Orthodontic tooth movement causes tooth pain hypersensitivity by periodontal acidification

Ayaka Osada

Nihon University Graduate School of Dentistry

Major in Orthodontics

(Directors: Profs. Mitsuru Motoyoshi, Masamichi Shinoda

and Assist. Prof. Suzuro Hitomi)

Index

This thesis is composed of the following article and additional result of the percentage of FG-labelled ASIC3-IR TG cells/FG-labelled TG cells on day 7 after elastic insertion. (Fig. 3D)

Osada A, Hitomi S, Nakajima A, Hayashi Y, Shibuta I, Tsuboi Y, Motoyoshi M, Iwata K and Shinoda M (2021) Periodontal acidification contributes to tooth pain hypersensitivity during orthodontic tooth movement. Neurosci Res, in press.

Abstract

Tooth movements associated with orthodontic treatment often cause tooth pain. However, the detailed mechanism remains unclear. Here, I examined the involvement of periodontal acidification caused by tooth movement in mechanical tooth pain hypersensitivity. Elastics were inserted between the first and second molars to move the teeth in Sprague-Dawley rats. Mechanical head-withdrawal reflex threshold to first molar stimulation and the pH of the gingival sulcus around the tooth were measured. The expression of acid-sensing ion channel 3 (ASIC3) in trigeminal ganglion neurons and phosphorylation of ASIC3 in the periodontal tissue were analyzed. The mechanical head-withdrawal reflex threshold to first molar stimulation and pH in the gingival sulcus decreased on day 1 after the elastic insertion. These decreases recovered to the sham level by buffering periodontal acidification. Periodontal inhibition of ASIC3 channel activity reversed the decreased mechanical head-withdrawal reflex threshold to first molar stimulation. On day 1 after elastic insertion, the tooth movement did not change the number of ASIC3 immunoreactive trigeminal ganglion neurons innervating the periodontal tissue but increased phosphorylated-ASIC3 levels in the periodontal tissue. Periodontal acidification induced by tooth movement causes phosphorylation of ASIC3, resulting in mechanical pain hypersensitivity in mechanically forced tooth.

Introduction

Oral functions, including mastication, swallowing, and speech, are essential for living. However, malocclusion, an abnormal alignment of the teeth, frequently causes oral dysfunction, leading to a lower quality of life [1]. Orthodontic treatment can improve these abnormalities by tooth movement through mechanical force application using fixed or removable appliances. However, mechanical forces sometimes induce tooth pain [2]. The pain intensity peaks on day 1 after tooth movement and gradually decreases [3,4]. In previous reports, the release of pro-inflammatory substances, such as substance P, interleukin (IL)-1β, and tumor necrosis factor-α, from leukocytes and fibroblast proliferation has been observed in the tooth movement model [5]. Furthermore, ischemia has been observed in the periodontal tissue around mechanically forced teeth [6] and has been reported to cause acidification in the tissue [7]. Although acidification has been suggested to be involved in orthodontic pain [6,7], the detailed mechanism remains unclear.

Acid-sensing ion channels (ASICs) are sodium-selective neuronal cation channels that are activated by extracellular protons and several ligands [8,9]. In particular, ASIC3 has been reported to have a higher sensitivity to extracellular protons other than ASICs [8,10]. Ischemia-induced acidosis causes mechanical pain, which is suppressed by ASIC3 inhibition [11]. Transient receptor potential vanilloid 1 (TRPV1) is also an acid sensor [12]. Both ASIC3 and TRPV1 are proton-sensing ion channels and are activated by extracellular pH lower than pH 7.2 and 5.3, respectively [8,13,14]. These ion channels contribute to orthodontic pain [2,15].

In this study, to clarify the involvement of ASIC3 signaling in orthodontic tooth pain, I examined mechanical pain sensitivity in mechanically forced teeth, acidification and ASIC3 phosphorylation in the periodontal tissue, and ASIC3 expression in trigeminal ganglion (TG) neurons.

Materials and Methods

Animals

Male Sprague-Dawley rats (n = 150, 250–350 g, Japan SLC, Hamamatsu, Japan) were used. The present studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and guidelines of the International Association for the Study of Pain [16]. The Animal Experimentation Committee of Nihon University approved all experiments in this study (protocol numbers: AP18DEN009 and AP20DEN014). All animals were kept under environmental conditions (temperature, 21–23°C; humidity, 40–60%) with free access to food and water. All procedures were performed during the light phase (12 h light/dark cycle). The number of animals and animal suffering was minimized in all experiments. All the experiments were conducted under blinded conditions.

Orthodontic elastic insertion (Waldo's method)

Rats were anesthetized with intraperitoneal administration of butorphanol (2.5 mg/kg; Meiji Seika Pharmaceutical, Tokyo, Japan), medetomidine (0.375 mg/kg; Xenoac, Fukushima, Japan), and midazolam (2.0 mg/kg; Sand, Tokyo, Japan). An orthodontic elastic (M8, Tomy International, Tokyo, Japan) was inserted between the right maxillary first and second molars (M1 and M2, respectively) and trimmed into small pieces (width, 3 mm; height, 1.5 mm; depth, 0.6 mm) [17]. The rats that received elastic insertion were defined as the elastic-induced tooth movement model (ELS). Sham rats underwent the same procedure without elastic insertion.

Measurement of mechanical pain sensitivity

The rats have received graded mechanical stimulation to the occlusal surface of M1 using an electronic von Frey (0 to 100 g, 4 g/s; Bioseb, Chaville, France) under light anesthesia with 2% isoflurane (Mylan, South point, PE, USA) inhalation and mechanical head-withdrawal reflex threshold to M1 stimulation (MHWRT) was measured. Before measurement of the MHWRT, the depth of anesthesia was confirmed by observing the hind paw-withdrawal reflex to noxious pinch stimulation (150 g) and identical breathing pattern, according to a previous report [18]. Before (Pre) and on days 1 to 7 after the elastic insertion and sham treatment, the minimum mechanical intensity required to evoke a head-withdrawal reflex was defined as MHWRT. The mechanical stimuli were applied 3 times at 1-min intervals. The averaged MHWRT in each rat was determined.

Micro-computed tomography (mCT) analysis

To evaluate the changes in the distance between M1 and M2, mCT analysis was performed using an R-mCT2 system (Rigaku, Tokyo, Japan) on pre and days 1 to 7 after elastic insertion. Under anaesthetic condition with intraperitoneal administration of butorphanol at 2.5 mg/kg, medetomidine at 0.375 mg/kg, and midazolam at 2.0 mg/kg, the rats were placed on the instrument stage after removal of the inserted orthodontic elastic from the maxillary molars. Then, mCT images of M1 and M2 were obtained under the conditions that the exposure parameters were 90 kV and 100 μA. The mCT images were reconstructed using i-View software (Morita, Osaka, Japan), and the shortest distance between the distal surface of M1 and mesial surface of M2 was measured. After each measurement, the elastic was replaced between M1 and M2.

Measurement of pH in the gingival sulcus

To confirm the acidity of the periodontal tissue in the model, the pH of the buccal gingival sulcus of M1 was measured using a pH meter (D-51; HORIBA, Kyoto, Japan) with a needle pH electrode (21-guage, MI-407B, Microelectrodes, NH, USA) and a flexible reference electrode (MI-402, Microelectrodes) under deep anesthesia with intraperitoneal administration of butorphanol at 2.5 mg/kg, medetomidine at 0.375 mg/kg, and midazolam at 2.0 mg/kg. Before measurement, the pH meter was calibrated with a standard solution (pH 4.01, pH 6.86, and pH 9.18; HORIBA). On days 1 to 7 after the elastic insertion and sham treatment, the pH electrode was inserted into the gingival sulcus of M1. The reference electrode was placed subcutaneously on the left femoral region. Then, the pH was measured three times, and the averaged pH values were taken as the pH in each rat.

Drug administration

Two microliters of the ASIC3 channel blocker APETx2 (0.17 mM diluted in saline, 07APE002, Smartox Biotechnology, Saint-Egrève, France); 0.01 M phosphate buffer saline (PBS); and phosphate buffer (PB) at pH 7.4, 7.0, and 6.5 were submucosally administered into the periodontal tissue around M1 five times (just after elastic insertion at 18:00 and on days 1 and 2 after the elastic insertion at both 9:00 and 21:00) under anesthesia with 2% isoflurane.

FluoroGold injection

To label primary sensory neurons innervating the periodontal tissue around the M1, 2 μL of the retrograde neuronal tracer FluoroGold (FG, 2% in saline; Fluorochrome,

Denver, CO, USA) was injected into the periodontal tissue close to M1 using a 31-gauge needle under 2% isoflurane anesthesia. To enable complete retrograde tracing to the neuronal soma, FG injection was performed on 4 days before an orthodontic elastic was inserted between M1 and M2.

Immunohistochemistry

On day 1 and 7 after elastic insertion or sham treatments, the rats were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under deep anesthesia with 5% isoflurane inhalation and an intraperitoneal administration of butorphanol (2.5 mg/kg), medetomidine (0.375 mg/kg), and midazolam (2.0 mg/kg). The TGs and right maxillary tissues were dissected and post-fixed with the same fixative for several days at 4°C. These tissues were then transferred to 20% sucrose in PBS overnight for cryoprotection. The TGs were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Tokyo, Japan) at -20°C and sectioned at a thickness of 10 μm using a cryostat (Leica, Tokyo, Japan). The TG sections were mounted on MAS-coated glass slides (Matsunami, Tokyo, Japan) and dried overnight. After rinsing in 0.01 M PBS, the TG sections were incubated in 3% normal goat serum for 1.5 h at room temperature (RT), and in rabbit polyclonal anti-ASIC3 antibody (1:300, ASC-018; Alomone Labs, Jerusalem, Israel) overnight at 4°C. After washing in 0.01 M PBS, the TG sections were incubated with Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (IgG, 1:300; A11034, Thermo Fisher Scientific, Waltham, MA, USA) for 3 days at 4°C. The TG sections were rinsed in 0.01 M PBS and cover-slipped using a mounting medium (PermaFluor, Thermo Fisher Scientific). ASIC3-immunoreactive (IR) cells in the TG were analysed using BZ-9000 (Keyence, Osaka, Japan). The number of FG-labelled

ASIC3-IR cells in the second branch of the TG in five sections from each rat were manually counted.

The periodontal tissue was removed with the teeth from the maxilla and decalcified in K-CX (Falma, Tokyo, Japan) at 4°C for 5 days. Then, it was cut into 10-μm thick slices using a microtome (Yamatokouki, Saitama, Japan) after being embedded in paraffin. Periodontal tissue with tooth sections was mounted on MAS-coated glass slides (Matsunami) and dried overnight. The sections were stained with hematoxylin and eosin and used for IL-1β immunohistochemistry. For immunohistochemistry, the ImmunoCruz rabbit ABC Staining System (sc-2018, Santa Cruz Biotechnology, Dallas, TX, USA) was used according to the manufacturer's protocol. The sections were incubated with H_2O_2 for 10 min, washed with 0.01 M PBS, and blocked with 1.5% blocking serum for 1 h at RT. The sections were incubated with goat polyclonal anti-IL-1β antibody (15 μg/mL, R&D Systems, Minneapolis, MI, USA) overnight at 4°C. The sections were washed with 0.01 M PBS and incubated with a biotinylated secondary antibody with biotinylated rabbit anti-goat IgG antibody (1:500; BA-5000, Vector, Burlingame, CA, USA) for 1.5 h. After washing with 0.01 M PBS, the sections were incubated for 30 min with AB enzyme reagent. Then, the sections were washed with 0.01 M PBS and incubated in peroxidase substrate for 5 min. The sections were washed with 0.01 M PBS, dehydrated in alcohol/xylene, and coverslipped. Immunostaining tissue sections were observed under a microscope BZ-9000 (Keyence).

Immunoprecipitation and western blotting

The rats were deeply anesthetized according to the above-described method and perfused transcardially with chilled saline. The periodontal tissue, including the

periodontal ligaments, was dissected 1 day after elastic insertion. The tissues were homogenized in radioimmunoprecipitation assay buffer (Nacalai Tesque, Kyoto, Japan) supplemented with protease inhibitors (1:100; TaKaRa, Kusatsu, Japan) and a phosphatase inhibitor (1:100; Nacalai Tesque) at 4°C. After centrifugation for 30 min at 4°C at 13,000 g, the supernatants were collected. Protein concentration was determined using a BCA protein assay kit (TaKaRa). For immunoprecipitation, the ASIC3 antibody was reacted with Dynabeads Protein G (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, 1 μg of rabbit polyclonal anti-ASIC3 antibody (ASC-018; Alomone Labs) was incubated with dynabeads and Tween-20 for 10 min with rotation to form a magnetic bead–antibody complex. The anti-ASIC3 conjugated dynabeads were further reacted with the lysate of the periodontal tissue samples (75 μg). Then, the dynabeads-antibody-antigen complex was separated by a magnet and washed three times with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST).

The immunoprecipitated samples and homogenized samples without immunoprecipitation were mixed with Laemmli sample buffer solution (Bio-Rad, Hercules, CA, USA) and heat-denatured for 5 min. The samples were loaded in each lane and electrophoresed by 4–20% Mini-PROTEAN TGX precast gels (Bio-Rad). The gels were then transferred onto a polyvinylidene difluoride membrane using a Trans-Blot Turbo (Trans-Blot Turbo Transfer pack; Bio-Rad). After rinsing with TBST, the membrane was incubated with 5% Blocking One-P (Nacalai Tesque) in TBST and incubated overnight at 4°C with rabbit polyclonal anti-ASIC3 antibody (1:1000, Alomone Labs), mouse monoclonal anti-phosphoserine antibody (1:1000; P5747, Sigma-Aldrich), and mouse monoclonal anti-β-actin antibody (1:200; Cat No. sc-69879, Santa Cruz Biotechnology). The membrane was incubated for 2 h at RT with horseradish peroxidase

(HRP)-conjugated rabbit anti-mouse polyclonal antibody (1:5000; Cat No. 315-035-003, Jackson ImmunoResearch, West Grove, PA, USA) or HRP-conjugated anti-rabbit antibody (1:5000; Cat No. 111-035-003, Jackson ImmunoResearch). Peroxidase activity was detected using Western Lightning ELC Pro (PerkinElmer, Waltham, MA, USA). The intensity of each band was analyzed using a ChemiDoc MP system (Bio-Rad) and normalized to β-actin or ASIC3 immunoreactivity. The band intensities for phosphorylated-serine (pSer) of immunoprecipitated samples were normalized to ASIC3 of immunoprecipitated samples. The band intensities for ASIC3 of samples without immunoprecipitation were normalized to β-actin of samples.

Statistical analyses

The Shapiro-Wilk normality test was conducted to check for normality for each group. The Brown-Forsythe test was performed to check for equality of variance. When the results of the Shapiro-Wilk normality test and Brown-Forsythe test did not show normality or homogeneity of variances ($p < 0.05$), non-parametric procedures were selected. The data are expressed as the mean ± standard error of the mean, where n represents the number of rats. An unpaired Student's *t*-test and a paired *t*-test were used to compare differences between the sham and ELS groups. Dunnett's and Tukey's post hoc tests were performed using one-way analysis of variance (ANOVA) for comparison. A two-way repeated-measures ANOVA was used to compare the time-dependent changes between the sham and ELS groups, followed by Dunnett's post hoc tests, Bonferroni's multiple comparisons tests, and Sidak's multiple comparison tests. Statistical analyses were performed using GraphPad Prism ver. 8 (GraphPad Prism Software, San Diego, CA, USA). Statistical significance was defined as a *p*-value of less than 0.05.

Results

Profiles of ELS

Profiles of orthodontic ELS was shown in Figure 1A. The orthodontic elastic was inserted between the right maxillary M1 and M2 in rats (Figure 1B). Many inflammatory cells were observed in the periodontal ligament of ELS (Figure 1C). Greater IL-1β immunoreactivity was observed on the tension side of the periodontal ligament after elastic insertion (Figure 1D). From the mCT images, the distance between M1 and M2 in the model rats gradually increased after elastic insertion (Figure 1E lower and F). The distance between the distal palatal root of M1 and alveolar bone was shorter on the pressure side and longer on the tension side than in the sham group after elastic insertion.

Periodontal acidification and mechanical pain sensitivity after elastic insertion

The MHWRT was lower than that in the sham group on days 1, 3, and 5 after elastic insertion (Figure 2A). To evaluate whether tooth movement causes acidification of the periodontal ligament, the pH of the gingival sulcus was measured. The pH in the buccal gingival sulcus of M1 was decreased at 1 and 2 days after elastic insertion compared with that in the sham group (Figure 2B). To buffer the acidity, PBS was repeatedly injected into the periodontal tissue. The PBS injections suppressed the decrease in pH after the elastic insertion (Figure 2B).

Moreover, the PBS injection significantly recovered the decreased MHWRT on day 1 after elastic insertion to the sham level (Figure 2C). To examine whether acidification in the periodontal ligament causes mechanical allodynia in mechanically forced teeth, the

MHWRT was measured following submucosal periodontal injection of solutions at pH 7.4, 7.0, and 6.5 in naive rats. The MHWRT decreased 5 min after injection of pH 6.5 acidic solution and recovered 10 min after the injection. However, injections of pH 7.4 and pH 7.0 acidic solutions did not change the MHWRT (Figure 2D), indicating that acidification in the periodontal ligament causes mechanical allodynia in the mechanically forced tooth. In addition, the pH in the gingival sulcus around M1 was 7 and 7.4, 5 min after injecting pH 6.5 and pH 7.0 acidic solution, respectively (Figure 2E), suggesting that the acidic solution was buffered by internal bicarbonate (HCO_3^-). Repetitive PBS injections did not change the mean distance between M1 and M2 in the model (Figure 2F), indicating that buffering with PBS in the periodontal tissue around M1 does not affect tooth movement.

ASIC3 contribution in mechanical allodynia in the mechanically forced tooth

In Fig. 2B, the pH in the gingival sulcus on day 1 after elastic insertion was decreased to around 7.0. ASIC3 is known to be activated by extracellular pH lower than pH 7.2 [8,13,14], suggesting ASIC3 activation in ELS. Therefore, I determined whether ASIC3 in the periodontal tissue is involved in mechanical allodynia in ELS. Injection of ASIC3 inhibitor APETx2 in the periodontal tissue of the mechanically forced tooth significantly increased the decreased MHWRT compared with vehicle (saline) injection on days 1 and 3 after elastic insertion (Figure 3A). Next, ASIC3 expression in the TG neurons innervating the periodontal tissue of mechanically forced teeth was analyzed 1 and 7 days after elastic insertion. ASIC3 was expressed in the TG neurons innervating the periodontal tissue of M1 in sham and ELS (Figure 3B), consistent with previous studies [19]. There was no significant difference in the total number of FG-labelled TG

neurons in the second branch region between the sham and ELS groups (data not shown). No difference was also observed in the percentage of FG-labelled ASIC3-IR neurons against total FG-labelled neurons between the sham and ELS groups on days 1 and 7 (Figure 3C and 3D, respectively), suggesting that molar tooth movement after elastic insertion does not alter the number of ASIC3-IR neurons innervating the periodontal tissue close to the mechanically forced tooth.

ASIC3 has been reported to be phosphorylated by protein kinase C (PKC) in the intracellular C-terminal domain (Ser-523) [20]. To examine whether ASIC3 is phosphorylated following elastic insertion, proteins from the periodontal tissue around M1 were immunoprecipitated with an anti-ASIC3 antibody, and phosphorylated-ASIC3 was detected by anti-pSer antibody (Figure 3E). The relative amount of pSer/ASIC3 in the periodontal tissue increased in the ELS group (Figure 3F).

Discussion

In previous reports, ASIC3 was shown to be involved in the pathogenesis of orthodontic tooth movement-induced pain caused by the installation of coil-spring and orthodontic elastic between the teeth [2,19,21]. In agreement with these reports, I found that ASIC3 in the periodontal tissue contributed to tooth movement-induced pain in the ELS model. In summary, infiltration of inflammatory cells in the periodontal ligament and acidification in the periodontal tissue around the mechanically forced tooth was induced on day 1 after elastic insertion. In addition, the elastic insertion induced phosphorylation of ASIC3 in the periodontal tissue, including the cervical gingiva and periodontal ligament, without changing the number of ASIC3-IR TG neurons innervating the periodontal tissue close to the mechanically forced tooth. Importantly, buffering of the periodontal acidification attenuated mechanical allodynia in the mechanically forced tooth following elastic insertion without interfering with the distance of tooth movement.

In this study, inflammatory cells and the release of IL-1β were observed in the periodontal ligaments around M1 after elastic insertion. Previous studies have reported that macrophage-lineage cells, such as monocytes, macrophages, and dendritic cells, have been increased in elastic-inserted tooth movement models [22,23]. Inflammatory mediators, including IL-1β, stimulate osteoblasts and osteoclasts, leading to tooth movement via periodontal bone remodeling [2,24,25]. Furthermore, IL-1 receptor antagonism was shown to reduce the distance of tooth movement and the number of tartrate-resistant acid phosphatase-positive osteoclasts during orthodontic tooth movement in mice [26]. The present results and previous studies suggest that inflammation in the periodontal ligament is essential for tooth movement via bone

remodeling by osteoclasts and osteoblasts. In this study, the pH in the gingival sulcus around M1 decreased on day 1 after elastic insertion. Repetitive submucosal injection of PBS in the periodontal tissue around the mechanically forced tooth suppressed the decrease in pH, indicating that PBS injection can buffer the periodontal acidification. Application of orthodontic force to the teeth has been shown to decrease the cross-sectional areas of blood vessels on the pressure side of the periodontal ligament of force-received teeth, leading to ischemia [2,6]. Ischemia/hypoxia induces anaerobic respiration, causing an increase in CO_2 concentration and local acidosis [2]. Since osteoclasts release protons for bone decalcification, the protons may also be involved in the periodontal pH decrease.

ASIC3 and TRPV1 are known as proton sensors [27]. These two ion channels are activated by a decline in extracellular pH to lower than pH 7.2 and 5.3, respectively [8,14,26]. In this study, the pH in the gingival sulcus decreased from over 7.4 to approximately 7.0, on day 1 after elastic insertion, which is sufficient acidification to activate ASIC3 [14]. Injection of acidic solution (pH 6.5) into the periodontal tissue around M1 of the naive rats decreased the MHWRT in M1 at 5 min, which was returned to the pre-injection level at 10 min after the injection. In contrast, injection of acidic solution at pH 7.0, almost the same acidity as that of the periodontal tissue after elastic insertion, did not affect the MHWRT. Moreover, the pH in the gingival sulcus was increased to approximately 7.0 at 5 min after injection of acidic solution of pH 6.5, and it almost returned to the physiological level (pH 7.4) at 10 min after the injection. Likely, the injected low-pH solution was immediately buffered by an internal buffer, such as HCO_3^-. Together with previous data, the present results suggest that periodontal acidification around pH 7.0 after elastic insertion activates ASIC3 expressed in the

nociceptive afferents in the periodontal ligament, resulting in mechanical pain hypersensitivity in the mechanically forced tooth.

ASIC3 contains a PKC-induced phosphorylation site in the C-terminal domain (Ser-523) [20]. Phosphorylation of ASIC3 enhances the peak current of ASIC3 by modulating the interaction between ASIC3 and the adaptor protein Na^+/H^+ exchanger regulatory factor-1 [20]. ASIC3 is expressed in small and medium nociceptive neurons in the dorsal root ganglion (DRG), and its phosphorylation contributes to the channel functions associated with nociception [20]. In this study, ASIC3 phosphorylation was observed in periodontal tissue on day 1 after elastic insertion. Together with some previous findings that ASIC3 is an ion channel directly responsible for mechanical nociception [10], the results suggest that phosphorylation of ASIC3 in the periodontal nociceptive afferents enhances mechanical pain hypersensitivity in mechanically forced teeth.

ASIC3 has reported to be sensitized by $G\alpha_{q/11}$ coupled receptors such as protease-activated receptor 2 (PAR2) via phospholipase C (PLC) activation followed by PKC activation [28,29]. PAR2 is activated by proteases, including tryptase and elastase released from mast cells and neutrophils in the inflammatory state [30,31,32,33]. I observed many inflammatory cells in the periodontal ligament after elastic insertion, suggesting that ASIC3 phosphorylation occurs via PAR2 activation in the periodontal ligament. Furthermore, inhibition of IL-1 receptor 1, a receptor of the IL-1 family including IL-1β, prevented the increase of ASIC3 in the DRG in ischemia/hypoxia model mice [11]. In this study, the expression of IL-1β in the periodontal ligament on the pressure side of M1 was observed. Although the report indicates that IL-1β is involved in ASIC3 upregulation, the number of ASIC3-IR TG neurons did not change after elastic insertion in the study. It is possible that the expression of IL-1β was not sufficient to

increase ASIC3-IR TG neurons; however, further investigations are needed to clarify the involvement of IL-1β in ASIC3 phosphorylation. Recently, endothelin-1 application has been reported to enhance of ASIC3 currents in the rat primary sensory neurons [34]. Endothelin-1 is known to bind to two G-protein-coupled receptors, endothelin-A receptor (ET_AR) and endothelin-B receptor (ET_BR), and only ET_AR contributes to ASIC3 sensitization [34]. Since endothelin-1 expression in the periodontal ligament has increased 3 h after continuous loading to a rat molar tooth [35], the ASIC3 sensitization was likely to occur via ET_AR in the model.

In the present study, the decrease in MHWRT following elastic insertion was suppressed by ASIC3 inhibition in nerve terminals in the periodontal tissue. ASIC3 has mechanical sensitivity, in addition to proton sensing [2], suggesting that ASIC3 also acted as a mechano-sensor in the ELS. TRPA1, TRPV4, PIEZO1 and PIEZO2 are also known to be as mechano-sensitive channels and these are observed in the periodontal ligament [3,36,37]. TRPA1 and TRPV4 have been activated/sensitized during inflammation, which caused inflammatory pain [38]. In agreement with previous studies [2,25], I observed inflammation in the periodontal ligament following continuous orthodontic mechanical force to tooth. Therefore, these various mechano-sensitive channels as well as ASIC3 likely contributes to the mechanical allodynia in ELS.

I used APETx2 as an ASIC3 inhibitor. APETx2 is known as a selective inhibitor ASIC3, which also has been reported to attenuate the tetrodotoxin-resistant voltage-gated sodium channel 1.8 ($Na_v1.8$) activity in DRG neurons [39]. $Na_v1.8$ is localized in primary afferent nociceptors and contributed to producing and propagating action potentials [40]. Possibly, the administration of APETx2 into the periodontal tissue affected negatively to $Na_v1.8$ activity in the present study. Further study is needed to clarify this possibility.

Mechanical allodynia in mechanically forced teeth continued until day 5 after the elastic insertion. Since the significant decrease in pH in the gingival sulcus was continued only 2 days after insertion, other factors are probably involved in the long-lasting mechanical allodynia. The orthodontic force increases nerve growth factor (NGF) in the nerve endings in the periodontal ligament, and local neutralization of NGF was shown to result in a depressed bite force, an indicator of mechanical allodynia [7]. The orthodontic force also induces oxidative stress in the periodontal ligament, causing orthodontic pain [3]. Presumably, NGF and oxidative stress are also involved in mechanical allodynia in mechanically forced teeth.

I found that orthodontic force causes mechanical allodynia in mechanically forced tooth, most likely because of ASIC3 activation in the nociceptive afferents in the periodontal tissue by mechanical stimulation to the tooth and acidification. Moreover, ASIC3 excitability was probably enhanced by ASIC3 phosphorylation after elastic insertion. Clinically, nonsteroidal anti-inflammatory drugs (NSAIDs) are prescribed to patients suffering from orthodontic pain. However, tooth movement is inhibited by NSAIDs by reducing the number of osteoclasts [41]. This study ameliorated orthodontic pain without preventing tooth movement by buffering the periodontal acidity induced by orthodontic treatment. This could lead to effective orthodontic pain management without undesirable side effects.

Acknowledgements

I am grateful to Prof. Mitsuru Motoyoshi for giving the opportunity to perform this study, Prof. Masamichi Shinoda and Assist. Prof. Suzuro Hitomi for their instruction of this study. I also thank colleagues in Department of Orthodontics for their support and colleagues in Department of Physiology for their technical advice and assistance.

References

1. Zhang M, McGrath C, Hagg U (2006) The impact of malocclusion and its treatment on quality of life: a literature review. Int J Paediatr Dent 16, 381–387.

2. Long H, Wang Y, Jian F, Liao LN, Yang X, Lai WL (2016) Current advances in orthodontic pain. Int J Oral Sci 8, 67–75.

3. Morii A, Miyamura Y, Sago MI, Mizuhara M, Shikayama T, Naniwa M, Hitomi S, Ujihara I, Kuroishi KN, Gunjigake KK, Shiga M, Morimoto Y, Kawamoto T, Ono K (2020) Orthodontic force-induced oxidative stress in the periodontal tissue and dental pulp elicits nociception via activation/sensitization of TRPA1 on nociceptive fibers. Free Radic Biol Med 147, 175–186.

4. Wang J, Jian F, Chen J, Ye NS, Huang YH, Wang S, Huang RH, Pei J, Liu P, Zhang L, Zhao ZH, Chen QM, Lai WL, Lin YF (2012) Cognitive behavioral therapy for orthodontic pain control: a randomized trial. J Dent Res 91, 580–585.

5. Yamaguchi M, Takizawa T, Nakajima R, Imamura R, Kasai K (2009) The Damon System and release of substance P in gingival crevicular fluid during orthodontic tooth movement in adults. World J Orthod 10, 141–146.

6. Noda K, Nakamura Y, Kogure K, Nomura Y (2009) Morphological changes in the rat periodontal ligament and its vascularity after experimental tooth movement using superelastic forces. Eur J Orthod 31, 37–45.

7. Long H, Shan D, Huang R, Liu H, Zhou Y, Gao M, Jian F, Wang Y, Lai W (2019) Bite force measurements for objective evaluations of orthodontic tooth movement-induced pain in rats. Arch Oral Biol 101, 1–7.

8. Kellenberger S, Schild L (2015) International union of basic and clinical pharmacology. Xci. structure, function, and pharmacology of acid-sensing ion channels and the epithelial Na^+ channel. Pharmacol Rev 67, 1–35.

9. Li WG, Xu TL (2011) ASIC3 channels in multimodal sensory perception. ACS Chem Neurosci 2, 26–37.

10. Wemmie JA, Price MP, Welsh MJ (2006) Acid-sensing ion channels: advances, questions and therapeutic opportunities. Trends Neurosci 29, 578–586.

11. Ross JL, Queme LF, Lamb JE, Green KJ, Ford ZK, Jankowski MP (2018) Interleukin 1 beta inhibition contributes to the antinociceptive effects of voluntary exercise on ischemia/reperfusion-induced hypersensitivity. Pain 159, 380–392.

12. Holzer P (2009) Acid-sensitive ion channels and receptors. Handb Exp Pharmacol 283–332.

13. Ambalavanar R, Dessem D (2009) Emerging peripheral receptor targets for deep-tissue craniofacial pain therapies. J Dent Res 88, 201–211.

14. Deval E, Lingueglia E (2015) Acid-sensing ion channels and nociception in the peripheral and central nervous systems. Neuropharmacology 94, 49–57.

15. Wang S, Chung, MK (2020) Orthodontic force induces nerve injury-like transcriptomic changes driven by TRPV1-expressing afferents in mouse trigeminal ganglia. Mol Pain 16, 1–16.

16. Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.

17. Waldo CM, Rothblatt JM (1954) Histologic response to tooth movement in the laboratory rat; procedure and preliminary observations. J Dent Res 33, 481–486.

18. Furukawa A, Shinoda M, Kubo A, Honda K, Akasaka R, Yonehara Y, Iwata K (2018)

Endothelin signaling contributes to modulation of nociception in early-stage Tongue Cancer in Rats. Anesthesiology 128, 1207–1219.

19. Gao M, Yan X, Lu Y, Ren L, Zhang S, Zhang X, Kuang Q, Liu L, Zhou J, Wang Y, Lai W, Long H (2021) Retrograde nerve growth factor signaling modulates tooth mechanical hyperalgesia induced by orthodontic tooth movement via acid-sensing ion channel 3. Int J Oral Sci 13, 18.

20. Deval E, Friend V, Thirant C, Salinas M, Jodar M, Lazdunski M, Lingueglia E (2006) Regulation of sensory neuron-specific acid-sensing ion channel 3 by the adaptor protein Na^+/H^+ exchanger regulatory factor-1. J Biol Chem 281, 1796–1807.

21. Yang H, Shan D, Jin Y, Liang H, Liu L, Guan Y, Chen C, Luo Q, Yang Y, Lai W, Long H (2020) The role of acid-sensing ion channel 3 in the modulation of tooth mechanical hyperalgesia induced by orthodontic tooth movement. Neuroscience 442, 274–285.

22. Nakamura K, Sahara N, Deguchi T (2001) Temporal changes in the distribution and number of macrophage-lineage cells in the periodontal membrane of the rat molar in response to experimental tooth movement. Arch Oral Biol 46, 593–607.

23. Xie R, Kuijpers-Jagtman AM, Maltha JC (2011) Inflammatory responses in two commonly used rat models for experimental tooth movement: comparison with ligature-induced periodontitis. Arch Oral Biol 56, 159–167.

24. Hienz SA, Paliwal S, Ivanovski S (2015) Mechanisms of bone resorption in periodontitis. J Immunol Res 2015, 615486.

25. Kobayashi M, Horinuki E (2017) Neural mechanisms of nociception during orthodontic treatment. J Oral Sci 59, 167–171.

26. Salla JT, Taddei SR, Queiroz-Junior CM, Andrade Junior I, Teixeira MM, Silva TA, (2012) The effect of IL-1 receptor antagonist on orthodontic tooth movement in mice.

Arch Oral Biol 57, 519–524.

27. Sun WH, Chen CC (2016) Roles of proton-sensing receptors in the transition from acute to chronic pain. J Dent Res 95, 135–142.

28. Salzer I, Ray S, Schicker K, Boehm S (2019) Nociceptor signalling through ion channel regulation via GPCRs. Int J Mol Sci 20, 2488.

29. Wu J, Liu TT, Zhou YM, Qiu CY, Ren P, Jiao M, Hu WP (2017). Sensitization of ASIC3 by proteinase-activated receptor 2 signaling contributes to acidosis-induced nociception. J Neuroinflammation 14, 150.

30. Ito M, Ono K, Hitomi S, Nodai T, Sago T, Yamaguchi K, Harano N, Gunnjigake K, Hosokawa R, Kawamoto T, Inenaga K (2017) Prostanoid-dependent spontaneous pain and PAR2-dependent mechanical allodynia following oral mucosal trauma: involvement of TRPV1, TRPA1 and TRPV4. Mol Pain 13, 1–17.

31. Nodai T, Hitomi S, Ono K, Masaki C, Harano N, Morii A, Sago-Ito M, Ujihara I, Hibino T, Terawaki K, Omiya Y, Hosokawa R, Inenaga K, (2018) Endothelin-1 elicits TRP-mediated pain in an acid-induced oral ulcer model. J Dent Res 97, 901–908.

32. Ossovskaya VS, Bunnett NW (2004) Protease-activated receptors: contribution to physiology and disease. Physiol Rev 84, 579–621.

33. Zhao P, Lieu T, Barlow N, Sostegni S, Haerteis S, Korbmacher C, Liedtke W, Jimenez-Vargas NN, Vanner SJ, Bunnett NW (2015) Neutrophil elastase activates protease-activated receptor-2 (PAR2) and transient receptor potential vanilloid 4 (TRPV4) to cause inflammation and pain. J Biol Chem 290, 13875–13887.

34. Wu L, Liu TT, Jin Y, Wei S, Qiu, CY, Hu WP (2020) Endothelin-1 enhances acid-sensing ion channel currents in rat primary sensory neurons. Acta Pharmacol Sin 41, 1049–1057.

35. Sims MR (2001) Endothelin-1 expression in the microvasculature of normal and 3-hour continuously loaded rat molar periodontal ligament. Eur J Orthod 23, 647–662.

36. Gao Q, Cooper PR, Walmsley AD, Scheven BA (2017) Role of piezo channels in ultrasound-stimulated dental stem cells. J Endod 43, 1130–1136.

37. Jin Y, Li J, Wang Y, Ye R, Feng X, Jing Z, Zhao Z (2015) Functional role of mechanosensitive ion channel piezo1 in human periodontal ligament cells. Angle Orthod 85, 87–94.

38. Dai Y (2016) TRPs and pain. Semin Immunopathol 38, 277–291.

39. Blanchard MG, Rash LD, Kellenberger S (2012) Inhibition of voltage-gated Na^+ currents in sensory neurones by the sea anemone toxin APETx2. Br J Pharmacol 165, 2167–2177.

40. Bhattacharya A, Wickenden AD, Chaplan SR (2009) Sodium channel blockers for the treatment of neuropathic pain. Neurotherapeutics 6, 663–678.

41. Arias OR, Marquez-Orozco MC (2006) Aspirin, acetaminophen, and ibuprofen: their effects on orthodontic tooth movement. Am J Orthod Dentofacial Orthop 130, 364–370.

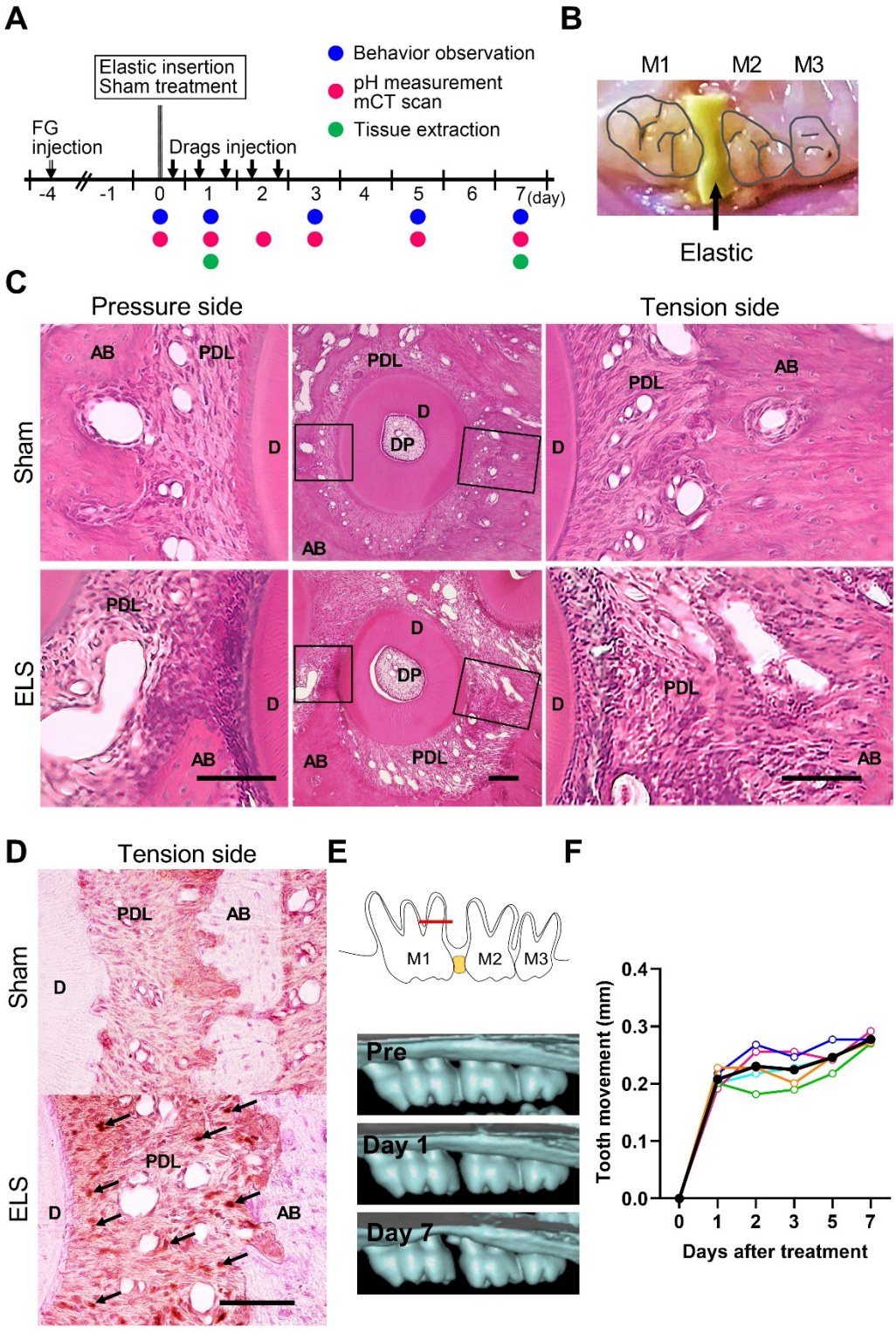

Figure 1. Profiles of orthodontic elastic-induced tooth movement model (ELS). (A) Time course of the experiments. FG, fluorogold. (B) Representative occlusal view of the elastic insertion between the right maxillary first molar (M1) and second molar (M2). (C)

Histological appearance of the ipsilateral periodontal tissue in the distal palatal root of M1 in the sham and ELS groups on day 1 following elastic insertion. The squares in the middle microphotographs indicate the area of the high magnitude microphotograph on the pressure and tension sides. Scale bars indicate 100 μm. (D) Expression of interleukin (IL)-1β in the periodontal ligament of the distal palatal root of M1 in the sham and ELS groups on day 1 after elastic insertion. The arrows represent IL-1β immunoreactivity. Scale bar indicates 100 μm. AB, alveolar bone; PDL, periodontal ligament; DP, dental pulp; D, dentin. (E) Schematic sagittal view of elastic (yellow) insertion between the right maxillary M1 and M2. Red line indicates sliced level in the distal palatal root of M1 shown in the images in (C) and (D) (upper). Reconstructed mCT images of the maxillary molars on days 0, 1, and 7 after elastic insertion (lower). (F) Daily change in distance between M1 and M2 after elastic insertion. Each colour line indicates individual distance between M1 and M2 in each rat (n = 5).

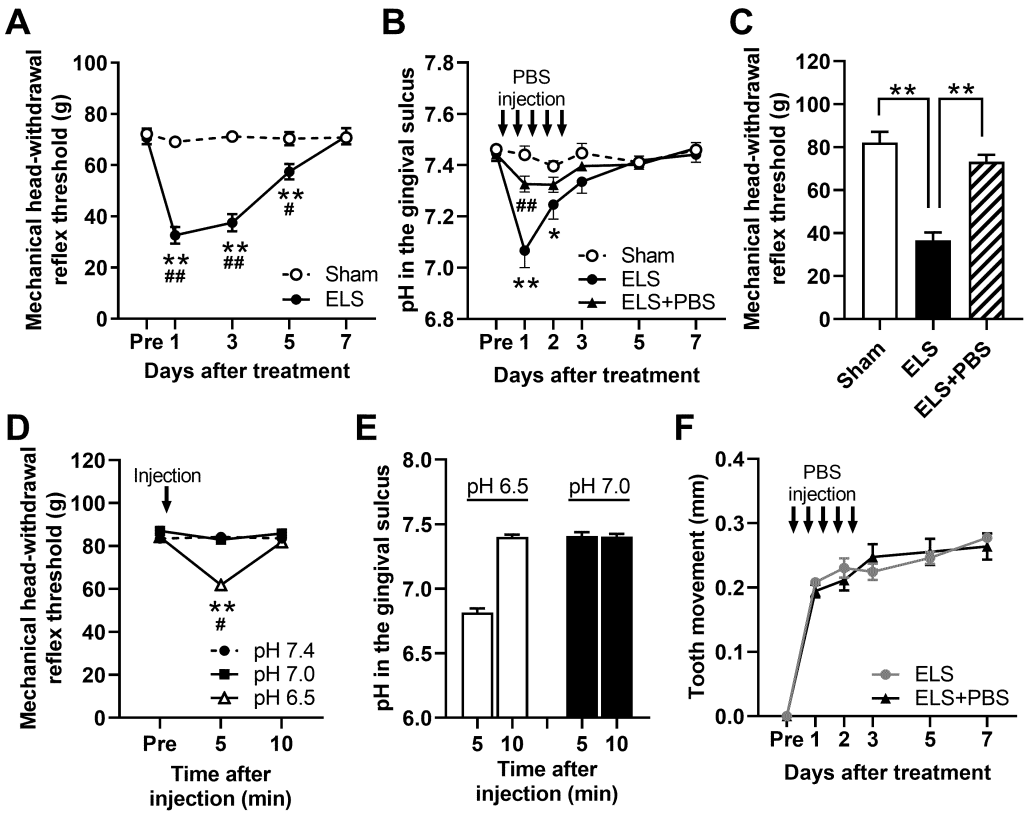

Figure 2. Change in MHWRT and pH in the gingival sulcus in sham and orthodontic elastic-induced tooth movement model (ELS). ELS + PBS, PBS administration into the periodontal tissue around the right maxillary first molar (M1) in ELS. (A) Changes in MHWRT of the occlusal surface of M1 in the ELS and sham groups (n = 9 in sham, n = 8 in ELS). $^{**}p < 0.01$, compared with sham; Two-way ANOVA followed by Bonferroni's multiple comparison test. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with pre; one-way repeated-measures ANOVA followed by Dunnett's multiple comparisons test. (B) pH in the gingival sulcus of M1 in the sham, ELS, and ELS + PBS groups (n = 6 in sham and ELS, n = 5 in ELS + PBS). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with sham and $^{\#\#}p < 0.01$ compared with ELS; Two-way ANOVA followed by Sidak's multiple comparison test. (C) MHWRT in the sham, ELS, and ELS + PBS groups on day 1 after treatments (n = 7 in sham, n = 5 ELS, n = 6 in ELS + PBS). $^{**}p < 0.01$; One-way ANOVA followed by Dunnett's multiple comparisons test. (D) MHWRT following submucosal periodontal injection of pH 7.4, 7.0, and 6.5 solution the in naive rats (n = 6 in pH 7.4 and pH 6.5, n = 8 in pH 7.0). $^{**}p < 0.01$, compared with sham; Two-way ANOVA followed by Bonferroni's multiple

comparison test. $^{\#}p < 0.05$, compared with pre; one-way repeated-measures ANOVA followed by Dunnett's multiple comparisons test. (E) Time course change in pH in the gingival sulcus of M1 following submucosal injection of pH 7.0 or 6.5 acidic solution (n = 5 in each group). (F) Daily change in distance between M1 and the right maxillary second molar in the ELS and ELS + PBS groups (n = 5 in ELS, n = 3 in ELS + PBS).

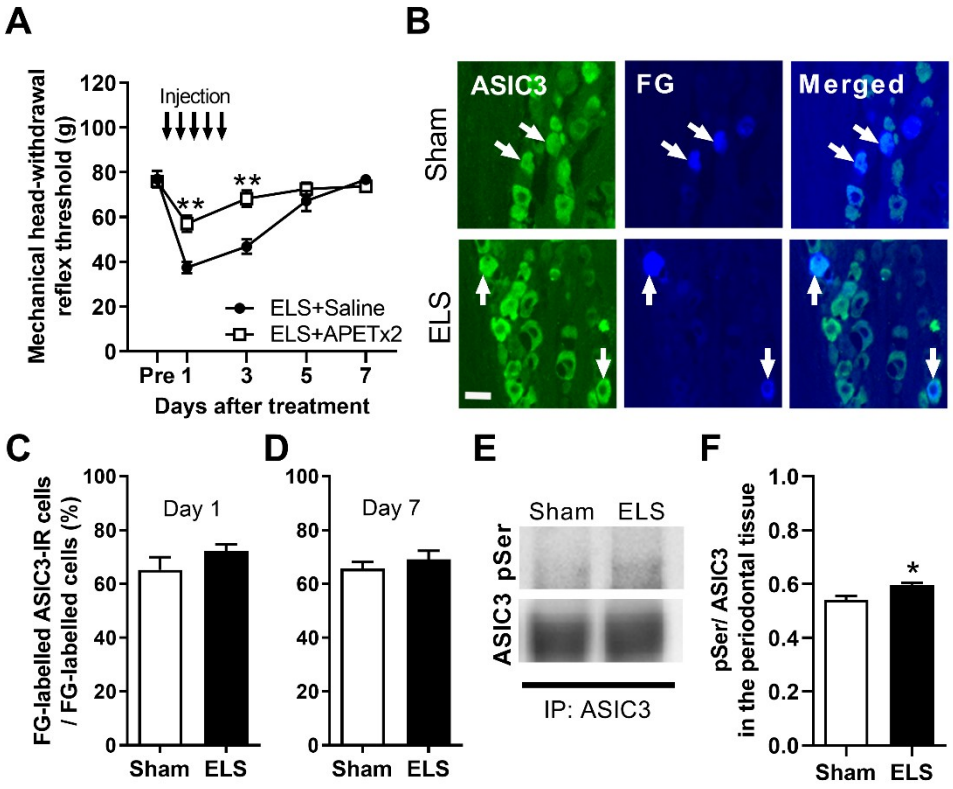

Figure 3. Involvement of ASIC3 in mechanical allodynia induced by orthodontic elastic-induced tooth movement model (ELS). (A) Daily change in the mechanical head-withdrawal reflex threshold to M1 stimulation following submucosal administration of APETx2 and vehicle (saline) in the ELS model n = 6 in each group. $^{**}p < 0.01$ compared with ELS + saline; Two-way ANOVA followed by Bonferroni's multiple comparison test. (B) Representative ASIC3 immunofluorescence images of the second branch (maxillary innervation) in trigeminal ganglion (TG) on day 1 after elastic insertion in the sham and ELS groups. Scale bars indicate 50 μm. White arrows represent FluoroGold (FG)-labelled ASIC3-immunoractive (IR) TG neurons. (C) Percentage of FG-labelled ASIC3-IR TG cells/ FG-labelled TG cells on day1 after elastic insertion. (D) Percentage of FG-labelled ASIC3-IR TG cells/FG-labelled TG cells on day7 after elastic insertion. (E) ASIC3 phosphorylation in the periodontal tissue in the ELS and sham groups. The bands represent ASIC3 and phosphorylated-serine (pSer) residue of ASIC3 in the periodontal tissue following immunoprecipitation with anti-ASIC3 antibody. (F) Relative amount of pSer/ASIC3 in the periodontal tissue (n = 5 in each group). $^{*}p < 0.05$ compared with sham, unpaired *t*-test.