Abstract: Pulpitis often causes referred pain in opposing teeth. However, the precise mechanism underlying ectopic pain associated with tooth-pulp inflammation remains unclear. We performed the present study to test the hypothesis that functional interactions between satellite glial cells (SGCs) and trigeminal ganglion (TG) neurons are involved in ectopic orofacial pain associated with tooth-pulp inflammation. Digastric muscle electromyograph (D-EMG) activity elicited by administration of capsaicin into the upper second molar pulp (U2) was analyzed to evaluate noxious reflex responses. D-EMG activity was significantly increased in rats with lower first molar (L1) inflammation relative to saline-treated rats. Significantly increased expression of glial fibrillary acid protein (GFAP), a marker of activated glial cells, and connexin 43 (Cx43), a gap-junction protein, was observed in activated SGCs surrounding U2-innervating TG-neurons after L1-pulp inflammation. Daily administration of Gap26, a Cx43-inhibiting mimetic peptide, into the TG significantly suppressed capsaicin-induced D-EMG activity enhancement and reduced the percentage of fluorogold-labeled (U2-innervated) cells that were surrounded by GFAP-immunoreactive (IR) and Cx43-IR cells after L1-pulp inflammation. These findings indicate that tooth-pulp inflammation induces SGC activation and subsequent spread of SGC activation in the TG via Cx43-containing gap junctions. Thus, remote neuron excitability becomes enhanced in the TG following tooth-pulp inflammation, resulting in ectopic tooth-pulp pain in the contralateral tooth.

Keywords: referred pain; pulpitis; endodontics; toothache.

Introduction

Pulpitis often causes ectopic or referred pain in neighboring or opposing teeth (1,2). Clinicians often struggle to make a definite diagnosis, and misdiagnosis may occur, resulting in inappropriate treatment (3,4). However, details of the mechanism underlying ectopic tooth pulp pain associated with tooth pulp inflammation remain unknown.

Noxious stimulation of the tooth pulp can evoke digastric muscle activity (5-7). Accordingly, changes in the value of reflex electromyogram (EMG) recordings after application of capsaicin to the tooth pulp can be a
consequence of activation of intra-pulpal high threshold afferents, including C-fibers (7,8).

Satellite glial cells (SGCs) that surround neurons in the trigeminal ganglion (TG) or dorsal root ganglion (DRG) modulate neuronal excitability after peripheral inflammation or nerve injury (9,10). These excited neurons can release various molecules into the extracellular space (11,12), that can interact with specific receptors on SGCs in the vicinity of the neurons and induce SGC activation (13). This activation of SGCs can trigger opening of connexin 43 (Cx43)-containing gap junctions that link neighboring SGCs (14). Signals from activated SGCs can be transmitted among SGCs via gap junctions, leading to continuous propagation of SGC activation from the site of injury to uninjured more distant locations (14). The distantly located and activated SGCs subsequently release molecules that facilitate the excitability of remote neurons innervating uninjured and distant tissues, leading to the development of secondary hyperalgesia (9,11). Thus, it is highly likely that the overexpression of connexin 43 accompanied by SGC activation following pulp inflammation is involved in hyperalgesia of the opposing tooth.

To test this hypothesis, EMG recordings of the digastric muscle (D-EMG) were generated to evaluate changes in the noxious reflex following noxious stimulation to the intact tooth pulp (upper second molar: U2) accompanied by opposing pulp inflammation (lower first molar: L1). Additionally, immunohistochemical analysis of glial fibrillary acid protein (GFAP) and Cx43 expression in the trigeminal ganglion (TG) was performed to assess whether GFAP- and Cx43-expressing cells surrounded U2-innervated neurons. Furthermore, the effects of a selective gap junction blocker (Gap26) on U2 hyperalgesia were examined.

Materials and Methods

Animals

Male Sprague-Dawley rats (n = 47, Japan SLC, Hamamatsu, Japan) weighing between 250 and 350 g were used. They were housed at 23°C in a specific pathogen-free vivarium under a 12/12 h light/dark cycle with food and water provided ad libitum. The Animal Experimentation Committee of Nihon University approved this study (animal protocol number: AP14D032, and AP17D001). Experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the guidelines detailed in a previous report (15).

All animal experiments were carried out in accordance with institutional and national guidelines for the care and use of experimental animals, which are in compliance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European Communities Council Directive of 24 November 1986 (86/609/EEC) or the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). All efforts were made to minimize animal suffering, to minimize the number of animals used, and to utilize alternatives to in vivo techniques if available.

Rats with L1 tooth pulp inflammation

Initially, light inhaled isoflurane (2%, Mylan, Canonsburg, PA, USA) anesthesia was administered, and then the rats were deeply anesthetized by intraperitoneal (i.p.) administration of butorphanol (2.5 mg/kg, Meiji Seika Pharma, Tokyo, Japan), medetomidine (0.375 mg/kg, Zenoaq, Fukushima, Japan), and midazolam (2.0 mg/kg, Sandoz, Tokyo, Japan) dissolved in saline solution. The procedure used to induce L1 pulp inflammation has been reported previously (16). In brief, the unilateral L1 pulp was exposed, and complete Freund’s adjuvant (CFA) was administered into the L1 pulp using paper points. On day 3 after CFA administration, the D-EMG was recorded or the rats were perfused for immunohistochemical analysis.

D-EMG recording

On day 3 after administration of CFA into the L1 pulp, rats were lightly anesthetized with 2-3% isoflurane and the U2 pulp was exposed. Then, a pair of bipolar wire electrodes (stainless steel wire coated with enamel; inter-electrode distance, 5 mm; Narishige, Tokyo, Japan) was inserted into the ipsilateral digastric muscle. A D-EMG recording was performed as reported previously (8). In brief, following insertion of the electrodes into the digastric muscle and exposure of the U2 tooth pulp, the isoflurane concentration was reduced and maintained at 0.8-1.2% throughout the experiment. Then, the tip of a dental paper point saturated with capsaicin (3 µm: diluted with 7% Tween 80, 100% ethanol, and saline; Wako, Osaka, Japan) or vehicle was administered into the U2 tooth pulp. A D-EMG recording was taken for 20 min before and after the administration of capsaicin. Then, the amplification, rectification, and integration values of D-EMG activity were analyzed. The area under the curve (AUC) of D-EMG activity was calculated using Spike 2 software (CED, Cambridge, UK). Before or after capsaicin administration, the AUC value of D-EMG activity was averaged every minute. The mean D-EMG activity for each 1-min period was then plotted on a graph. The baseline D-EMG activity was calculated from the average AUC value of D-EMG activity 3 min before capsaicin administration; baseline values were defined...
as 100%. The averaged D-EMG activity for 1 min was normalized at each time point.

**Immunohistochemistry**

TG neurons that innervated the U2 pulp were visualized using fluorogold (FG), a retrograde tracer, dissolved in saline (Fluorochrome, Denver, CO, USA). To identify TG neurons innervated by U2, a dental paper point soaked with FG was administered into the U2-tooth pulp. On day 3 after the application of CFA (L1) and FG (U2), the rats were anesthetized with saline solution mixed with 2.5 mg/kg butorphanol, 0.375 mg/kg medetomidine, and 2.0 mg/kg midazolam. Then, transcardiac perfusion was performed with saline, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). The ipsilateral TGs were removed and immersed in 4% PFA in 0.1 M PB for post-fixation. At 24 h post-fixation, the TGs were soaked in a solution of 20% sucrose dissolved in 0.01 M phosphate-buffered saline (PBS) for 24 h for cryoprotection. TissueTek (Sakura Finetek, Tokyo, Japan) was used to embed the TGs; the embedded TGs were then sectioned along the horizontal axis of the TG at a thickness of 15 μm. This immunohistochemical procedure was performed on every tenth section (5 sections per TG). TG sections were mounted on microscope slides (Matsunami, Tokyo, Japan) and incubated in a solution of mouse anti-GFAP (1:800, cat. SAB1405864; Sigma-Aldrich, St. Louis, MO, USA) or rabbit anti-Cx43 (1:300, cat. C6219; Sigma-Aldrich). The sections were immersed in secondary antibody solution of Alexa Fluor 488-conjugated goat anti-mouse IgG (1:300 in 0.01 M PBS; Thermo Fisher, Waltham, MA, USA) or Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:300 in 0.01 M PBS; Thermo Fisher). After coverslipping the TG sections with mounting medium, immunoreactive (IR) cells were observed under a fluorescence microscope. The triple-labeled (FG, GFAP, and Cx43) cells were identified using a BZ-9000 system (Keyence, Tokyo, Japan), and the number of cells was then carefully counted and analyzed. The cells that exhibited two-fold or more saturation when compared to the averaged background were considered to be positive. FG-labeled neurons with GFAP-IR cells were counted in cases where the neuron was surrounded by GFAP-IR cells for more than a half of the outer circumference of the neuron. To quantify and analyze IR cells in the TG of the second branch area, the formula shown in the legends for Figs. 2 and 4 was used.

**Administration of Gap26 into the TG**

A midline skin incision (length: 2 cm) was made from the head to the neck. Then, a small hole (pore diameter, 1 mm) was drilled into the skull above the TG. A guide cannula was inserted through the hole to reach the TG; the tip of the cannula was positioned 9 mm below the skull surface. Then, three stainless-steel screws and dental resin were used to fix the abutment of the cannula.

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**Fig. 1** Reflex electromyogram recording of the digastic muscle (D-EMG) and satellite glial activation following pulp inflammation. (A) Typical examples of D-EMG following capsaicin administration to the upper second molar (U2) in rats administered with vehicle or complete Freund’s adjuvant (CFA) into the lower first molar (L1) on day 3. (B) The mean area under the curve of integrated D-EMG following administration of capsaicin into the U2 pulp in L1 vehicle- or CFA-treated rats; *, vehicle vs. CFA; #, Pre (−1 min) vs. Post (0 min) following capsaicin administration; *, P < 0.05; **, ##, P < 0.01.
on the skull surface, as reported previously (17). Saline (1 μL/day) or Gap26 (1 μL/day, 3 mM; R&D Systems, Minneapolis, MN, USA) was administered into the TG once per day for 3 consecutive days (day 0 through day 2; n = 6 in each group). D-EMG measurements or immunohistochemistry were also performed on day 3 to examine the effect of Gap26 on hyperalgesia in the U2 tooth pulp following the induction of L1 pulp inflammation.

**Statistical analysis**

Non-parametric data presented in box plots with median, quartiles (middle 50%), and range. The Mann-Whitney U test was used to compare the D-EMG activity between groups, and the percentage of positive cells between groups (Fig. 1B: n = 6, Fig. 2C: n = 6, Fig. 3B: n = 5, per group Fig. 4B: n = 5, per group). The threshold for statistical significance was set at P < 0.05.

**Results**

**D-EMG responses after induction of L1 pulp inflammation**

Large D-EMG activities were observed after capsaicin administration into the U2 pulp in L1 vehicle- or CFA-treated rats (Fig. 1A). The relative AUC values for D-EMG activity 0-1 min after capsaicin administration into the U2 pulp were significantly greater in L1 CFA-treated rats than in vehicle-administered rats. The corresponding AUC values for D-EMG activity 0-1 min after capsaicin administration into the U2 pulp in L1 vehicle- and CFA-treated rats were significantly greater than those at −1-0 min (n = 6 in each group) (Fig. 1B).

**SGC activation and Cx43 expression after induction of L1 pulp inflammation**

Following the application of FG (U2) and DiI (L1), no TG neurons were co-labeled with FG and DiI, indicating that U2/L1-inervating neurons were not present in the TG (Fig. 2A). Many FG-labeled neurons surrounded by GFAP-IR and Cx43-IR cells were observed in the TG in CFA-treated rats on day 3, compared with the TG in vehicle-treated rats (Fig. 2B). The percentage of FG-labeled cells surrounded by GFAP-IR and Cx43-IR cells was significantly greater in L1 CFA rats than in vehicle-treated rats (n = 6 in each group) (Fig. 2C).

**Effects of Gap26 administration into the TG on D-EMG activity**

A small D-EMG response was observed in Gap26-treated
rats relative to vehicle-treated rats after U2 pulp injection of capsaicin (Fig. 2A). The increased AUC values for D-EMG responses at 0-1 min after U2 application of capsaicin were significantly suppressed by daily administration of Gap26 (0.5 µL/day for 3 days) into the TG of L1 CFA-treated rats when compared with vehicle-treated rats (n = 5 per group) (Fig. 3B).

**Effect of Gap26 administration into the TG on Cx43 expression**

A reduction in the number of FG-labeled cells surrounded by GFAP-IR and CX43-IR cells was observed in the TG after daily administration of Gap26 relative to that found in vehicle-treated rats (Fig. 4A). The percentage of FG-labeled cells surrounded by GFAP-IR and CX43-IR cells was significantly reduced in Gap26-treated rats (n = 5 per group) (Fig. 4B).

**Discussion**

This study represents the first documented attempt to investigate the involvement of satellite glial cell activation and overexpression of Cx43 in opposing tooth pulp pain after induction of tooth pulp inflammation. Although it is well known that ectopic pain occurs following tooth pulp inflammation, the precise underlying mechanism has remained unclear (1,2). To clarify the mechanism of pulpal pain following pulp inflammation, we investigated changes in the expression of GFAP and Cx43 in the TG.

Hypersensitivity of the upper tooth pulp was observed after the onset of lower tooth pulp inflammation. SGC activation and overexpression of Cx43-IR cells surrounding TG neurons that innervated the upper tooth pulp were evident following lower tooth pulp inflammation. No TG neurons innervated either the upper or lower tooth pulp. Administration of Gap26 into the TG significantly suppressed hypersensitivity of the opposing tooth pulp and decreased the number of GFAP-IR and CX43-IR cells that surrounded TG neurons innervating the opposing tooth. These findings indicate that SGC activation and Cx43 overexpression in the TG play pivotal roles in the development of ectopic tooth pulp pain associated with pulp inflammation.

Approximately 6% of TG neurons innervate the neighboring maxillary molars (8) and 9.2% of TG neurons innervate both the tongue and mandibular molar tooth pulp (16). However, no TG neurons innervating the upper and lower molar tooth pulps were observed in the present
study. These findings suggest that multiple innervation of TG neurons in the orofacial region exists only in the same branches of TG neurons.

In rats, SGCs surrounding TG neurons are known to be activated after peripheral inflammation in the orofacial region, such as the whisker pad or tooth pulp (8,9,11). The activated SGCs release various molecules that can bind to receptors expressed in the ganglion neurons, thus facilitating neuronal excitability (13). Furthermore, inhibition of SGC activation causes a significant reduction in neuronal excitability and nocifensive reflexes (8,17,18). Meanwhile, Cx43-containing gap junctions are overrepresented in SGCs of the TG or DRG after both nerve injury and repeated water avoidance-induced stress (19). Gap26 (connexin mimetic) peptides located on the first extracellular loop of Cx43 can rapidly inhibit hemichannels, which close Cx43-containing gap junctions after some delay (20,21). The overexpression of GFAP and Cx43 after nerve injury can be significantly suppressed by administration of connexin mimetic peptides into the TG (18). In the present study, we observed a significant increase in the number of neurons surrounded by activated SGCs, and the spread of SGC activation leads to enhanced remote neuronal excitability, resulting in U2 tooth pulp hypersensitivity.
the TG. Together with previous data, the present findings suggest that SGC activation followed by upregulation of CX43-containing gap junctions can cause further activation of remote SGCs after the induction of L1-pulp inflammation. These activities enhance the excitability of TG neurons that innervate the non-inflamed U2 pulp (Fig. 5). Overall, these data indicate that satellite glia-neuron interactions can drive hyperalgesia of the non-inflamed U2 tooth pulp (11,22,23).

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Conflict of interest
The authors have no conflict of interest related to the authorship and/or publication of this article.

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