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Involvement of transient receptor potential vanilloid 1 in ectopic pain following inferior alveolar nerve transection in rats

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ABSTRACT

Chronic pain often develops in the orofacial region after inferior alveolar nerve (IAN) injury. In animal models IAN injury often causes severe neuropathic pain-like behavior in the IAN-innervated region as well as the adjacent region that includes the whisker pad skin. However, the basis for the spreading of pain to adjacent facial areas after IAN injury is still unknown. In this study we determined if the transient receptor potential vanilloid 1 (TRPV1) was associated with altered nocifensive behavior evoked by stimulation of the whisker pad skin following IAN transection. Grooming behavior after capsaicin injection into the whisker pad region was significantly increased after IAN transection and the increase in the behavior was reversed by systemic administration of a TRPV1 antagonist. The number of phosphorylated extracellular signal-regulated kinase immunoreactive (IR) neurons in trigeminal spinal subnucleus caudalis and upper cervical spinal cord following capsaicin injection into the whisker pad region was significantly greater in IAN-transected rats than sham-operated rats. The number of TRPV1-IR trigeminal ganglion (TG) neurons innervating the whisker pad skin was also increased significantly after IAN transection. The present findings suggest that an increase in TRPV1 expression in TG neurons innervating the whisker pad skin after IAN transection may underlie the spreading of pain to the adjacent whisker pad skin.

The inferior alveolar nerve (IAN), a branch of the third division of the trigeminal nerve, is occasionally injured during dental surgery such as impacted tooth extraction or dental-implant implantation [22]. IAN injury often causes persistent numbness or pain that can spread to adjacent orofacial regions [12,13,17]. The exact mechanism of such ectopic orofacial pain remains unclear. A better understanding of the mechanisms for ectopic orofacial pain abnormalities induced by IAN injury may aid in the effort to develop effective treatments.

Nerve injury leads to persistent alterations in the properties of adjacent uninjured unmyelinated fibers due to redistribution and altered expression of several ion channels responsible for membrane excitability [8]. It has been reported that the IAN transection enhances nocifensive behavior and increases nociceptive neuronal excitability in the trigeminal spinal subnucleus caudalis (Vc) and peripheral trigeminal ganglion (TG) neurons [26]. For instance, A-fiber activity in the infraorbital nerve (ION), a branch of the second division of the trigeminal nerve, was significantly enhanced by IAN transection as indicated by an increase in background activity and mechanically evoked responses [11,34]. The head-withdrawal threshold to mechanical stimulation of the ipsilateral whisker pad skin innervated by ION was decreased following IAN transection [21]. Moreover, the response of primary afferent neurons innervated by the ION elicited by mechanical stimulation of the ION-innervated region was increased after IAN transection due to the decrease of potassium currents [34].

The transient receptor potential vanilloid 1 (TRPV1) belongs to the TRPV subfamily of the large TRP ion channel superfamily and is highly expressed in primary afferent neurons. TRPV1 is well known to contribute to nociceptive processing in physiological and pathological pain conditions [31]. TRPV1 is activated by noxious heat (>43 °C), extracellular acidification, various lipids and capsaicin [3,32]. Capsaicin is an irritant that activates TRPV1 on C-fibers and small diameter Aδ-fibers [29] and causes severe pain in the administrated regions [25].

Extracellular signal-regulated kinase (ERK) which is a member of the mitogen-activated protein kinase (MAPK) family is activated by calcium influx and is served as a marker for excitation in Vc and upper cervical spinal cord (C1) neurons [20,35]. These

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findings indicate that ERK phosphorylation in Vc and C1 neurons could be a reliable indicator of neuronal activation induced by noxious stimulation of the orofacial regions.

In the present study, we examined changes in nocifensive behavior and the expression of phosphorylated-ERK (pERK)-immunoreactive (IR) cells in Vc and C1 caused by capsaicin injection into the whisker pad region after IAN transection. In addition, we examined changes in the expression of TRPV1 in TG neurons that supply the whisker pad skin to determine if this receptor plays a role in orofacial pain following IAN transection.

Fifty-four male Sprague-Dawley rats (250–350 g) were used in this study. They were exposed to a light-dark cycle (L:D 12:12 h) and kept in a temperature-controlled room (23 °C) with food and water ad libitum. This study was approved by the Animal Experimentation Committee at Nihon University School of Dentistry and procedures were performed according to the guidelines of the International Association for the Study of Pain [38].

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p. Schering-Plough, Tokyo, Japan), and placed on a warm mat. IAN transection was performed as described before [26]. Briefly, a small incision was made on the surface of the left facial skin over the masseter muscle and tissue was dissected to expose the alveolar bone. The bone covering the IAN was removed and the exposed IAN was lifted, transected and placed back in the mandibular canal without any discernable gap between the cut nerve ends. For control, a sham operation was performed, which was identical except IAN transection.

Prior to behavioral testing, rats were placed in a plastic cage (30 cm × 30 cm × 35 cm) for 20-min adaptation interval. Rats were injected 10 μl capsaicin (10 mM, Wako, Osaka, Japan) dissolved in 7% ethanol and 7% Tween 80 in 0.9% saline into the left whisker pad region, and the number of grooming was counted over the following 8-min period. This procedure was performed on IAN-transected rats on days 3, 14 and 30 following IAN transection ($n = 6$) and sham rats on day 14 after sham operation ($n = 4$).

To assess the involvement of TRPV1 in capsaicin-induced nocifensive behavior, IAN-transected rats were given the TRPV1 antagonist, N-(3-methoxyphenyl)-4-chlorocinnamide (SB366791, Sigma–Aldrich, St. Louis, MO), intraperitoneally. SB366791 (1.0 mg/kg) was dissolved in 10% dimethylsulfoxide in 0.9% saline. On day 14 following IAN transection, rats were injected 10 μl of capsaicin (10 mM) into the whisker pad region 30 min after intraperitoneal administration of SB366791 in a volume of 1.3 ml and the number of grooming was counted over the following 8-min period ($n = 6$).

We used pERK immunoreactivity as a marker of the neuronal activation and NeuN as a neuron-specific marker in Vc and C1. IAN-transected rats were anesthetized with sodium pentobarbital (50 mg/kg) and 10 μl of capsaicin was injected subcutaneously into the whisker pad region with Hamilton syringe (Hamilton, Reno, NV) on day 3, 14 or 30 after IAN transection ($n = 5$ in each group). The same injection procedure as IAN-transected rats was performed in sham rats on day 14 ($n = 5$). Five min after injection, rats were perfused through the aorta with 500 ml 0.9% saline followed by 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The whole brain including medulla and cervical cord was removed and post-fixed in the same fixative for 3 days at 4 °C. The tissues were transferred to 20% sucrose (w/v) in phosphate-buffered saline (PBS) for several days for cryoprotection. Thirty-micron-thick sections were cut with a freezing microtome. Free-floating tissue sections were rinsed in PBS, 3% normal goat serum in PBS for 1 h, and incubated in rabbit anti-phospho-p44/42 MAPK antibody (1:1000, Cell Signaling, Danvers, MA) for 72 h at 4 °C. The sections were incubated in biotinylated goat anti-rabbit IgG (1:600; Vector Labs, Burlingame, CA) for 2 h at room temperature. After washing, sections were incubated in peroxidase-conjugated avidin-biotin complex (1:100; ABC, Vector Labs) for 1 h at room temperature washed in 0.05 M Tris Buffer (TB), and then incubated in 0.035% 3,3′-diaminobenzidine-tetra HCl (Sigma–Aldrich), 0.2% nickel ammonium sulfate and 0.05% peroxide in 0.05 M TB (pH 7.4). Sections were washed in PBS, serially mounted on gelatin-coated slides, dehydrated in alcohols and cover slipped. The number of pERK-IR cells in Vc and C1 were counted from every fourth section [20], and a mean number of pERK-IR cells (three sections/rat) was obtained from each animal. For NeuN and pERK double labeling, tissue preparation was performed on day 14 in IAN-transected rats as described above. The sections were incubated in rabbit anti-phospho-p44/42 MAPK antibody (1:300) for 3 days at 4 °C, and then mouse anti-NeuN antibody (1:1000; Chemicon, Temecula, CA) for 1 h at room temperature and secondary antibodies (AlexaFluor488-conjugated goat anti-rabbit IgG and AlexaFluor568-conjugated goat anti-mouse IgG (1:1000 in 0.01 M PBS; Invitrogen, Carlsbad, CA)) for 2 h at room temperature in a dark room. The sections were mounted on slides and cover slipped in PermaFluor (Sigma–Aldrich) and examined under a fluorescence microscope.

TRPV1 expression in TG neurons innervating the whisker pad skin on day 14 after IAN transection was compared with sham rats. The TG neurons that supply the whisker pad skin were identified by retrograde labeling with 100 mg/ml (in 100% ethanol) of 1,1′-dioctadecyl-3,3,3′,3-tetramethylindocarbocyanine methanesulfonate (DiI; Molecular Probes, Eugene, OR). DiI injection (10 μl) into the ipsilateral whisker pad region was performed with a 30-gauge needle 3 days before perfusion by 4% paraformaldehyde for immunohistochemistry. After perfusion, the ipsilateral TG was removed and embedded in Tissue Tek (Sakura Finetechnical, Tokyo, Japan) and stored until cryosectioning at −20 °C. TGs were cut in the horizontal plane along the long axis of the ganglion at a thickness of 16 μm. Every tenth section, four sections per TG per rat were collected for analyses. Sections were thaw-mounted onto MAS-coated Superfrost Plus microscope slides (Matsunami, Tokyo, Japan) and dried at dark room temperature overnight. TG sections were incubated with rabbit anti-TRPV1 polyclonal antiserum (1:200, Alomone, Jerusalem, Israel) and then reacted with AlexaFluor488-conjugated goat anti-rabbit IgG (1:1000 in 0.01 M PBS). The sections were coverslipped in mounting medium and examined under a fluorescence microscope. Using appropriate filters, double- (DiI and TRPV1) labeled cells were identified and analyzed using a BZ-9000 system (Keyence, Tokyo, Japan). Cells expressing a two-fold or greater increase in intensity above average background were considered positive for TRPV1 immunoreactivity. No specific labeling was observed in the absence of primary antibody. The total number of DiI-labeled cells and DiI-labeled TRPV1-IR cells in four sections of TG was counted in each animal ($n = 5$ in each). The ratio of TRPV1-IR cells in each animal was calculated by the following formula: 100 × total number of DiI-labeled TRPV1-IR cells in four sections of TG/total number of DiI-labeled cells in four sections of TG. Mean percentages of TRPV1-IR cells in DiI-labeled cells were calculated from the ratio of five animals.

Data were expressed as mean ± SEM. Statistical analyses were performed by Student's t-test and one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison tests where appropriate. Differences were considered significant at p value < 0.05.

The number of capsaicin-evoked grooming on day 14 was significantly increased in IAN-transected rats (82.0 ± 8.7) compared with sham rats (43.0 ± 14.1) (Fig. 1). The increase in grooming behavior on day 14 was reversed to the value of sham rats at 30 min after SB366791 administration (28.2 ± 5.0).

The pERK-IR cells induced by capsaicin injection into the whisker pad region were double-stained with NeuN in IAN-transected rats, indicating that pERK-IR cells expressed in Vc and C1