Involvement of the NMDA-Nitric Oxide Pathway in the Development of Hypersensitivity to Tactile Stimulation in Dental Injured Rats

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ABSTRACT—To investigate mechanisms in pathological pain conditions as the hyperalgesia and allodynia observed after dental surgery, we employed a rat dental-injury model involving the simultaneous pulpectomy to a lower incisor and extraction of an ipsilateral upper incisor. We found that hypersensitivity to tactile stimulation developed on both ipsilateral and contralateral sides in the dental-injured rats 5 days after the surgery and that this lasted for at least 30 days. Recovery from hypersensitivity to tactile stimulation was achieved by the intraperitoneal (i.p.) administration of MK-801 (0.05 mg/kg) or \( \text{NG} - \text{monomethyl-L-arginine monoacetate (L-NMMA: 10 – 100 mg/kg)} \), but not attained by \( \text{NG} - \text{monomethyl-D-arginine monoacetate (D-NMMA: 100 mg/kg)} \). This recovery effect of L-NMMA (50 mg/kg) was inhibited by pretreatment with L-arginine (600 mg/kg). In the trigeminal nucleus caudalis (SpVc), the changes in nitric oxide (NO) levels invoked by the intravenous (i.v.) administration of \( \text{N-methyl-D-aspartate (NMDA; 10 mg/kg)} \) were found to be significantly larger in the dental-injured rats than in sham-operated rats. The number of neuronal NO synthase (nNOS)-positive neurons increased in layers I-II and III-IV in the SpVc on both sides of the dental-injured rats. These results suggest that hypersensitivity to tactile stimulation developed following dental injury, and that NMDA receptor/NOS/NO production pathways in the SpVc may be involved in pathological conditions.

Keywords: Dental injury, Hypersensitivity to tactile stimulation, Trigeminal nucleus caudalis, Nitric oxide, NMDA receptor

Such pathophysiological pain conditions as allodynia, hyperalgesia and phantom pain may be produced after nerve injury or due to chronic inflammation. It has been reported that changes in nitric oxide (NO) levels following the activation of \( \text{N-methyl-D-aspartate (NMDA)} \) receptor mechanisms after nerve injury may contribute to these sensory alterations (1 – 4). The activation of these NMDA receptors in the central nervous system initiates an influx of \( \text{Ca}^{2+} \), thus inducing various \( \text{Ca-dependent intracellular processes (5)} \). Accordingly, nitric oxide synthase (NOS) has been shown to be \( \text{Ca}^{2+}-\text{dependently activated by NMDA receptors, with NO being synthesized from L-arginine (6, 7)} \). Furthermore, NO has also been shown to enhance the release of excitatory amino acids (8 – 11). In other studies, such states of pathological pain as tactile hypersensitivity and allodynia, etc., were shown to be reversibly blocked by the administration of (NOS) inhibitors (12) and NMDA-receptor antagonists (13). These observations support the idea that NO systems linked with the activation of NMDA-receptor mechanisms play an important role in the development of nerve injury-evoked pathological pain. On the other hand, conflicting results have recently been reported indicating that the systemic treatment with a specific pharmacological inhibitor of neuronal NOS (nNOS) failed to prevent or reverse allodynia in nerve-injured rats (14). These findings suggest that the conditions surrounding pathological pain induced by nerve injury in experimental animal models are complex and may be influenced by a variety of factors.

Pathological pain states have been produced in experimental animal models through the performance of such traumatic surgical procedures as nerve ligation (15 – 18) and by the undertaking of such metabolic treatments as the creation of streptozotocin-induced diabetes (19, 20). In human oral and facial regions, hyperalgesia and allodynia are sometimes observed in connection with such dental procedures as pulpectomy and tooth-extraction (21 – 23).
In our study, the first aim was the creation of experimental animal models with states of pathological pain in the orofacial region. This was achieved by the performing dental extractions and pulp treatments. The second aim of this study was to investigate the involvement of the NMDA receptor/NOS/NO production pathway in the mechanisms of development and/or maintenance of these states of pathological pain. As NO-selective electrodes capable of directly measuring NO production (24 – 26) have been developed, using these NO-selective electrodes and immunohistochemical staining methods, we measured the changes in NO and nNOS levels in the trigeminal nucleus caudalis (SpVc), the location of the central terminals of the primary afferent nerves from oral and facial regions, and a region with the function of mediating nociceptive transmissions to higher levels of the central nervous system such as the thalamus.

MATERIALS AND METHODS

Male Sprague-Dawley (SD) rats (approx. 200 – 250 g in body weight) were used as subjects. All surgical and experimental procedures were reviewed and approved by the Osaka University Graduate School of Dentistry Intramural Animal Care and Use Committee and conformed to the guidelines of the International Association for the study of Pain (27).

Surgery

Animals were randomly divided into sham-operation and dental-injury operation groups. The dental-injury group animals were then anesthetized with pentobarbital (50 mg /kg, i.p.), and the left upper incisor was first extracted, after which pulpectomy was performed on the lower left incisor using reamers and K-files following resection at the level of the alveolar bone using a diamond bar, because the lower incisors are imbedded deeply into the alveolar bone, making it impossible to extract them without heavy damage to the mandible. The root canal was then disinfected with sodium hypochlorite and oxydol 2 or 3 times. In the case of the sham-operated animals, subjects were placed under general anesthesia and then allowed to recover. Both dental-injured- and sham-operated animals were fed on a powder diet and given a free supply of water. The nutritional value of this food was equivalent to a solid diet. Animals were kept in a 12-h light-dark cycle environment.

Behavioral experiments

A series of von Frey filaments of increasing bending force (4.5 – 1479 mg) were used to determine the development of paresthesia to tactile stimulation. Each rat was accommodated in a plastic cage (0.5 × 0.3 × 0.2 m), and then von Frey filaments were delivered from above to the surface of the skin around the whisker and cheek regions. Senses in these areas are transmitted by the trigeminal nerve. A series of filaments, beginning with one that had a target force of 8 mg, were applied in a consecutive sequence to the oro-facial surface with a force causing the filament to bend. Animals withdrawing, touching or scratching their faces when the von Frey filaments were delivered, were considered to have shown positive responses. Each von Frey filament was applied 3 times at approx. 1-s interval. If the first filament showed no response, another filament with a higher bending force was delivered within 2 min. This procedure was repeated until positive responses occurred. The lowest von Frey filament to elicit a positive response was considered as indicating the threshold value. Behavioral tests were performed before operations and once every five days after the operation for approx. 1 month. Data were expressed as a filament rank number. The weakest (8 mg bending force) and strongest (1.4 g bending force) von Frey filaments used in the present study are equivalent to 1 and 9 filament rank numbers, respectively. The relationship between bending force (filament pressure) and the filament rank number is shown in Fig. 1.

Administration of drugs in behavioral experiments

Two weeks after operations, we used the rats that developed nerve injury-evoked tactile hypersensitivity and that had physically recovered from their surgeries for the behavioral experiments with the von Frey filaments. \(N^\text{G}-\text{monomethyl-L-arginine monoacetate (L-NMMA)}\) (saline, 10, 50, 100 mg/kg), \(N^\text{G}-\text{monomethyl-D-arginine monoacetate (D-NMMA)}\) (100 mg/kg), MK801 (saline, 0.05, 0.1, 0.5 mg /kg) or \(L\)-arginine (600 mg/kg) was administered intra-peritoneally (i.p.) to subjects; and the effects of these drugs on withdrawal thresholds were determined using the von
Frey filaments. Withdrawal thresholds were measured every 30 min for a period of 5 h after drug injection. Drugs were diluted in physiological saline.

**NO levels in the SpVc**

We measured the changes in NO levels in the SpVc 2 weeks after dental injuries, because at this point, rats were thought to have undergone sensory changes and to have physically recovered from their surgeries.

Male SD rats were anesthetized with urethane (1 g/kg, i.p.) and then paralyzed with alcuronium (1 mg/kg, i.p.). Experiments were performed under artificial ventilation. Animals were placed in a stereotaxic apparatus, and NO levels were measured in accordance with the methods described by Ichimori et al. (24) using an NO-selective electrode (Nitric Oxide Monitor model NO-501; Inter Medical Co., Tokyo) inserted into the superficial layers of the SpVc on the ipsilateral side. In brief, the working electrode consisted of a Pt/Ir alloy wire coated with a three-layer membrane consisting of KCl, an NO-selective nitrocellulose resin (pyroxyline lacquer), and a gas-permeable silicon membrane. The counter electrode was made of carbon fiber and was placed on neck muscles near the working electrode. A +0.4 V to +0.8 V voltage was applied to the working electrode to permit the electrochemical oxidation of NO. The pA-order redox current between the working electrode and the counter electrode was detected with a current-voltage converter circuit in a high input impedance preamplifier. Currents indicating the levels of NO at the superficial layers of the SpVc were continuously recorded with a pen recorder (HITACHI 561-3003; Hitachi, Ltd., Tokyo). The position of the tip of the working electrode was decided based on the atlas of Pellegrio and Cushman (28) (P: 1.0 mm from the obex, L: 1.3 – 1.5 mm, H: 1.0 – 1.5 mm). A polyethylene tube was then inserted into the femoral vein to allow the intravenous administration of NMDA. After surgical procedures, the animals were given a 1 – 2 h rest, and then intravenously injected with NMDA. Changes in NO currents were measured for at least 2 h after each administration.

**Immunohistological staining of nNOS-positive neurons in the SpVc**

nNOS staining was performed as described by Dun et al. (29). In brief, at the end of the survival period, anesthetized rats were perfused intracardially with heparinized saline followed by a 4% solution of paraformaldehyde in a 0.1 M sodium phosphate buffer containing saline (PBS, pH 7.4). The medulla oblongata was removed, post-fixed in the same fixative for 12 h, and then immersed in PBS containing 15 – 20% sucrose. Frozen transverse sections (60 n) were then cut 24-h later. Sections were rinsed several times in 0.02 M PBS, treated with PBS containing 0.3% H2O2 to block endogenous peroxidase activity, and preincubated in 3% normal goat serum (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature. Subsequently, the sections were then incubated with the primary antibody serum, rabbit polyclonal anti-nNOS serum (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), for 16 h at 4°C under gentle agitation. After several washes with PBS, sections were incubated with biotinylated goat anti-rabbit IgG (1:300, Vector Laboratories, Inc.) for 2 h and subsequently with ABC complex (Vector Laboratories, Inc.) for 30 min at room temperature before being rinsed extensively in 0.1 M PB. Sections were immersed in a medium containing 0.05% 3,3’diaminobenzidine tetrahydrochloride and 0.003% H2O2 in a 0.05 M Tris-HCl buffer (pH 7.2), rinsed in 0.1 M PBS and distilled water, mounted on gelatin-coated slides, air dried, and cover-slipped.

Counts of nNOS-positive neurons were made in two regions, laminae I/II and laminae III/IV, of 10 random sections per animal, and the average number of nNOS-positive neurons was determined. A comparison of the values in each region was made between the sham-operated rats and the dental-injured rats.

**Drugs**

The drugs and chemicals used were as follows: (+)-MK-801 hydrogen maleate (Sigma Chemical Co., St. Louis, MO, USA); L-NMMA and D-NMMA (Wako Pure Chemical Industries, Ltd., Osaka); NMDA (Nacalai Tesque, Inc., Kyoto); and L-arginine (Peptide Institute, Inc., Minoh). These drugs were dissolved in saline, and then the pHs of all solutions were adjusted to pH 7.4.

**Statistical analyses**

In behavioral tests, filament rank numbers were obtained from withdrawal thresholds at each measured post-surgery time interval on the basis of Fig. 1. Filament rank numbers obtained before operations were used as control values (100%) and data were expressed as a percentage of this value for each side of each animal. The statistical significance of the differences was assessed with an analysis of variance (ANOVA) followed by a Dunnett’s multiple comparison test. In the experiment on immunohistochemical staining of nNOS-positive neurons in the SpVc, an unpaired Student’s t-test was used. A P value of less than 0.05 was considered statistically significant.

**RESULTS**

**Behavioral experiments**

In sham-operated rats, the withdrawal thresholds to tactile stimulation around the whisker and cheek regions were 276 ± 35 mg (n = 74, S.E.M.) and 230 ± 30 mg (n =
On the left and right sides, respectively. As is shown in Fig. 2A, there was no change in the withdrawal thresholds to tactile stimulation on either side in rats following sham-operations. On the other hand, reductions in the withdrawal thresholds to tactile stimulation developed on the ipsilateral side of dental-injured rats from day 5 after surgery and lasted for at least 30 days. On the contralateral side, reductions in withdrawal thresholds were also observed (Fig. 2B).

As a general observation, dental-injured rats showed small reductions in body weight in the 10-day post-operative period, but after this, they gradually regained their lost weight. In addition, acute inflammation was observed as a result of the surgical procedures, but this disappeared promptly. On the other hand, secondary infections involving suppuration were seldom observed. Based on the above observations, in the following experiments, we therefore used rats after first allowing them around a 2 week period to physically recover from the dental surgical procedures and because at around this period, all rats showed signs of nerve injury-evoked tactile hypersensitivity.

**Administration of MK801 and L-NMMA**

To examine whether MK801, a noncompetitive NMDA receptor antagonist, and L-NMMA, a NOS inhibitor, could modulate the expression of dental injury-evoked tactile hypersensitivity, we administered these drugs to dental-injured rats and sham-operated rats. In saline-treated experimental subjects, no changes in the withdrawal thresholds to von Frey filaments were observed in the sham-operated rats or in the dental-injured rats ($P>0.05$) (Figs. 3 and 4). On the other hand, the administration of MK-801 (0.05 mg/kg, i.p.) produced significant increases ($P<0.05$) in withdrawal thresholds on both ipsilateral and contralateral sides (Fig. 5B). We failed to observe a dose-dependent effect for MK-801 on tactile hypersensitivity in the 3-h period after injections, because motor deficits were observed at doses higher than 0.05 mg/kg.

In the operated rats, the administration of L-NMMA produced increases in the withdrawal thresholds on both the ipsilateral side and the contralateral side in a dose-dependent manner (Fig. 4: A and B), but D-NMMA (100 mg/kg) did not have any modulatory affect. Maximal effects of L-NMMA on hypersensitivity to tactile stimulation were observed 1 h after injections. In the sham-operated rats, neither 100 mg/kg doses of D- or L-NMMA showed any changes in withdrawal thresholds when compared with those of the saline-treated rats (Fig. 3). Pretreatments with L-arginine (600 mg/kg, i.p.), a NOS substrate, 5 min prior to the administration of L-NMMA (50 mg/kg, i.p.) reduced the inhibitory effects of the L-NMMA from 253.6 ± 20.7% (n = 20, means ± S.E.M.) to 156.1 ± 20.4% (n = 20, mean ± S.E.M.) and from 217.4 ± 20.2% (n = 20, mean ± S.E.M.) to 152.8 ± 20.6% (n = 20, mean ± S.E.M.) on the ipsilateral and contralateral sides, respectively, 1 h after L-NMMA injections (Fig. 6: A and B). We did not observe motor
Fig. 3. Effect of the systemic administration of L-NMMA on withdrawal reactions to tactile stimulation with von Frey filaments in the sham-operated rats. L-NMMA was intraperitoneally administered at a dose of 100 mg/kg. Rank numbers obtained 20 min before drug administrations were used as control values (100%), and data are expressed as a percentage of these values for each side. Values in panels A and B indicate the means ± S.E.M. on the left and the right sides, respectively. Data were obtained from 10 sham-operated rats.

Fig. 4. Effect of the systemic administration of L-NMMA on withdrawal reactions to tactile stimulation with von Frey filaments in the dental-injured rats. This experiment was conducted using rats in the period 1 to 2 weeks after operation. L-NMMA was intraperitoneally administered at various doses. Rank numbers obtained 20 min before drug administrations were used as control values (100%), and data are expressed as a percentage of these values for each side. Values in panels A and B indicate the means ± S.E.M. for the ipsilateral and the contralateral sides, respectively. Data were obtained from 10 dental-injured rats for each dose. \(^*\) represents a significant difference in withdrawal responses before and after operations (\(P<0.05\), Dunnett’s test) for each side of the dental-injured rats at each time measured.
**Fig. 5.** Effect of the systemic administration of MK-801 on withdrawal reactions to tactile stimulation with von Frey filaments. This experiment was conducted using rats in the period one to two weeks after operation. MK-801 was intraperitoneally administered at a dose of 0.05 mg/kg. Rank numbers obtained 20 min before drug administrations were used as control values (100%), and data are expressed as a percentage of these values for each side. Values in panels A and B indicate the means ± S.E.M. obtained from 10 sham-operated rats and 10 dental-injured rats, respectively. # represents a significant difference in withdrawal responses before and after MK-801 administration (P<0.05, Dunnett’s test) for each side of the dental-injured rats at each time measured.

**Fig. 6.** Effect of the systemic administration of L-arginine on withdrawal reactions to tactile stimulation with von Frey filaments. This experiment was conducted using rats in the period 1 to 2 weeks after operation. L-arginine was intraperitoneally administered at a dose of 600 mg/kg 5 min before L-NMMA (50 mg/kg, i.p.). Rank numbers for each side obtained 20 min before the administration of L-arginine were used as control values (100%), and data are expressed as a percentage of these values. Values in panels A and B indicate the means ± S.E.M. for the ipsilateral and contralateral sides, respectively. # represents a significant difference in withdrawal responses between L-NMMA and L-NMMA + L-Arginine administered rats (P<0.05, Dunnett’s test) for each side of the dental-injured rats at each time measured.
deficiencies or any other side effects at any dose of the L-NMMA or L-arginine investigated in this study.

**NO levels in the SpVc**

When the experiment started, the measured relative NO currents were adjusted to 0 pA in each animal and the NO tracings obtained were observed to be stable. Changes in NO levels are represented by the relative pA-order redox current. As is shown in Fig. 7, relative NO currents began increasing in the period 5 min after the i.v. administrations of NMDA (10 mg/kg), and this increase reached a maximum in the interval 10 to 20 min following these administrations. After this, the currents then declined gradually. Maximum NO currents were 12 ± 2 pA (n = 20, mean ± S.E.M.) and 5 ± 1 pA (n = 24, mean ± S.E.M.) in the sham-operated rats and the dental-injured rats, respectively. The changes in relative NO currents evoked by NMDA showed similar patterns in both rat groups during the 50-min period after the NMDA administrations, whereas in the post 60-min period, for the sham-operated rats, the relative NO currents were observed to fall below zero and then decreased with time reaching −18 ± 5 pA at 2 h after the NMDA injections. For the dental-injured rats, relative NO currents began to increase after about 50 min and continued to be elevated, reaching 17 ± 5 pA at 2 h after the NMDA injections.

**Immunohistological staining of nNOS-positive neurons in the SpVc**

In the sham-operated rats, nNOS-positive staining was observed mainly in small- and occasionally in medium-sized neurons as is shown in Fig. 8.

In sham-operated rats, the average numbers of nNOS-positive neurons on the left and right sides were not significantly different (n = 10, S.E.M., 16.0 ± 1.0 (left side, I/II), 13.7 ± 0.5 (left side, III/IV), 15.8 ± 1.0 (right side, I/II), 13.9 ± 1.0 (right side, II/IV)). One week after operations, the average number of nNOS-positive neurons increased significantly to about 50% on both the ipsilateral and contralateral sides in laminae I/II of the SpVc of the dental-injured rats when compared with the sham-operated rats. It was also observed that the number of nNOS-positive neurons in laminae III/IV increased by about 35% on the ipsilateral and contralateral sides of the dental-injured rats (Figs. 8 and 9).

No morphological changes in nNOS-positive neurons were observed in the SpVc on the ipsilateral or contralateral sides in either dental-injured or sham-operated rats.

**DISCUSSION**

Loose and tight nerve ligation, (13, 15, 17, 18) and nerve transection (30–32) have been developed as procedures for studying neuropathic pain following peripheral nerve injury. We assume that the surgical procedures used in this study are similar to nerve transection. Therefore, to examine whether or not such neuropathological forms of pain as tactile allodynia developed, we measured thresholds through responses including withdrawing and the touching or scratching of the face evoked by tactile stimulation applied to the surface of the skin around the whiskers and cheeks, a region innervated by the same trigeminal nerve as the treated teeth. In the present study, the withdrawal thresholds to tactile stimulation in sham-operated rats were 276 and 230 mg (n = 74) on the left and right sides, respectively. On the other hand, the threshold to mechanical stimulation with von Frey filaments in the rat paw has previously been found to be about 15 g (33). These findings and our present results suggest that the sensitivity to mechanical stimulation in the whisker pad region may be much higher than that of the paw.

Concerning the time-course of the behavioral tests, the experiment was started day 5 after operation to allow time for the influence of surgery. Following surgical pulpectomy
procedures to the lower incisor and extraction of the upper incisor, reductions in withdrawal thresholds to mechanical stimulation developed about 5 days after surgery and lasted for nearly 30 days. This development is similar to the time-course of thermal hyperalgesia obtained in the loose-ligated sciatic nerve model (34). Namely, we have already reported that marked thermal hyperalgesia was observed in sciatic nerve-ligated animals in the period from 3 days through to 2 weeks after surgery, which was followed by a gradual return to control levels by post-surgery day 35. However, because of the extremely low threshold to mechanical stimulation required to produce escape behavior in this study, we can not exclude the possibility that the responses observed following tactile stimulation may not necessarily have been due to pain.

Concerning the reasons for the reduction in withdrawal thresholds to mechanical stimulation that arose on the contralateral side, it is thought that because trigeminal nerve sensory perception is not clearly divided in the midline structure between right and left upper incisors, when the left upper incisor was treated, a branch of the trigeminal nerve belonging to the right area was also damaged in spite of careful operating procedures. Such “mirror image” mechanical hypersensitivity has also been observed in lumbar dorsal root constrictions (35) and in ligations of the sciatic nerve (36, 37), when these operations were restricted to one side. We cannot explain the mechanisms underlying the mirror image increase in mechanical sensitivity following dental injury. Some neural pathways and/or signal transduction pathways in the central nervous system including the spinal dorsal horn and the SpVc, which are not understood clearly, may be activated to maintain a sensory balance between each side following damage of sensory neurons, and consequently, tactile hypersensitivity might arise on the non-operated side. In regard to this, it has been reported that 2 weeks following dorsal root constriction, the numbers of c-fos immunoreactive neurons in the spinal cord were elevated on both the ipsilat-

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**Fig. 8.** nNOS-positive neurons in the trigeminal nucleus caudalis 1 week after operation. A: nNOS staining of the left side of the sham-operated rat. B: nNOS staining of the right side of the sham-operated rat. C: nNOS staining ipsilateral to the operated side. D: nNOS staining contralateral to the operated side. Bar: 250 μm, I/II: laminae I/II, III/IV: laminae III/IV. The broken line between I/II and III/IV indicates the border between laminae II and III.
eral side and the contralateral side (non-injured side) (17, 18).

The mechanisms by which hypersensitivity is induced in nerve-injured rats are not known, but they may involve plasticity changes in the trigeminal ganglia and/or in the SpVc. Several lines of evidence support the hypothesis that the activation of NMDA receptors residing on postsynaptic dorsal horn neurons plays a critical role in the spinal mechanisms of such pathological pain states as hyperalgesia and allodynia (38). With respect to the mechanisms of the hypersensitivity to tactile stimulation observed in our study, although further experiments are needed to elucidate whether or not the tactile hypersensitivity evoked by pulpectomy and the extraction of incisors is pathophysiologically similar to the allodynia and thermal hyperalgesia observed in sciatic nerve injured-rats, as is shown in Fig. 5, the administration of the NMDA receptor antagonist, MK-801, produced increases in the withdrawal thresholds on both the ipsilateral and contralateral side. Our results resemble the conclusions drawn from a recent study showing that treatment with a NMDA receptor antagonist partially blocks the development and maintenance of allodynia evoked after nerve injury (13, 34, 39, 40).

There is some evidence to support the hypothesis that NMDA-receptor-linked nNOS activation in the spinal cord may be involved in neuropathic pain processes (12, 13, 20, 41, 42). The activation of NMDA receptors in the central nervous system initiates an influx of Ca\(^{2+}\), inducing Ca\(^{2+}\)-dependent intracellular processes. nNOS is a Ca\(^{2+}\)/calmodulin-dependent enzyme that is stimulated by the activation of NMDA receptors. In our result for the behavioral experiments, the NOS inhibitor L-NMMA inhibited hypersensitivity to tactile stimulation on both the ipsilateral and contralateral sides, in a dose-dependent manner, but the inactive enantiomer, D-NMMA did not. The inhibitory effect of L-NMMA was blocked by the NOS substrate L-arginine. In sham-operated rats, treatment with the same substrates did not produce any changes in the tactile withdrawal latencies. Similar findings have been observed in rats with tonic or persistent pain, a kind of neuropathic pain (loose ligation of the sciatic nerve) (43), or with chronic allodynia-like conditions (44). Persistent thermal (43) or mechanical hyperalgesia (44) has also been reported as being reversed by the administration of a NOS inhibitor, and this was observed in our study. Although the changes of nNOS level and NOS activity in the SpVc has been studied during only a limited time course, however, a good correlation among increases in nNOS levels, NOS activity and escape re-

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**Fig. 9.** Changes in numbers of nNOS-positive neurons in the trigeminal nucleus caudalis 1 week after operation. All data are presented as means ± S.E.M. obtained from at least 10 sham-operated and 10 dental-injured subjects. * indicates significant increases in the number of stained neurons comparing sham-operated and dental-injured rats for each side of each region (P<0.05, Dunnett’s test).
sponses has also been observed. For example, the change in relative NO current was markedly larger in dental-injured animals than in sham-operated animals (Fig. 7). Furthermore, the immunohistochemical study showed that nNOS-positive neurons increased significantly in laminae I/II of the SpVc (Figs. 8 and 9). These findings lead to the idea that the sustained production of NO in the SpVc may be required for the tactile hypersensitivity induced in dental-injured models.

However, a recent study has indicated conflicting results reporting that the systemic treatment with a specific pharmacological inhibitor of nNOS failed to prevent or reverse allodynia in the Kim and Chung model (36) of nerve-injured rats (14). The reasons for this are not known, but this variance in the effects of NOS inhibitors could reflect the differences in such pathophysiological conditions as pain and/or paresthesia. Namely, in normal rats, the withdrawal threshold to tactile stimulation in the whisker pad region was sixty times more sensitive than that of the paw. In view of these facts, our results indicate that the diversity of the participation of NO may depend on pathological conditions: In the orofacial region, the NMDA/NO pathway is more closely related to the development and/or maintenance of tactile hypersensitivity than it is to that of allodynia and hyperalgesia. In this connection, there is some evidence that the nNOS neurons in the brainstem trigeminal sensory nuclear complex have more NMDA receptors than the non-nNOS neurons (45). The primary afferent neurons innervating the tooth pulps use glutamate as a major transmitter (46).

As for the increase in NOS level after the injury, there is also evidence that the transection of the inferior alveolar nerve innervating the lower jaw and the lower incisor did not cause any significant changes in the number of NOS-positive neurons compared with the control value in the ipsi- and contralateral SpVc, while injection of complete Freund’s adjuvant or exposure injury of the tooth pulps caused inflammation in the affected area and both of these nerve inflammatory insults caused a significant increase in the number of NOS-positive neurons in the SpVc on both sides one week after operation. These findings lead to possibility that inflammatory state rather than nerve injury could augment the number of NOS-positive neurons bilaterally in our dental injured model. Currently, precise knowledge about the reason why NOS neurons in the SpVc were sensitive to inflammatory insults, but not nerve injury is unavailable.

In addition to laminae I/II, increases in nNOS-positive neurons were observed in laminae III/IV, suggesting that not only nociceptive-specific neurons existing in the superficial layers but that also non-nociceptive and spinothalamic tract neurons, such as those of the wide dynamic range-type (WDR) neurons, might be involved in this pathophysiological state.

This study shows that the NMDA receptor/NOS/NO release cascade is involved in hypersensitivity to tactile stimulation (paresthesia) induced following dental injury. However, the mechanisms existing downstream of the NMDA receptor/NO pathway underlying the development of dental injury-induced paresthesia still remain unclear.

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