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Participation of NMDA and non-NMDA glutamate receptors in the formalin-induced inflammatory temporomandibular joint nociception

Gwi Y Yang, Ju H Lee, and Dong K. Ahn*

Department of Oral Physiology, School of Dentistry, Kyungpook National University, Daegu, Korea

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It has been well known that excitatory amino acids, primarily glutamate, are involved in the transmission of nociception in pathological and physiological conditions in the spinal and brainstem level. Recently, peripheral glutamate also play a critical role in the peripheral nociceptive transmissions. The present study investigated the role of N-methyl-D-aspartic acid (NMDA) or non-NMDA ionotropic glutamate receptors in formalin-induced TMJ pain. Experiments were carried out on male Sprague-Dawley rats weighing 220-280 g. Intra-articular injection was performed under halothane anesthesia. Under anesthesia, AP-7 (10, 100 μM, 1 mM/20 μL), a NMDA receptor antagonist, or CNQX disodium salt (0.5, 5, 50, 500 μM/20 μL), a non-NMDA receptor antagonist, were administered intra-articularly 10 min prior to the application of 5% formalin. For each animal, the number of behavioral responses, such as rubbing and/or scratching the TMJ region, was recorded for nine successive 5-min intervals. Intra-articular pretreatment with 1 mM of AP-7 or 50 μM CNQX significantly decreased the formalin-induced scratching behavioral responses during the second phase. Intra-articular pretreatment with 500 μM of CNQX significantly decreased the formalin-induced scratching behavior during both the first and the second phase. These results indicate that the intra-articular administration of NMDA or non-NMDA receptor antagonists inhibit formalin-induced TMJ nociception, and peripheral ionotropic glutamate receptors may play an important role in the TMJ nociception.

*Corresponding author: Dong-Kuk, Ahn Department of Oral Physiology, School of Dentistry, Kyungpook National University, 188-1 Sam Deok 2ga, Chung-gu, Daegu (700-412), Korea Tel: +82-53-660-6840; Fax: +82-53-421-4077; email: dkahn@knu.ac.kr

1. Introduction

It has been well known that excitatory amino acids, primarily glutamate, are involved in the transmission of nociception in pathological (Seltzer *et al.*, 1991) and physiological conditions (deGroot *et al.*, 2000; Gerber *et al.*, 1991) in the spinal level. Excitatory amino acids also contribute to the development of central sensitization and persistent nociception (Coderre and Melzack, 1992). Like the spinal cord level, glutamate plays an important role in pain transmission in the brain stem level that innervates the orofacial area (Clements *at al.*, 1991; Watkins and Evans, 1981). Recent studies indicate that glutamate receptors play a role in the peripheral nociceptive transmissions. Glutamate receptors were expressed in peripheral ends of small diameter primary afferents (Coggeshall and Carlton, 1998; Carlton *et al.*, 1995). Intra-articular injection of carrageenan or a subcutaneous injection of formalin produced inflammatory pain resulting from the released glutamate in the peripheral sites in rats (Omote *et al.*, 1998; Lawand *et al.*, 1997). Subcutaneous injection of glutamate also produced dose-related pain behavior as well as edema formation in the paw of mouse (Beirith *et al.*, 2002). Furthermore, intra-muscular administration of glutamate excited and sensitized the masseter muscle afferent fibers through activation of the peripheral excitatory amino acid receptors in anesthetized rats (Cairns *et al.*, 2002a). These results suggest that the peripheral excitatory amino acid receptors play an important role in the nociceptive processing of peripheral tissues.

TMJ pain is one of the chief complaints of patients with temporomandibular disorder. Curiously, despite the rise of cultural studies as an academic discipline, few have attempted to address TMJ nociception. An injury to the TMJ that produced inflammatory changes in the TMJ and its surrounding tissues resulted in TMJ pain. Several previous

data indicate involvement of peripheral glutamate receptors in the orofacial pain transmission. Intra-venous injection of MK801, a NMDA receptor antagonist, attenuated electromyographic responses of the masseter muscle evoked by intra-muscular injection of mustard oil (Yu *et al*., 1996). Glutamate or capsaicin injected into the rat's TMJ also produced increases in digastric and masseteric electromyographic activity and the electromyographic activity was significantly attenuated by the pretreatment with NMDA receptor antagonists (Lam *et al*., 2005; Cairns *et al*., 2002a). Although there was participation of the peripheral glutamate receptors in the orofacial nociceptive processing, the role of NMDA or non-NMDA receptors in the formalin-induced inflammatory TMJ nociception remains unclear.

The aim of the present study is to evaluate the role of peripheral NMDA and non-NMDA glutamate receptors in the formalin-induced inflammatory TMJ nociception. For this purpose, an intra-articular injection of formalin into the TMJ-induced behavior was examined after the intra-articular administration of a NMDA or a non-NMDA receptor antagonist.

2. Materials and Methods

2.1. Animals

All procedures involving the use of animals were approved by the Institutional Care and Use Committee of the School of Dentistry, Kyungpook National University and were carried out in accordance with the ethical guidelines for the investigation of experimental pain in conscious animals of the International Association for the Study of Pain. Experiments were carried out on 120 male Sprague-Dawley rats weighing 220-280 g. Animals were maintained under a constant temperature ($23 \pm 1^{\circ}C$) and lighting conditions with a 12 hr light/dark cycle. Food and water were freely available. All behavioral responses were measured by an experimenter who was blind to the treatment group in each experiment.

2.2. Intra-articular injection of formalin into the TMJ

Each animal was placed in a Plexiglas box for a 30 min habituation period to minimize stress before the TMJ injection. Rats were not allowed access to food or water during the test. After the period of adaptation, each animal was anesthetized by an inhalation of 5% of halothane for the TMJ injection. Under anesthesia, a 30-gauge needle was introduced into the joint capsule of the left TMJ, as previously described (Ahn *et al*., 2005; Choi *et al*., 2005; Roveroni *et al*., 2001). Intra-articular injection of 5% formalin (50 μL) was made into the TMJ region. Animals usually recovered from anesthesia within 2 to 3 minutes after the TMJ injection. The number of noxious scratching behavior such as grooming, rubbing, and/or scratching the injected TMJ was recorded for 9 successive 5-min intervals (Ahn *et al*., 2005; Choi *et al*., 2005; Roveroni *et al*., 2001). To confirm the behavior resulted from the effect of the formalin injected into the outside of the TMJ regions, off-site injections were performed. The same volume of formalin was injected into the left masseter muscle. As a control, saline was injected into the TMJ region. The orofacial formalin-induced responses showed two distinct phases (Choi *et al*,. 2003a, b, c; Clavelou *et al*., 1995, 1989) that were separated by a time of relative inactivity with an early short-lasting response (0-10 min, 1st phase) and a continuous prolonged response (11-45 min, 2nd phase). We analyzed the total number of scratches in both the first and second phases as indices of TMJ nociception after the formalin injection (Ahn *et al*., 2005; Choi *et al*., 2005; Roveroni *et al*., 2001).

2.3. Intra-articular pretreatment with NMDA or non-NMDA receptor antagonists

To investigate the effects of NMDA or non-NMDA receptor antagonist on formalin-induced TMJ nociception, DL-2-Amino-7-phosphono-heptanoic acid (AP-7), a NMD A receptor antagonist, or 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX disodium salt), a non-NMDA receptor antagonist, was administered through a 30-gauge needle in the anesthetized rats. AP-7 and CNQX were dissolved in 0.9% sterile saline. After AP-7 (10, 100 μM, 1 mM/20 μL) or CNQX (0.5, 5, 50, 500 μM/20 μL) was administered intra-articularly 10 min prior to the application of formalin, we monitored the total number of scratches in both the first and second phases as indices of TMJ nociception. AP-7 was obtained from Sigma and CNQX disodium salt was obtained from Tocris-Cookson.

2.4. Verification of inflammation

To investigate whether the TMJ injection was restricted to the TMJ region, a plasma extravasation of Evans' blue dye evoked by the formalin injection was measured, as previously described (Choi *et al*., 2005; Cairns *et al*., 1998; Haas *et al*., 1992; Harada *et al*., 1971). At the conclusion of each experiment, the animals were anesthetized with pentobarbital sodium (40 mg/kg, ip) and Evans' blue dye (0.1%, 5 mg/kg) was injected into the right femoral vein. Ten minutes after the injection of Evans' blue dye, each rat was perfused through the heart with heparinized normal saline. Joint tissues were dissected from the left side, weighed and stored at $-20^{\circ}C$ until analyzed. The tissues were incubated overnight in a 7 : 3 mixture of acetone and 0.5% sodium sulphate solution at room temperature with intermittent shaking. After incubation, the samples were centrifuged at 300 rpm for 10 min and the supernatant was separated. The samples were analyzed for the amount of dye present by measuring absorbance at 620 nm spectrophotometrically. The recovery of the extravasated dye per gram weight of tissue (μg/g) was calculated by comparing the absorbance of the supernatant with a standard curve. The

standard curve was generated from a series of the same extraction solution mixed with the known amounts of Evans' blue dye.

2.5. Statistical analysis

Statistical analysis of the behavioral data was carried out with a one-was analysis of variance (ANOVA) followed by a Bonferroni post-hoc analysis. Comparisons between the two means were performed by a Student's T-test. In all statistical comparisons, $p < 0.05$ was used as the criterion for statistical significance. All data are presented as mean ± SEM.

3. Results

Evans' blue dye (0.1%, 5 mg/kg) was injected systemically to determine the extent of the spread of inflammation induced by the injection of formalin in the TMJ. Fig. 1 illustrates the average of the extravasated Evans' blue dye concentration in the tissues obtained from the TMJ treated by the formalin or saline. The extravasated concentration of Evans' blue dye was 3.8 ± 0.8 μg in the saline-treated group. Intra-articular injection of formalin significantly inc- reased the extravasated concentration of Evans' blue dye (15.7 ± 3.0 μg $P < 0.05$) as compared with the vehicle (saline) -treated group. However, the intra-muscular or contralateral TMJ injection of formalin did not affect the extravasated concentration of Evans' blue dye, as compared with the vehicle-treated group. Intra-articular injection of 50 μL of 5% formalin significantly produced noxious scratching behavioral responses, which lasted for 40 min (Fig. 2). An intra-articular injection of 50 μL of formalin significantly increased the responses to total of 26 ± 7 scratches and 118 ± 14 scratches in the first (0-10 min) and second phase (11-45 min), respectively, as compared with the vehicle-treated group.

We examined the effects of the intra-articular pretreatment with NMDA or non-NMDA receptor antagonists on the behavioral responses produced by the formalin injection. Fig. 3 illustrates the effects of the intra-articular administration of AP-7, a selective NMDA receptor antagonist, on the

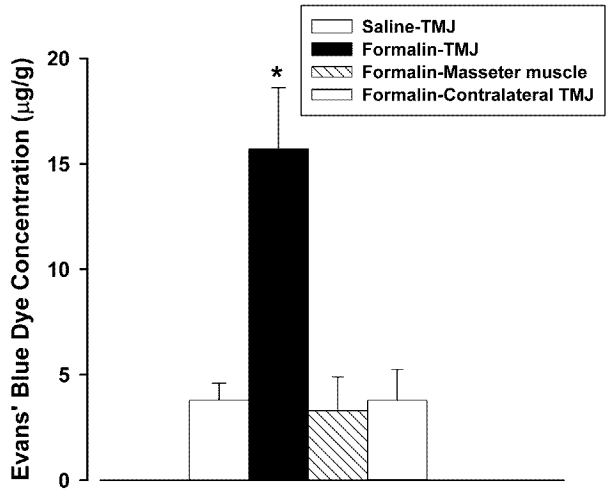

Fig. 1. Spectrophotometric measurement (620 nm) of plasma protein extravasation using Evans' blue dye bound to protein in the TMJ. Formalin-induced TMJ inflammation was indicated by plasma extravasation. Saline-TMJ; saline injected into TMJ, Formalin-TMJ; formalin injected into TMJ, Formalin-masseter muscle; formalin injected into masseter muscle, Formalin-contralateral TMJ; dye concentration of contralateral TMJ. *P < 0.05, Saline- vs. Formalin-treated group.

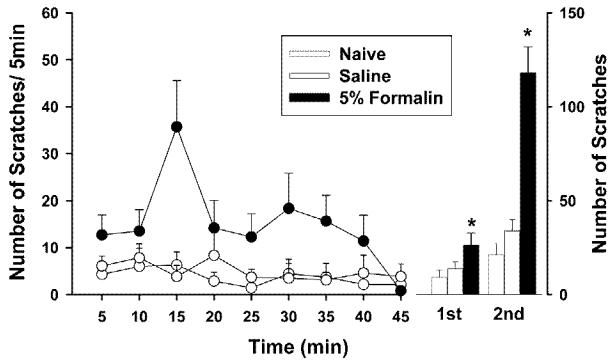

Fig 2. Microinjection of 50 μL of 5% formalin into the TMJ significantly produced noxious scratching behavioral responses. Left: Time course of the formalin-induced behavioral responses. The number of scratching behavioral responses was measured for nine successive 5-min intervals. Right: Formalin-induced scratching behavioral responses exhibit two phases with an early short lasting response (1st phase; 0-10 min) and a continuous prolonged response (2nd phase; 11-45 min). There were 8 animals in each group. *P < 0.05, Saline- vs. Formalin-treated group.

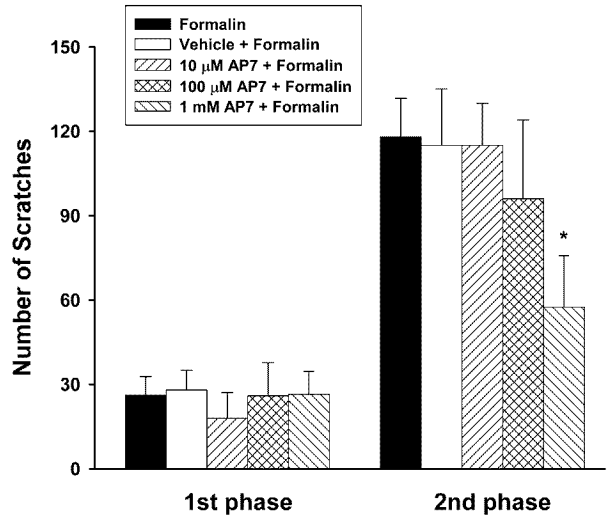

Fig. 3. Effects of AP-7, a selective NMDA receptor antagonist, on the formalin-induced behavioral responses in TMJ. Intra-articular injection of 1 mM AP-7 decreased the number of scratches produced by the formalin injection in the 2nd phase. There were 8 animals in each group. *P < 0.05, Vehicle- vs. AP7-treated group.

number of scratches produced by the formalin. The vehicle (saline) did not affect the formalin-induced nociceptive behavior. Neither the intra-articular administration of 10 μM nor 100 μM of AP-7 affected the formalin-induced scratching behavior. However, 1 mM of AP-7 injected intra-articularly significantly decreased the number of scratches by 52% (58 ± 18 in the number scratches, $p < 0.05$) in the second phase produced by the formalin injection, as compared with the vehicle-treated group.

The effects of CNQX, a non-NMDA receptor antagonist, injected intra-articularly on the formalin-induced TMJ nociception are illustrated in Fig. 4. Neither vehicle nor 0.5 μM or 5 μM of CNQX affect the formalin-induced nociceptive scratching behavior. However, the intra-articular injection of 50 μM CNQX significantly inhibited the number of scratches by 64% (43 ± 10 in the number scratches, $p < 0.05$) in the second phase produced by the formalin injection, as compared with the vehicle-treated group. A high dose of CNQX (500 μM) significantly decreased the number of scratches by 75% and 63% (7 ± 5 and 39 ± 15 in the number of scratches, $p < 0.05$) in both the 1st and 2nd phases, respectively. Neither the intra-articular injection of 1 mM of AP-7 nor 50 μM of CNQX produced any significant scratching behavioral responses in the intra-articular administration of the saline-treated animals, as compared with the vehicle-treated group (Fig. 5).

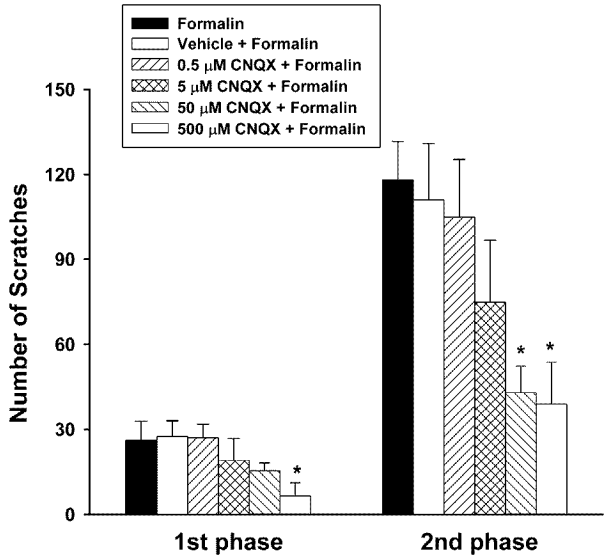

Fig. 4. Effects of CNQX, a potent non-NMDA receptor antagonist, on the formalin-induced behavioral responses in TMJ. Intra-articular injection of 50 μM CNQX decreased the number of scratches produced by the formalin injection in the 2nd phase. Pretreatment with 500 μM CNQX decreased the number of scratches produced by the formalin injection in the 1st and 2nd phase. There were 8 animals in each group. *P < 0.05, Vehicle- vs. CNQX-treated group

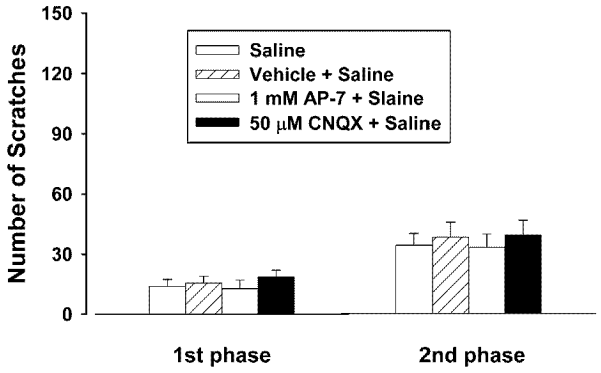

Fig. 5. The number of scratching behavioral responses produced by the intra-articular administration of saline, vehicle, AP-7 or CNQX. Intra-articular administration of vehicle, AP-7 or CNQX did not produce significant scratching behavioral responses compared with the saline-treated group. There were 8 animals in each group.

4. Discussion

The present study demonstrated that the intra-articular administration of NMDA or non-NMDA ionotropic glutamate receptor antagonists decreased the formalin-induced scratching behavior in the TMJ. Glutamate is a major excitatory amino acid neurotransmitter in the central nervous system (CNS). Glutamate produces its action by acting on N-methyl-D-aspartic acid (NMDA), a non-NMDA ionotropic [± α-amino-3-hydroxy-5-methylisoxazole - 4-propionic acid (AMPA)/kainate], and metabotropic receptors (Watkins *et al.*, 1990). AMPA receptors mediate the majority of rapid excitatory synaptic transmission in the CNS. Recent studies have indicated that the activity and synaptic distribution of these receptors is dynamically regulated and could be crucial for the short- and long-term modification of synaptic efficacy. It is well known kainate receptor play an important role in the synapse of hippocampal mossy fiber (Castillo *et al.*, 1997; Vignes and Collingridge, 1997) and this class of glutamate receptors is present at both pre- and postsynaptic neurons. Pre- and post-synaptic receptors can regulate transmission at many synapses by short-and long-term plastic phenomena (Lerma, 2003). Moreover, the number of AMPA receptors expressed at synapses can be rapidly modulated by long-and short-term changes in synaptic activity (Song and Huganir, 2002). In contrast, NMDA receptor mechanisms more prolonged excitatory post-synaptic potential mediation (Monaghan *et al.*, 1989) and play an important role for the induction of synaptic plasticity and several neuropsychiatric disorders (Dingledine *et al.*, 1999; Hollmann and Heinemann, 1994).

Several previous studies provide the evidence for the participation of glutamate receptors in the nociceptive signaling and central sensitization in chronic pain conditions in the spinal cord level (Dickenson *et al.*, 1997; Coderre *et al.*, 1993). Nociceptive stimulation produced the release of glutamate from presynaptic terminals in the spinal cord, suggesting that released glutamate is involved in pain

processing (Yoshimura and Yonehara, 2006). Administration of glutamate receptor antagonists into the spinal cord attenuated behavioral signs of neuropathic pain (Fisher *et al*., 2002; Mao *et al*., 1993).

Ionotropic glutamate receptor subtypes, NMDA and non-NMDA receptors, have been found to exist not only in the central nervous system but also in the peripheral nervous system (Shigemoto *et al*., 1992; Monaghan *et al*., 1989). The present study demonstrated that both NMDA and non-NMDA ionotropic glutamate receptor antagonists blocked the formalin-induced TMJ nociception in the peripheral sites. Peripheral ionotropic glutamate receptors in pain conditions have been studied in the previous several studies. Administration of formalin produced the release of glutamate and aspartate in the peripheral sites, which contribute to nociception and inflammatory pain (Omote *et al*., 1998). Intra-plantar injection of NMDA, AMPA or kainate produced mechanical allodynia and mechanical hyperalgesia (Zhou *et al*., 1996). Moreover, intra-plantar injection of MK-801, a NMDA receptor antagonist, delayed the onset time of mechanical hyperalgesia in rats with a lumbar 5 spinal nerve lesion (Jang *et al*., 2004). Both NMDA and non-NMDA receptor antagonists injected into the inflamed paws significantly reduced the thermal hyperalgesic response in the carrageenan-treated rats (Jackson *et al*., 1995). These studies suggest that peripheral NMDA or non-NMDA receptors mediate the production of nociception and hyperalgesia. In the present study, we investigated the participation of peripheral NMDA or non-NMDA receptors in the intra-articular administration of formalin-induced TMJ nociception. Intra-articular administration of AP-7, a NMDA receptor antagonist, or CNQX disodium salt, a non-NMDA receptor antagonist, attenuated the formalin-induced scratching behavior. These results indicate that both peripheral NMDA and non-NMDA glutamate receptors play an important role in modulation of formalin-induced TMJ nociception.

Participation of peripheral ionotropic glutamate receptors was further supported by a previous study. Intra-articular injection of either a NMDA or a non-NMDA glutamate receptor antagonist attenuated the thermal hyperalgesia and the mechanical allodynia produced by the injection of glutamate or by the injection of a mixture of kaolin and carrageenan into the joint of rats (Lawand *et al*., 1997). However, a non-NMDA receptor played a pivotal role in mediating the peripheral inflammation of related pain, although both NMDA and non-NMDA antagonists injected subcutaneously into the receptor fields inhibited C fiber-evoked responses of dorsal horn neurons (Wang *et al*., 2000). Moreover, pretreatment with 6,7-dinitroquinoxaline-2,3-dione, a non-NMDA receptor antagonist, abolished the decrease in the threshold of air puffs, while pretreatment with dl-2-amino-5-phosphonvaleric acid, an N-methyl-d-aspartic acid (NMDA) receptor antagonist, did not affect IL-1beta-induced mechanical allodynia (Ahn *et al*., 2004). On the other hand, the role of a peripheral NMDA receptor in the transduction of nociceptive processing has been demonstrated. Intra-muscular administration of NMDA increased the discharges of afferent nerve that innervates the masseter muscle in rats (Cairns *et al*., 2003). Blockade of the peripheral NMDA receptors effectively inhibited the intra-muscular injection of MO-induced nociceptive responses as well as muscle edema (Ro, 2003). Therefore, the underlying mechanisms of exact role of peripheral ionotropic glutamate receptors in TMJ nociception need to be explored further.

Although TMJ nociception is an important clinical entity, it is poorly understood. This is caused by the lack of an appropriate animal model available to study TMJ pain. In order to establish an animal model, inflammatory inducing agents, such as complete Freund's adjuvant (Iwata *et al*., 1999; Zhou *et al*., 1999) or mustard oil (Cairns *et al*., 2002b), were injected into TMJ. Capsaicin injected into the rat's TMJ also evoked an increase in its jaw muscle electromyographic activity and produced an inflammatory response (Tang *et al*., 2004). The present study used a formalin-induced TMJ behavior in rats for the in evaluation of the TMJ nociception as previous studies (Choi *et al*., 2005; Roveroni *et al*., 2001). The present study demonstrated that microinjection of 5% formalin into the TMJ region produced nociceptive scratching behavioral responses. Formalin-induced TMJ inflammation was verified and quantified by measuring tissue Evans' blue extravasation. Evans' blue dye binds to plasma proteins normally contained within the vessels, but released with increased vascular permeability. Intra-articular administration of formalin significantly increased extravasated Evans' blue dye concentration compared with the saline-treated group. When the formalin was injected into the outside of the TMJ region or the right masseter muscle, it did not increase the extravasated Evans' blue dye concentration in the TMJ. Increases in extravasation with the Evans' blue dye concentration confirm that the intra-articular injection of formalin led to inflammation of the TMJ region that was not mimicked by injections outside the TMJ region.

Generally, behavioral responses produced by the formalin injection exhibited two phases separated by a period of relative inactivity with an early short-lasting response and a continuous prolonged response. However, injection of formalin into the TMJ region did not show two distinct phases because the early phase was apparently masked by the anesthesia that allowed the TMJ injections in the previous study (Ahn *et al*., 2005; 2007). We also used anesthetic induction for the TMJ injections becauses it is practically impossible and ethically unacceptable without anesthetic induction (Choi *et al*., 2005; Roveroni *et al*., 2001). We analyzed the total number of formalin-induced behavioral responses in the first and the second phases in the present study. Although we analyzed the total number of scratches in the first phase, the results are very limited to explain TMJ nociception. Participation of peripheral glutamate receptors has been introduced in previous study. Intra-

articular pretreatment with NMDA receptor antagonist, ketamine or memantine, prevented arthritic pain-related behavior induced by carrageenan injection into the knee joint (Zhang *et al.*, 2004). The arthritis that produced increases in release of endogenous glutamate in the knee joint resulted in activation of glutamate receptors located on primary afferent axons in the knee joint (Lawand *et al.*, 2000; Zhang *et al*, 2004). Pre-injection of NMDA receptor antagonists into the TMJ attenuated capsaicin-evoked digastric and masseter EMG activity (Lam et al., 2005) and pretreatment with CNQX, a non-NMDA receptor antagonist, abolished the IL-1β-induced mechanical allodynia in the orofacial area of rats (Ahn et al., 2004). The present study demonstrated that intra-articular injection of NMDA or non-NMDA receptor antagonist is effective in formalin-induced TMJ pain. These finding, taken together the previous studies, suggested that the activation of peripheral glutamate receptors play an important role in the transmission of nociceptive trigeminal responses including TMJ pain.

In summary, intra-articular injection of 5% formalin produced nociceptive pain behavior. Intra-articular administration of AP-7, a NMDA receptor antagonist, or CNQX, a non-NMDA receptor antagonist, inhibited formalin-induced TMJ behavior. These results suggest that peripheral ionotropic glutamate receptors play an important role in the nociceptive process associated with TMD and that NMDA or non-NMDA receptor antagonists may be of therapeutic values for the treatment of inflammatory pain in the TMJ.

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