptive transmission (Angus-Leppan 1994; Bereiter and Bereiter 1996; Razook et al. 1995; Takeda et al.1998a; Matsumoto et al. 1999). This is further confirmed by a recent report by Iwata et al. (1998) showing that the rostrocaudal distribution of c-*fos* expression cells in the first cervical segment of the cat spinal cord becomes more prominent by increasing the stimulus intensities of TP stimulation as well as by applying mustard oil into the TP.

Possible functional significance of the NMDA and non-NMDA receptors in C1 neurotransmission

Activation of excitatory amino acid receptors has been implicated in the mediation of central sensitization that leads to pain-related behavior manifested in the rat model of neuropathy (Davar et al. 1991; Mao et al. 1992). The NMDA receptor antagonist exhibits an antinociceptive effect in animal models of pain (Leem et al. 1996). For this reason, iontophoretic application of NMDA receptor antagonists preferentially suppresses the enhanced pinch-evoked response in peripheral nerve-injured rats, but non-NMDA receptor antagonists dominantly attenuate the enhanced brush-evoked response. The fact that the activation of C1 neurons responding to TP stimulation was mediated by excitatory amino acid acting at both NMDA and non-NMDA receptors agrees with the observation that pretreatment with either NMDA or non-NMDA receptor blockers induces a decrease in the number of c-*fos*-positive cells in the trigeminal subnucleus caudalis/upper cervical cord transition (Bereiter and Bereiter 1996). In the normal rat the majority of excitatory amino acid (EAA)-sensitive spinal neurons are also sensitive to both NMDA and non-NMDA receptor antagonists (Aanonsen et al. 1990), suggesting the coexistence of two subtypes of EAA receptors on spinal neurons under normal conditions. The NMDA receptors have an influence on polysynaptic input in the spinal dorsal interneurons (Davies and Watkins 1983), and many spinal dorsal interneurons are known to be glutaminergic (Todd et al. 1994). These observations are consistent with the report demonstrating that the widespread glutamate receptors are involved in nociception (Bereiter and Bereiter 1996). Thus, it is possible to explain the significant effect of both NMDA and non-NMDA receptor antagonisms on the TP-evoked C1 neuron activity.

Furthermore, it has been demonstrated that substance P (SP) and glutamate coexist in small dorsal root ganglion neurons as well as in small primary afferent terminals of the superficial dorsal horn (Battaglia and Rustioni 1988; Debiasi and Rustioni 1998). Indeed, there is an interaction between SP and glutamate receptors in processing spinal nociception (Song and Zhao 1994). Recent evidence has shown that presynaptic NMDA receptors lo-

cated on the terminals of small-diameter fibers facilitate and prolong the transmission of nociceptive messages through the release of both SP and glutamate (Liu et al. 1997). Since blockade of NK1 receptors is known to raise the threshold for JOR in the guinea-pig (Alia et al. 1998), it is possible that SP is involved in the neuronal transmission from the TP afferents to the C1 spinal neurons. However, we did not examine whether there is an interaction between NK1 and glutamate receptors. Further studies are needed to elucidate this possibility.

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