Experimental tooth movement changes spatiotemporal profiles of somatosensory and insular cortical responses to electrical stimulation of the periodontal ligament

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This thesis is based on the following two articles:

1) Orthodontic force facilitates cortical responses to periodontal stimulation.
   Horinuki E, Shinoda M, Shimizu N, Koshikawa N, Kobayashi M.
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2) Sequential changes in cortical excitation during orthodontic treatment.
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Aim and Scope

Somatosensory information derived from the periodontal ligaments (PDL) plays a critical role in identifying the strength and direction of occlusal force. The orthodontic force to move a tooth often causes uncomfortable sensations, including tooth pain and discomfort. However, it has remained unknown whether orthodontic treatment modulates higher brain functions, especially cerebrocortical activity.

To address this issue, I first elucidated the cortical region involved in sensory processing of the PDL, and then examined how experimental tooth movement (ETM) changes neural activity in the cortical regions. *In vivo* optical imaging was performed to identify the cortical responses evoked by electrical stimulation of the maxillary and mandibular incisor and the first molar PDL in the rat. In naïve rats, electrical stimulation of the mandibular PDL initially evoked neural excitation in the rostroventral part of the primary somatosensory cortex (S1), the ventrocaudal part of the secondary somatosensory cortex (S2), and the insular oral region (IOR). On the other hand, maxillary PDL stimulation initially elicited excitation only in S2/IOR rostrodorsally adjacent to the mandibular PDL-responding region. The maximum responses to mandibular and maxillary PDL stimulation were observed both in S1 and S2/IOR, and most of these responses overlapped. One day after ETM of the maxillary 1st molar by Waldo's method, the maximum response to stimulation of the maxillary molar PDL showed larger and broader excitation in S2/IOR, though the initial responses were not affected.

Second, I focused on the sequential changes in the cortical excitation induced by stimulation of the maxillary 1st molar PDL during ETM of the maxillary 1st molar using a closed coil spring. Optical imaging was performed 1-7 days after ETM. The ETM model rats showed facilitative cortical excitatory propagation in comparison to that of naïve control and sham rats one day after ETM, however, the facilitation of excitation gradually recovered to the control level 3-7 days after ETM. Sham rats that received wire fixation without orthodontic force tended to enhance cortical responses, though the differences between controls and sham rats were mostly insignificant. In addition, an immunohistochemical study was performed to examine the relationship between cortical responses and expression of inflammatory cytokines in the maxillary 1st molar PDL. The peak amplitude of optical signals responding to PDL stimulation was increased in parallel to the number of interleukin (IL)-1β- and tumor necrosis factor (TNF)-α-immunopositive cells, suggesting that at least in part, the enhancement of cortical responses are induced by PDL inflammation.

These findings suggest that ETM-induced facilitation of the cortical excitatory propagation responding to PDL stimulation one day after ETM recovers to the control level within a week. The time course of facilitation of the cortical responses is comparable to that of pain and discomfort induced by clinical orthodontic treatments.

Abbreviations

ETM: experimental tooth movement
IC: insular cortex
IL-1β: interleukin-1β
IOR: insular oral region
LTP: long-term potentiation
MCA: middle cerebral artery
PDL: the periodontal ligament
RF: rhinal fissure
S1: primary somatosensory cortex
S2: secondary somatosensory cortex
TNF-α: tumor necrosis factor-α

Chapter 1

Orthodontic force facilitates cortical responses to periodontal stimulation

Introduction

Orthodontic treatment contributes to improve oral functions, including mastication and speech, in addition to dental aesthetics (Proffit et al., 2013). However, orthodontic tooth movement is often accompanied by pain due to inflammation of the periodontal ligament (Saito et al., 1991; Meikle, 2006). These sensations tend to appear approximately one day after the application of orthodontic forces and last for a few days (Jones and Chan, 1992a, 1992b; Krishnan, 2007).

The periodontal ligament not only regulates occlusal force associated with muscle spindles in the closer jaw muscles (Zhang et al., 2003; Lund and Kolta, 2006) but also perceives nociception mediated by free nerve endings of Aδ and C fibers, which originate from the trigeminal ganglion (Byers, 1985) and project to the trigeminal spinal Vi and Vc (subnuclei interpolaris and caudalis, respectively; Sugimoto et al., 1997; Watanabe et al., 2002). A portion of these nociceptive fibers contain calcitonin gene-related peptide and/or galanin, whose expression is increased by ETM (Kvinnsland and Kvinnsland, 1990; Deguchi et al., 2006). Therefore, orthodontic treatment is likely to stimulate peripheral nociceptive receptors.

Effects of continuous mechanical pressure on the teeth have been explored by immunohistochemistry for neural activity-dependent markers, such as Fos and ERK (extracellular signal-regulated kinase). ETM upregulates Fos expression and/or phosphorylation of ERK in the rat Vi/Vc secondary sensory neurons (Kato et al., 1994; Yamashiro et al., 1997; Magdalena et al., 2004; Joviliano et al., 2008; Hasegawa et al., 2012). The upregulation of Fos is also observed in the parabrachial nucleus (Yamashiro et al., 1997; Hiroshima et al., 2001; Magdalena et al., 2004; Joviliano et al., 2008), periaqueductal gray matter (Magdalena et al., 2004; Joviliano et al., 2008), central amygdala, paraventricular nucleus of the hypothalamus, and thalamus (Yamashiro et al., 1998). These immunohistochemical findings indicate that ETM facilitates neural activity in the central nervous system, especially nociception-related regions. It is also worth noting that serotonergic (Yamashiro et al., 2001; Joviliano et al., 2008) and adrenergic systems (Magdalena et al., 2004) are excited by ETM, suggesting that the descending pain modulatory system may be activated.

Although the cerebral cortex assumes the final process of nociception—that is, the identification of pain profiles, such as location, strength, and duration—cortical regions

responding to stimulation of the periodontal ligaments have not been identified in the rat. A previous study revealed that somatosensory information from the dental pulps and tongue converge into the S2/IOR (Nakamura et al., 2015). If the somatotopic organization of the somatosensory cortex is taken into account, then a similar region in S2/IOR may respond to stimulation of the periodontal ligaments.

The present study aimed to elucidate the cortical regions that respond to electrical stimulation of the incisor and molar periodontal ligament in the mandible and maxilla, and it examined how ETM modulates the spatiotemporal profile of neural excitation in the periodontal ligament-responding cortical regions by optical imaging.

Materials and Methods

The experimental protocol used in this study was approved by the Animal Experimentation Committee at Nihon University. The animal treatments were performed in accordance with the ARRIVE guidelines and the institutional guidelines for the care and use of experimental animals described in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Animals and ETM

Twenty-four male Sprague-Dawley rats (203.8 ± 9.0 g; 6-7 weeks old) were divided into a naïve control group (n = 13) and a model group of ETM (n = 11). The model group received the insertion of a piece of an orthodontic elastic module (1 mm thickness, 3M Unitek, Tokyo, Japan; Waldo and Rothblatt, 1954) between the right maxillary first and second molars (Fig. 1B) under isoflurane anesthesia (3% to 5%). Twenty-four hours after the beginning of ETM, the rats were used for the optical imaging experiment. The results obtained from rats in which the orthodontic elastic module had been off before the subsequent operation were not included for the analyses (n = 5).

Optical Imaging

The optical imaging was performed using a voltage-sensitive dye as follows (Fujita et al., 2012; Nakamura et al., 2015).

The rat received atropine methyl bromide (1.4 g/kg, intraperitoneally) and was anesthetized with urethane (1.4 g/kg, intraperitoneally). Body temperature was monitored by a rectal probe (BWT-100, Bio Research Center, Osaka, Japan) and maintained at approximately 37 °C using a heating pad. The heart rate was maintained at physiologic levels (350 to 460 beats/min). Lidocaine (2% gel, AstraZeneca, Tokyo, Japan) was applied to the incisions to ensure complete analgesia. The anesthetized animal was fixed to a custom-made stereotaxic snout flame (Narishige, Tokyo, Japan) that was tilted 60° laterally to visualize the surface of the left insular cortex (IC) using a charge-coupled device (CCD) camera (MiCAM02,

Brainvision, Tokyo, Japan), and a craniotomy was performed to expose the IC and surrounding cortices (Fig. 1A).

The voltage-sensitive dye RH1691 (1 mg/ml, Optical Imaging, New York, NY, USA), which correlates with subthreshold membrane potential changes, in 0.9% saline was applied to the cortical surface for approximately one hour. Fluorescent changes in RH1691 were measured by the CCD camera system, which was mounted on a stereomicroscope (Leica Microsystems, Wetzler, Germany). The cortical surface was illuminated through a 632-nm excitation filter and a dichroic mirror using a tungsten-halogen lamp (CLS150XD, Leica Microsystems). The fluorescent emission was captured through an absorption filter ($\lambda >$ 650-nm longpass, Andover, Salem, MA, USA). The CCD camera had an imaging area (6.4 × 4.8 mm^2) that consisted of 184 × 124 pixels.

To remove signals due to acute bleaching of the dye, values in the absence of any stimuli were subtracted from each recording. The sampling interval was 4 ms, and the acquisition time was 500 ms. Forty consecutive images in response to the stimuli were averaged to reduce the noise.

Electrical Stimulation

For electrical stimulation, bipolar electrodes made from an enamel-coated copper wire (diameter = 100 μm, Tamagawadensen, Tokyo, Japan) were inserted into the periodontal ligament (the maxillary and mandibular incisors and the first molars; Fig. 1C). To ensure the insertion of the tip of the electrode into the periodontal ligament, adjacent gingiva was incised and detached from the alveolar bone. The stem of the electrode was fixed to the crown with dental cement (Estelite Flow Quick, Tokuyama Dental, Tokyo, Japan). To obtain stable optical responses, 5 trains of voltage pulses (80 μs, 50 Hz, 0.5 to 8 V) were applied at 0.05 Hz using a stimulator unit (STG2008, Multi Channel Systems, Reutlingen, Germany). Except for the experiment to obtain stimulation intensity-dependent responses, the stimulation intensity was set at 5 V.

Histology

Naïve rats (n = 4) and the ETM models (n = 4) were deeply anesthetized with sodium pentobarbital (100 mg/kg) and perfused with saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The maxilla was removed, post-fixed overnight, decalcified in K-CX (Falma, Tokyo, Japan) for 5 days, and neutralized in 5% sodium sulphate overnight. After cryoprotection in 20% sucrose in phosphate buffer, the maxilla was sectioned sagittally with a cryostat (CM1850, Leica Biosystems, Wetzlar, Germany) at 20 μm. The sections were thaw mounted onto slides. Maxillary sections were incubated with rabbit anti-IL-1β polyclonal antiserum (1:100, Abcam, Cambridge, UK), goat anti-TNF-α polyclonal antiserum (1:100, R&D Systems, Minneapolis, MN), or rabbit anti-F4/80 monoclonal antiserum (1:100, Abcam, Cambridge, UK) overnight. IL-1β is an inflammatory cytokine, and F4/80 is a biochemical marker for macrophage. TNF-α is a cytokine that is principally produced from

macrophage. For visualization of the antibodies, the sections incubated with the anti-IL-1β antiserum and anti-F4/80 antiserum were reacted with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200, Thermo Fisher Scientific, Waltham, MA, USA). The sections with the anti-TNF-α antiserum were reacted with Alexa Fluor 488-conjugated donkey anti-goat IgG (1:200, Thermo Fisher Scientific). The sections were cover-slipped in mounting medium and examined using a confocal microscope (FV1000, Olympus, Tokyo, Japan). Negative control sections that were processed without primary antiserum displayed no specific staining. A part of sections were stained with hematoxylin and eosin to examine inflammatory cell infiltration. The final schematic figures were generated in Adobe Illustrator (CS6, Adobe Systems, San Jose, CA, USA).

Data Analysis

Changes in the intensity of fluorescence (ΔF) in each pixel relative to the initial intensity of fluorescence (F) were calculated (ΔF/F) and the ratio processed with a spatial filter (9 × 9 pixels). A significant response was defined as a signal that exceeded 7 times the standard deviation of the baseline noise. The optical imaging data were processed and analyzed using Brain Vision Analyzer (Brain Vision LLC, Morrisville, NC, USA).

To quantify the spatiotemporal profiles of excitation, the initial and maximum excitation areas and the peak amplitude at the center of the initial response were quantified (Fig. 2). The initial response was obtained by outlining the evoked excitation in the first frame that exhibited a significant increase in the optical signal. The maximum response was defined as the outline of the excitatory response in the frame with the maximum amplitude of the optical signal in the center of the initial response (Fig. 1F; 18 ms).

Data are expressed as the mean ± SEM. Student's *t* test was used to compare the area and amplitude of responses between the controls and ETM models. The dependency of stimulation intensity was examined by comparing the area and amplitude of responses to 0.5 to 8 V, and Student's *t* test was used for the comparison between the controls and ETM models. Differences were considered significant when $P < 0.05$.

Results

Electrical stimulation was applied to the periodontal ligament of the maxillary and mandibular incisors and first molars in the controls and ETM models, and optical signals in the somatosensory cortex were recorded. As previously reported, sections of hematoxylin and eosin staining exhibited multinucleated cells and segmented cells in the periodontal ligament of the ETM models but not of the controls (Fig. 1D). Furthermore, the ETM models consistently showed immunopositive cells for IL-1β, F4/80, or TNF-α, which were seldom observed in control periodontal ligament (Fig. 1E). Periodontal stimulation consistently evoked cortical excitation in the following order: (1) a restricted region in S2/IOR caudal to

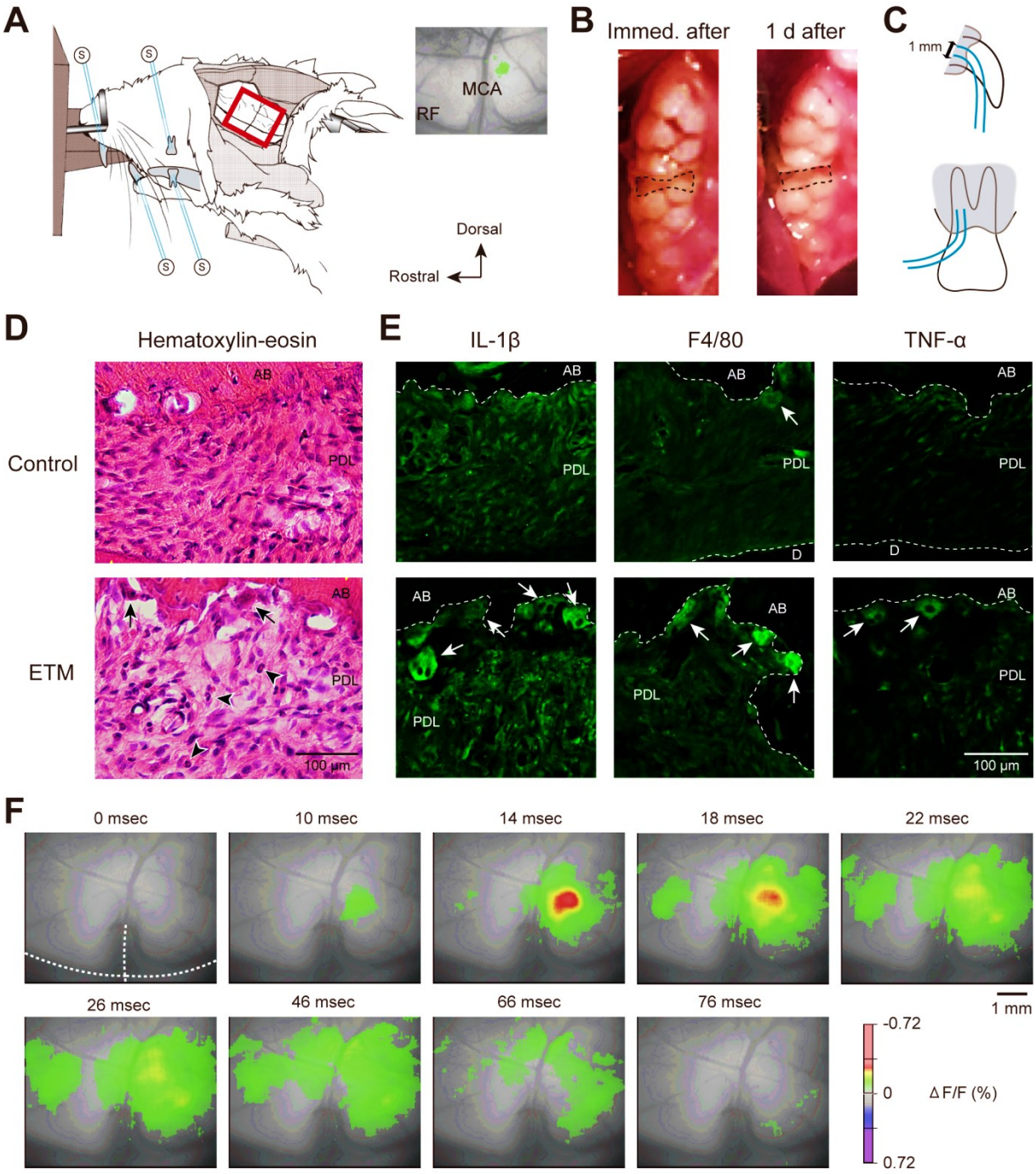

Figure 1. *In vivo* preparation for optical imaging and ETM. (**A**) A schematic drawing of the preparation for imaging of the somatosensory and insular cortices around the middle cerebral artery (MCA) and rhinal fissure (RF). Stimulation electrodes (S) were inserted into the maxillary and mandibular incisors and molars. The inset shows a CCD camera image of the cortical surface, indicated by the red square. (**B**) Photographs of maxillary molars immediately after (left) and one day after the insertion of a piece of elastic module between the right first and second molars (right). Dotted lines indicate the outlines of the upper surface of the elastic modules. (**C**) A schematic of the stimulation electrodes inserted into the periodontal ligament of the mandibular incisor and first molar. (**D**) The periodontal ligament (PDL) and alveolar bone (AB) in a control and an ETM model stained by hematoxylin and eosin. The images were obtained from the first molar. Note multinucleated cells (arrows) and segmented cells (arrowheads) in the ETM model. (**E**) Immunohistochemistry of IL-1β, F4/80, and TNF-α in the periodontal ligament of a control and an ETM model. Immunopositive cells (arrows) were identified in the ETM model. D, dentin. (**F**) Spatiotemporal pattern of the excitatory propagation corresponding to maxillary first molar periodontal stimulation (5 pulses at 50 Hz, 5 V) in a control rat. The ratio of ΔF/F was color coded, and the time from the onset of stimulation is shown at the top of each panel. F, initial intensity of fluorescence; ΔF, change in the intensity of fluorescence.

the middle cerebral artery (MCA) was activated; (2) the activated region subsequently expanded in a concentric manner; and (3) the excitation gradually decayed and disappeared in both the controls (Fig. 1F) and the ETM models. To elucidate the spatiotemporal profiles of excitation, quantitative analyses were performed using the initial and maximum responses.

Spatial Distribution Patterns of the Initial Responses

Figure 2A shows that the initial responses to periodontal stimulation of the maxillary and mandibular incisors and first molars were observed in the caudal region adjacent to the MCA. According to previous studies (Remple et al., 2003; Nakamura et al., 2015), this region corresponds to S2/IOR. In addition to S2/IOR, S1 was initially activated by mandibular incisor/molar stimulation. In S2/IOR, the initial response to maxillary periodontal stimulation was located in the dorsorostral part, whereas stimulation of mandibular periodontal ligament activated the ventrocaudal part (Fig. 2B).

Figure 3A shows a summary of the results of the initial responses in the controls. In agreement with the typical example shown in Figure 2A, the responses to maxillary periodontal stimulation were located in the dorsorostral part of S2/IOR. However, the cortical responses to mandibular stimulation were observed in the ventrocaudal part of S2/IOR; furthermore, S1 activation was frequently observed (91.7%). The mandibular incisor region tended to be located caudal to the molar region, whereas the maxillary incisor and molar regions nearly overlapped in S2/IOR. In terms of S1 responding to mandibular periodontal stimulation, the incisor and molar regions showed clear segregation (Fig. 3C).

Maximum Responses to Stimulation of the Periodontal Ligament

The maximum responses to the maxillary and mandibular periodontal stimulation were observed in both S1 and S2/IOR. Activation of S2/IOR began at the initial responding region and propagated in a concentric manner. Similarly, S1 activation was nearly independent from S2/IOR activation, but ultimately, the activated regions in S1 and S2/IOR were spatially continuous (Fig. 2A, B).

In contrast to the initial responses, the maximum responses to maxillary and mandibular periodontal ligament stimulation mostly overlapped in both S1 and S2/IOR (Figs. 2A, B, 3C).

ETM Changes Cortical Activation Map

Clinical studies have reported that orthodontic treatment for tooth movement elicits pain; the most severe pain occurs 24 to 48 hours after treatment but mostly disappears by approximately 5 days (Jones and Chan, 1992a, 1992b; Krishnan, 2007). Therefore, the present study compared cortical excitatory propagation in response to periodontal stimulation between controls and ETM models 24 hours after treatment.

The ETM model showed similar spatiotemporal profiles of excitatory propagation in the cerebral cortex: a consistent initial response in S2/IOR that expanded in a concentric manner (Fig. 2C, D). The summarized data demonstrate dorsorostral and ventrocaudal S2/IOR

activation in ETM models in response to maxillary and mandibular periodontal stimulation, respectively (Fig. 3B, D). In addition, similar results to those of the controls were observed, as mandibular incisor or molar periodontal stimulation often evoked an activation of S1 in the ETM models (Fig. 3B, D). The activated area was also comparable between the controls and the ETM models (Fig. 3E).

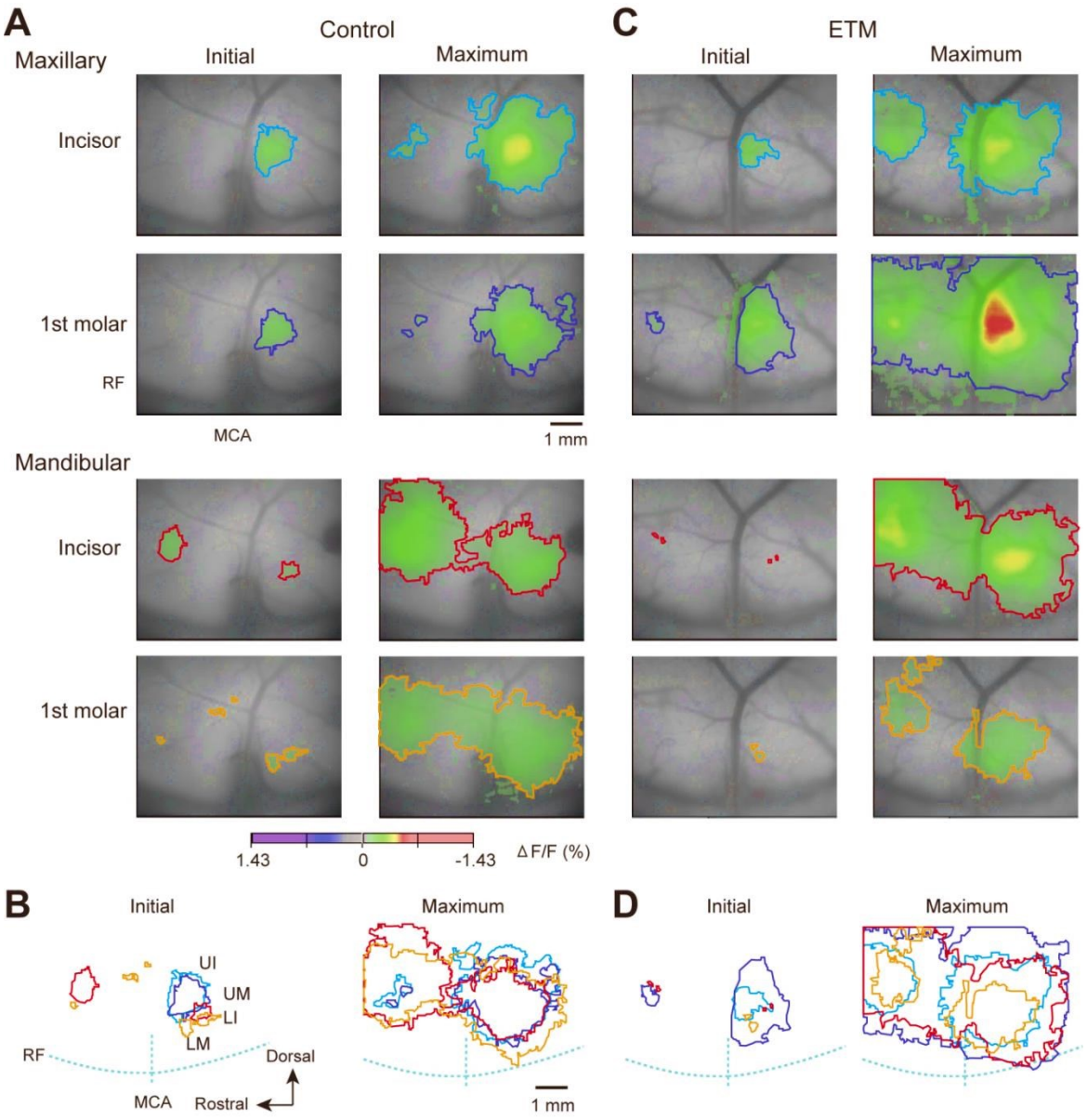

Figure 2. Optical signals in response to electrical stimulation of the periodontal ligament in a control and an ETM model. (**A**) Excitatory propagation induced by stimulation of the maxillary incisor, first molar, mandibular incisor, and first molar in a control rat (5 pulses at 50 Hz, 5 V). The first flame that exhibits excitation (initial) and that with the maximum amplitude of the optical signal at the center of excitation (maximum) are shown in the left and right columns, respectively. (**B**) Superimposed outlines of the optical responses shown in panel **A**. The colors correspond to those in panel **A**. (**C**) Excitatory propagation induced by stimulation of the periodontal ligament in the ETM models. (**D**) Superimposed outlines of the optical responses shown in panel **C**. The colors correspond to those in panel C. LI, lower (mandibular) incisor; LM, lower first molar; MCA, middle cerebral artery; RF, rhinal fissure; UI, upper (maxillary) incisor; UM, upper first molar.

In contrast to the initial responses, the maximum responses were critically altered in the ETM models. After ETM, the excitation area of maximum response to stimulation of the maxillary first molar, which was under pressure due to the insertion of an elastic module, was expanded (Fig. 2C, D). This expanded response was observed in both S1 and S2/IOR (Fig. 3B). Quantitative analysis of the activated area indicates that the ETM models showed a significant increased response to maxillary molar periodontal stimulation compared with that of the controls ($P < 0.05$, Student's t test; Fig. 3E). Excitatory areas that responded to maxillary and mandibular incisors also tended to be enlarged in the ETM models, although their P values were below the threshold for significance.

Additional analysis of the amplitude of the optical signals demonstrates that the peak amplitude of excitation in the center of the initial response in S2/IOR to maxillary molar stimulation was significantly larger in the ETM models than in the controls ($P < 0.01$, Student's t test; Fig. 3F, G). Furthermore, the excitation of the maxillary and mandibular incisors also showed larger amplitudes than those of the controls ($P < 0.05$, Student's t test; Fig. 3G).

Stimulation Intensity-Dependent Responses

The periodontal ligament involves multiple receptors, including Ruffini endings (Byers, 1985), Merkel cells (Tadokoro et al., 2002), and free nerve endings of Aδ and C fibers (Byers, 1985). In general, electrical stimulation at low and high intensity causes the activation of A and C fibers, respectively (Takemura et al., 2000; Fukui et al., 2007; Fujisawa et al., 2012); therefore, changing the stimulation intensity could reveal the type of fiber that is most sensitive to ETM. To examine this question, stimulation intensity-dependent activation were estimated in response to maxillary molar stimulation, which exhibited the largest enhancement of optical signals found in ETM models.

The excitation area and peak amplitude in response to maxillary molar stimulation showed an intensity-dependent increase in both the controls and the ETM models (Fig. 4). In terms of the initial response, there was no difference in the excitatory area between the controls and the ETM models. In contrast, the responses to larger stimulation intensity (≥ 4 V) showed a significant increase in the maximum excitation area and peak amplitude ($P < 0.05$, Student's t test; Fig. 4C-E). With low-intensity stimulation, little change was observed in the excitation area and peak amplitude between the controls and the ETM models.

Discussion

The findings in this study demonstrate that, in terms of the initial response, maxillary and mandibular periodontal stimulation activated dorsorostral and ventrocaudal regions of S2/IOR, respectively, in naïve controls and the ETM models. However, the maximum excitatory areas were observed in S1 and S2/IOR, and most areas overlapped. The ETM models showed larger

maximum responses than naïve controls, suggesting that orthodontic treatment is likely to facilitate nociceptive responses in S2/IOR.

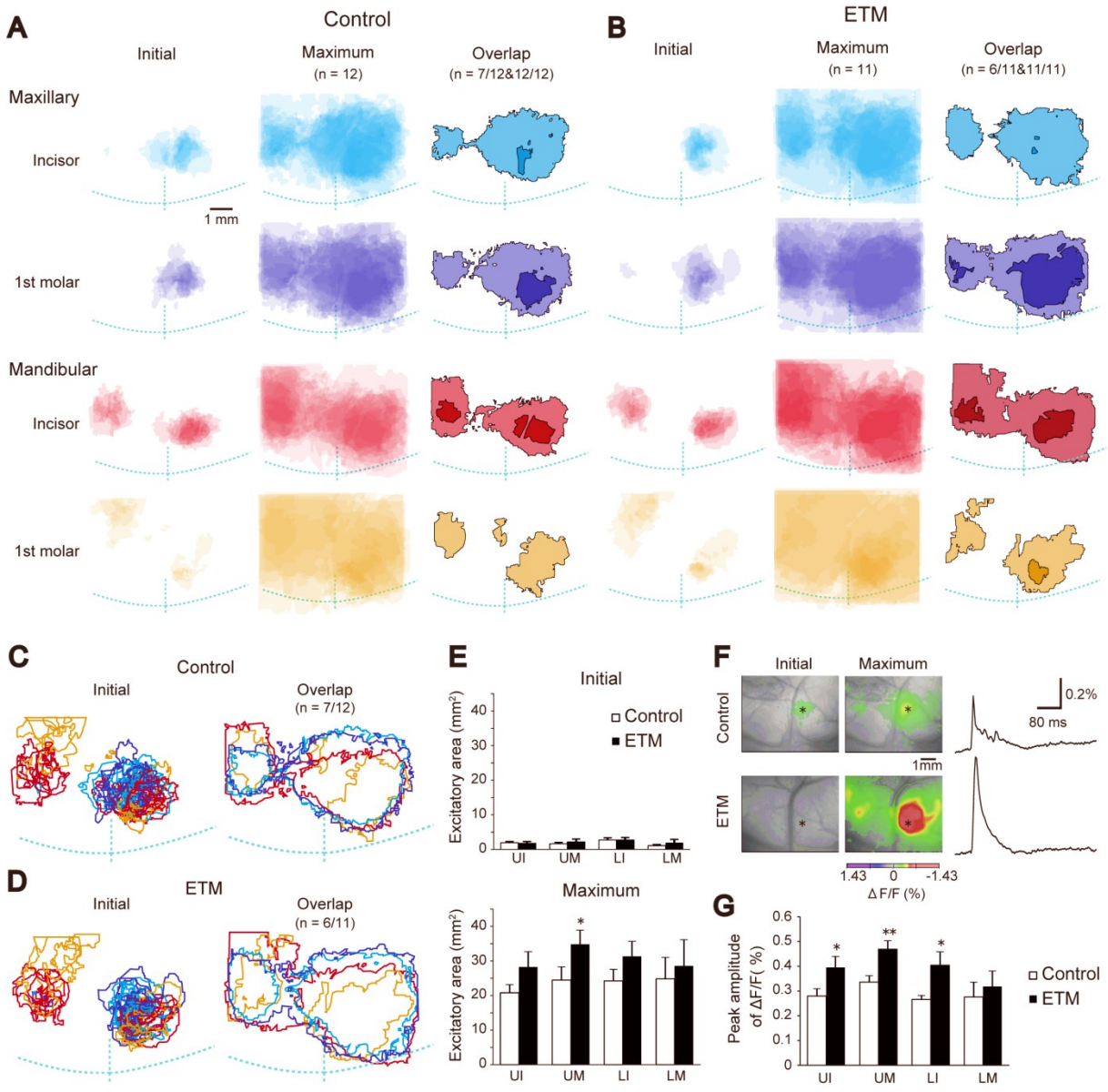

Figure 3. Spatial patterns of the initial and maximum responses to periodontal stimulation obtained from 12 controls and 11 ETM models. (**A**) Superimposed initial (left column) and maximum responses (middle column) to stimulation of the maxillary and mandibular incisors and first molar in the controls. Two-tone colored maps (right column) indicate the overlapped areas in 50% rats (light color) and 100% rats (deep color). (**B**) The initial responses and maximum responses of the ETM models. Superimposed outlines of the initial responses in the controls (**C**) and ETM models (**D**) are shown on the left. The maximum response areas, which overlapped in 7 of 12 rats among the controls (**C**) and in 6 of 11 rats among the ETM models (**D**) are shown on the right. (**E**) The effects of ETM on the initial (upper) and maximum excitation (lower) areas. The open and closed columns show the excitation areas in the controls and ETM models, respectively. *$P < 0.05$. (**F**) Spatiotemporal profiles of excitation evoked by stimulation of the maxillary first molar in a control and in an ETM model. The right traces show temporal profiles of the optical signals at the center of excitation (asterisks). (**G**) The effects of ETM on the peak amplitude of the optical responses in controls and ETM models. Responses to stimulation of the maxillary incisor, maxillary first molar, mandibular incisor, and mandibular first molar are shown as UI, UM, LI, and LM, respectively. *$P < 0.05$. **$P < 0.01$. LI, lower (mandibular) incisor; LM, lower first molar; UI, upper (maxillary) incisor; UM, upper first molar.

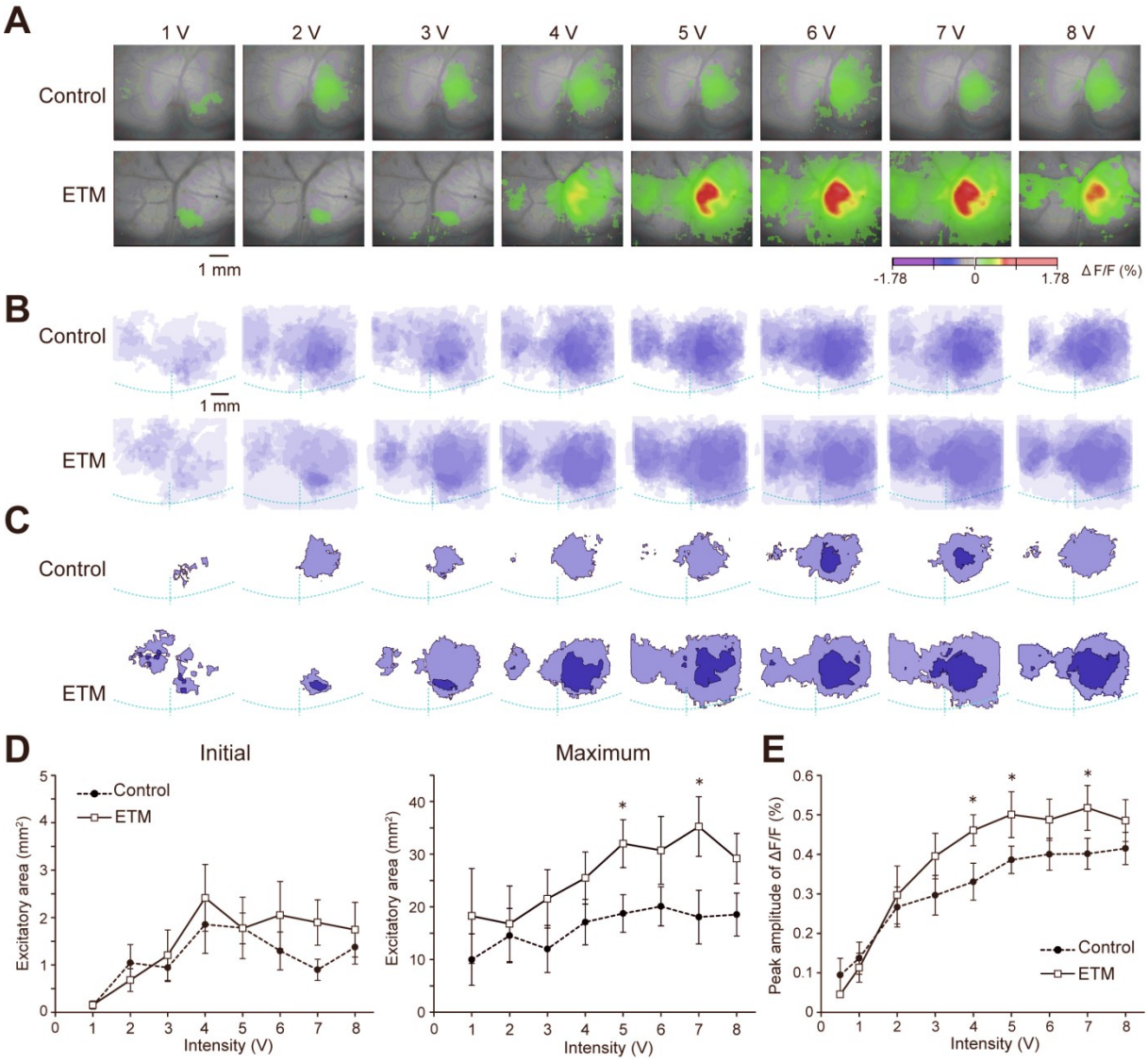

Figure 4. The intensity-dependent changes in the optical responses induced by stimulation of the maxillary first molar periodontal ligament. (**A**) Maximum optical responses to 1 to 8 V of stimulation of the maxillary first molar periodontal ligament (50 Hz) in a control (upper) and in an ETM model (lower). (**B**) Superimposed maximum responses in controls and ETM models. (**C**) Overlapped areas in 50% rats (light color) and 100% rats (deep color). (**D**) Initial and maximum excitation areas in the controls and ETM models were plotted against the stimulus intensity. **P* < 0.05. (**E**) Peak amplitude in the controls and ETM models were plotted against the stimulus intensity. Note that the peak amplitudes in response to 4 to 5 V of stimulation were significantly potentiated by ETM. **P* < 0.05.

Periodontal Sensation Is Principally Processed in S2/IOR

In agreement with previous finding that S2/IOR is the cortical region that most consistently responds to dental pulp stimuli (Nakamura et al., 2015), the present study demonstrated that periodontal stimulation invariably activated S2/IOR. Somatosensory information from oral region converges onto S2/IOR, which may integrate information to regulate oral functions. Indeed, rhythmic jaw movement is induced by repetitive electrical stimulation of IOR (Zhang and Sasamoto, 1990; Maeda et al., 2014). Interestingly, the gustatory cortex is located in the dorsal IC around the MCA, which is adjacent to the IOR (Yamamoto et al., 1984). Therefore, the spatial arrangement of periodontal sensation in S2/IOR may contribute to improving

palatability by adding texture sensation to taste.

It is worth noting the difference in the spatial profiles of the initial and maximum responses for each periodontal ligament. As previously reported, the application of an AMPA receptor antagonist to the IC surface diminishes the maximum responses (Fujita et al., 2010), suggesting that the maximum response involves a glutamatergic corticocortical circuit. Somatotopic organization in S2/IOR was controversial; the maximally activated areas nearly overlapped, whereas their initial responses showed distinctive nociceptive information processing. This finding suggests that thalamocortical inputs are somatotopically organized but that corticocortical projections are intermingled in S2/IOR. These spatiotemporal profiles may underlie the contradictory clinical finding that the identification of a tooth with fast pain is more accurate than that with slow pain (Torebjork and Ochoa, 1990; Lumb, 2002).

ETM Enhances Cortical Responses

ETM-dependent enhancement of the maximum responses was observed following middle- to high-intensity stimulation of the maxillary first molar periodontal ligament. Therefore, the ETM models may show a lower threshold to nociception, and as a result, they become sensitive to pain in response to stimuli that evoke nonnoxious somatosensation in the control. As a related speculation, enlargement of the responding area in the ETM models may reflect radiating pain. Furthermore, stimulation not only to the treated tooth but also to other teeth may induce nociception. These postulations are reasonable to explain the clinical findings that patients with orthodontic treatment often feel pain from mandibular and maxillary teeth contact.

Several studies have revealed that ETM activates serotonergic (Yamashiro et al., 2001; Joviliano et al., 2008) and adrenergic systems in the brainstem (Magdalena et al., 2004). There are 2 possible explanations for these phenomena: (1) ascending fibers project to the dorsal raphe and locus coeruleus, and (2) descending fibers from the cerebral cortex project to these regions. A previous study (Iida et al., 2010) demonstrated that the IC projects to the locus coeruleus in addition to several nociception-related areas in the brainstem. Thus, it is likely that the S2/IOR modulates noradrenergic but not serotonergic systems.

I consider that inflammation in the periodontal ligament possibly decreases the nociceptive threshold as previously suggested (Cunha et al., 1992; Luppanapornlarp et al., 2010). The histologic results of this study support this idea: expression of inflammatory cytokines and the marker of macrophage were increased in the ETM models. These inflammation-dependent biochemicals might spread to other part of oral regions and modulate cortical activities. However, it is an open issue whether periodontal inflammation without tooth movement induces facilitation of S2/IOR responses. In addition, it remains uncertain what types of receptors and fibers are activated by ETM. These questions should be examined in the future to develop an orthodontic treatment without pain.

Chapter 2

Sequential changes in cortical excitation during orthodontic treatment

Introduction

Clinical studies have reported that pain and discomfort following orthodontic tooth movement usually appear one day after applying orthodontic force, last for a few days, and disappear within a week (Ngan et al., 1989; Krishnan, 2007). A previous study reported that orthodontic force enhanced excitatory propagation in the S1 and the S2/IOR responding to electrical stimulation of the PDL one day after ETM (Horinuki et al., 2015). These facilitative effects on the cortical excitation may reflect higher sensitivity to pain responding to nonnoxious stimuli and radiating pain.

Recently, Sood et al. (2015) have demonstrated that orthodontic tooth movement induces neuroplastic changes revealed by intracortical microstimulation in the face primary motor and somatosensory cortices. The significant changes are observed in rats that receive continuous orthodontic force for one day, 7 days, and 28 days. Taking into account of the previous finding that tetanic stimulation of the IC effectively induces long-term potentiation (LTP) of excitatory propagation (Mizoguchi et al., 2011), there is a possibility that orthodontic force may cause neuroplastic changes in S2/IOR. If this is the case, orthodontic treatment might cause continuous hypersensitivity for noxious and nonnoxious stimuli to the orofacial region. However, the temporal profile of the facilitation of cortical responses triggered by orthodontic force remains an open issue.

Continuous mechanical force to teeth induces periodontal inflammation (Vandevska-Radunovic, 1999; Krishnan and Davidovitch, 2006) and facilitates the release of proinflammatory cytokines, IL-1β and TNF-α, from macrophages and endothelial cells in rat PDL (Baba et al., 2011; Davidovitch et al., 1988; Krishnan and Davidovitch, 2006) and rat maxillary alveolar bone tissue (Alhashimi et al., 2001). IL-1β and TNF-α lead to produce prostaglandins that act on silent nociceptors of C-fibers to activate second messenger pathways, which decreases nociceptor threshold (Ferreira et al., 1978; Verri et al., 2006). Therefore, PDL inflammation induced by ETM may play a role in the enhancement of cortical responses to PDL stimulation.

The present study investigated the sequential changes of activation in PDL-responding cortical regions after ETM by optical imaging to examine whether ETM induces long-term facilitation of cortical excitation. In addition, I explored the correlation between facilitation of cortical excitation and periodontal inflammation.

Materials and Methods

The experimental procedure used in this study was approved by the Animal Experimentation Committee of Nihon University. Animal experiments were performed in accordance with ARRIVE guidelines and the institutional guidelines for the care and use of experimental animals described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals and Experimental Tooth Movement

Fifty-six male Sprague-Dawley rats (202.8 ± 5.2 g; 6-7 weeks old) were divided into naïve control (n = 13), sham (n = 18), and ETM groups (n = 25). As shown in Figure 1A and C, 1-d, 3-d, and 7-d ETM received orthodontic treatment for one day, 3 days, and 7 days before optical imaging, respectively. Under isoflurane anesthesia (3% to 5%), shallow grooves were prepared around the crowns of the left and right maxillary incisors so they could be bound tightly with a stainless wire (Fig. 1A). The maxillary incisors and the left first molar were bound with a nickel-titanium coil spring (Sentalloy coil springs, 50 gf; Tomy) to apply the orthodontic force (Fig. 1A).

To examine the orthodontic force induced by the coil spring, a weight (200 g) on the electronic balance (GF-2000; A&D) was pulled up vertically with the coil spring, and the extended length of the spring was plotted against the force pulling up the weight (n = 10, Fig. 1D). The orthodontic force was estimated to be ~55 g with the coil spring set to 3 to 4 mm activated length. The distance between the first and second molars was measured by inserting the strips (50 μm thickness) to confirm that the ETM method described above certainly translocated the first molar (Fig. 1E).

The sham animals were composed of 1-d, 3-d, and 7-d sham, which received fixation of wires to the maxillary incisors and the right first molar for one day, 3 days, and 7 days before optical imaging, respectively (Fig. 1C).

Optical Imaging

The methods for optical imaging were described in Chapter 1.

Electrical Stimulation

Bipolar electrodes made from an enamel-coated copper wire (diameter = 100 μm; Tamagawadensen) were inserted into the mesial PDL of the maxillary first molars (Fig. 1B). To obtain stable optical responses, 5 trains of voltage pulses (80 μs, 50 Hz, 0.5 to 8 V) were applied at 0.05 Hz using a stimulator unit (STG2008, Multi Channel Systems, Reutlingen, Germany).

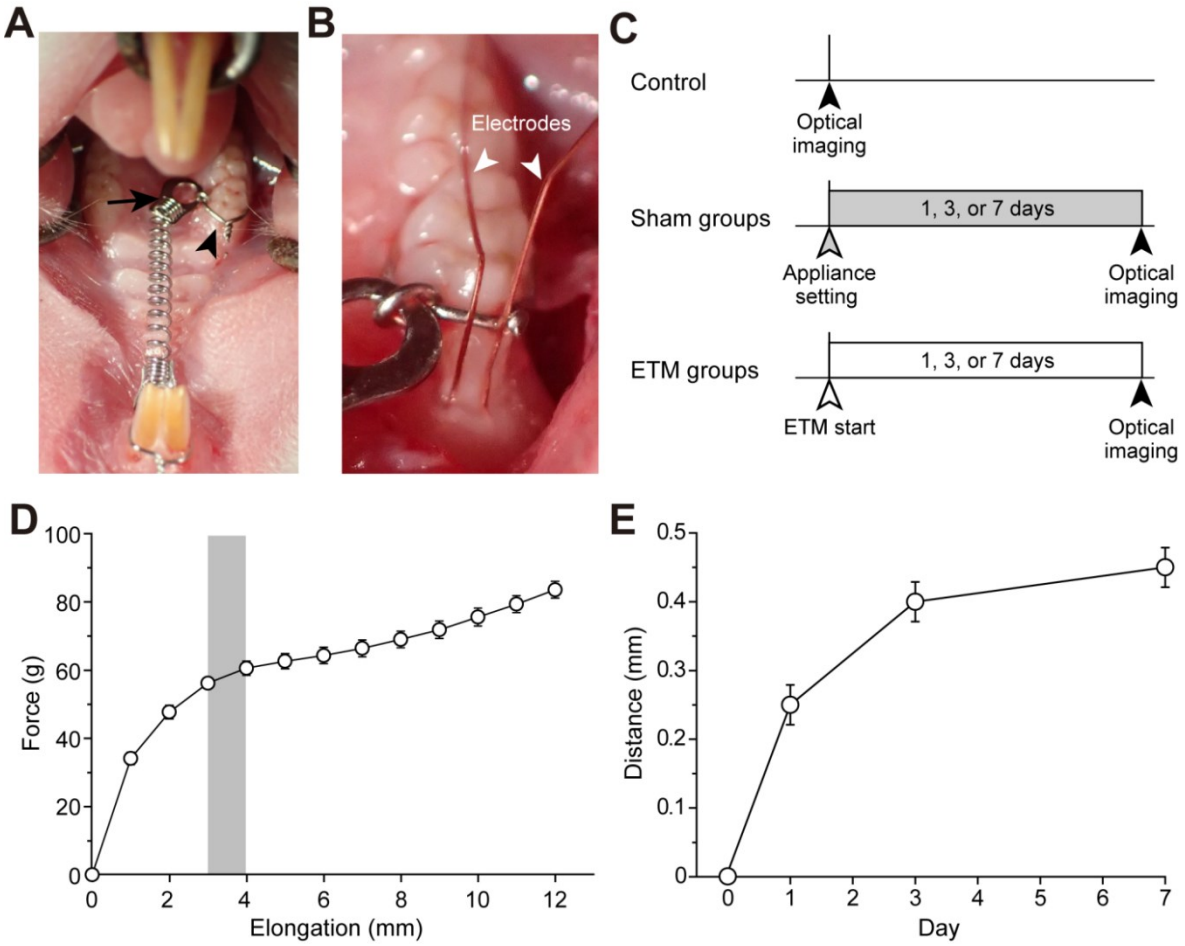

Figure 1. Methodology for experimental tooth movement. (**A**) A nickel-titanium coil spring fixed to the maxillary incisors and first molar. The head of the coil spring (arrow) was bonded to the cervical wire (closed arrowhead) of the first molar with dental cement to avoid rotation. (**B**) Stimulation electrodes (open arrowheads) were inserted into the medial PDL of the first molar. The photographs of (**A**) and (**B**) were taken without bonding using dental cement to show the fixation method clearly. (**C**) The scheme for the experimental schedule of sham treatment (gray stripe with gray arrowhead) and ETM (open stripe with open arrowhead) and optical imaging (closed arrowhead). (**D**) The load-deflection curve of the nickel-titanium coil springs (n = 10). The coil springs were used with 3 to 4 mm (shadowed region) elongation for ETM. (**E**) The distance between the maxillary first and second molars after application of the coil-spring in ETM models (n = 3).

Histology

A subgroup of rats was perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) after optical imaging to evaluate the expression of IL-1β and TNF-α in PDL. The methods for immunohistochemistry were same as those in Chapter 1 except for the secondary antiserum, Alexa Fluor 568-conjugated donkey anti-goat IgG (1:200; Thermo Fisher Scientific), to visualize TNF-α-immunopositive cells.

Data Analysis

The methods for data analysis of optical imaging were described in Chapter 1.

The number of IL-1β and TNF-α immunopositive cells in PDL was counted in the area between the mesial and distal roots, because the wire application to the crown induced inflammation in the adjacent PDL.

Data are expressed as mean ± SEM. Student's *t* test with Bonferroni correction was used to compare the area and amplitude of responses between controls, 1-d/3-d/7-d sham, and 1-d/3-d/7-d ETM (3 pairs for comparison): controls vs. sham, controls vs. ETM, and sham vs. ETM. Differences were considered significant when $P < 0.016$.

Results

The present study elucidated sequential changes in cortical excitation and IL-1β and TNF-α expression in controls, sham, and ETM models. Optical imaging focused on S1 and S2/IOR, where cortical excitation occurred in response to electrical stimulation of the mesial PDL of the maxillary first molar (Fig. 2). For evaluation of the effect of ETM, the initial and maximum responses were analyzed quantitatively (see the Materials and Methods).

Spatial Profiles of Cortical Excitation following ETM

In controls (n = 13), electrical stimulation of PDL evoked the initial response in the dorsorostral region of the cross point of the RF and MCA (Fig. 2A), where the somatosensory information of dental pulp and PDL converge (Horinuki et al., 2015; Nakamura et al., 2015; Nakamura et al., 2016). Histologically, the region corresponds to S2/IOR (Nakamura et al., 2015). The excitation then propagated in a concentric manner, and simultaneously, another excitation emerged in S1.

Both sham and ETM groups showed similar initial responses evoked by PDL stimulation in S2/IOR, and the maximum responses expanded in a concentric manner (Fig. 2B). However, the area and peak amplitude of the maximum responses were enhanced in comparison to those in controls (Fig. 2B, C). Among these groups, 1-d ETM showed the largest facilitation of cortical excitatory propagation. These facilitative effects recovered in the 7-d sham and 7-d ETM. These findings were quantitatively analyzed in the following sections.

Intensity-Dependent Spatial Profiles of S2/IOR Excitation

Electrical stimulation at low and high intensities is considered to activate A and C fibers, respectively (Takemura et al., 2000; Fukui et al., 2007) and, therefore, the dependency of stimulation intensity may provide additional information of ETM-induced changes in cortical excitatory propagation.

Figure 3A-G shows 2-tone colored maps that indicate the overlapping areas of the maximum responses in > 50% of rats (gray) and 100% of rats (black) for controls, sham, and ETM models. Stimulation at 1 V induced faint activation in S2/IOR in controls (Fig. 3A), and this activated region in S2/IOR tended to locate ventral to those activated by stimulation at ≥ 2 V.

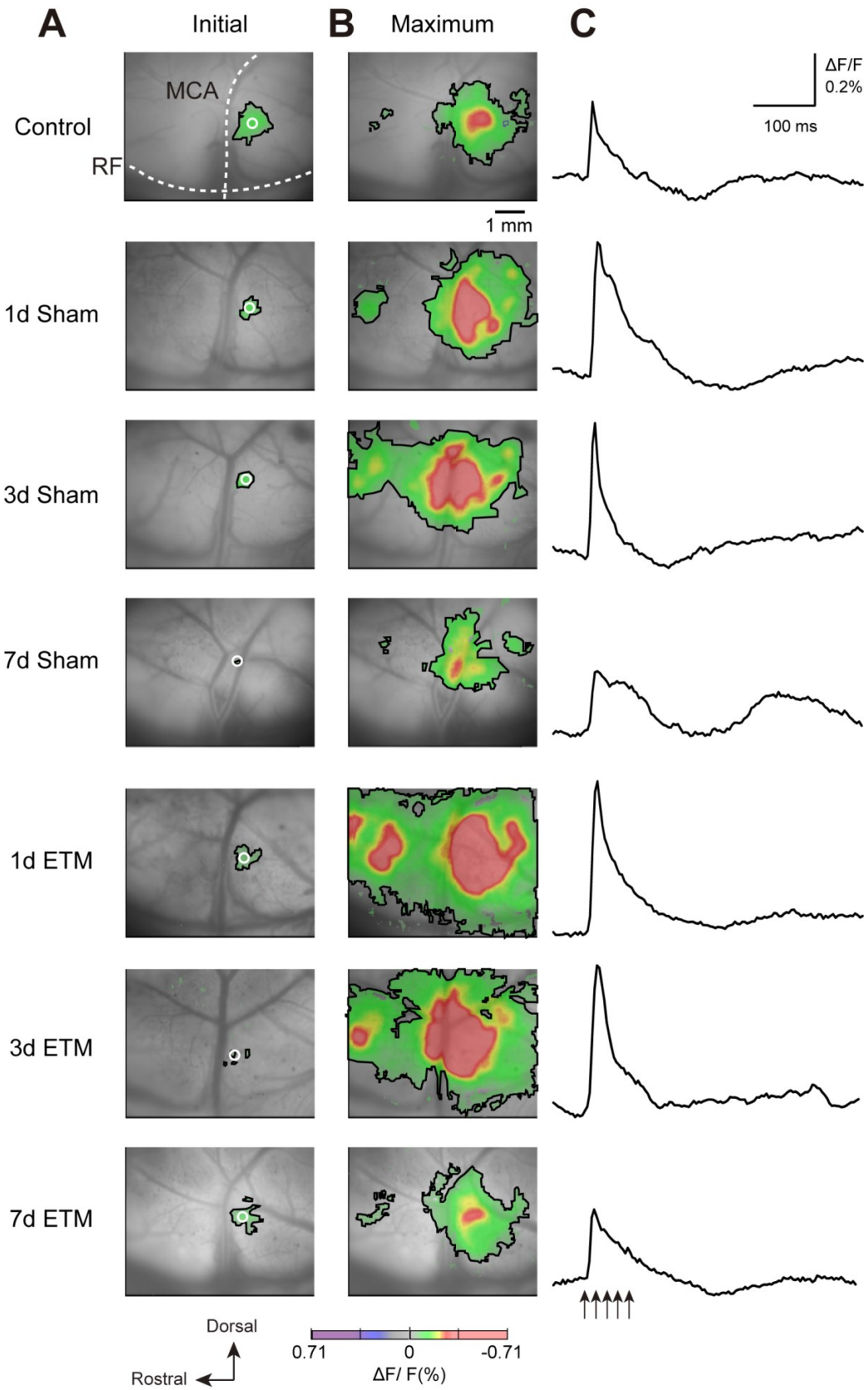

Figure 2. Color-coded optical signals evoked by electrical stimulation to the maxillary first molar PDL in control, sham, and ETM models. (**A**) Initial responses as the first flames that exhibit significant excitation. (**B**) Maximum responses as the maximum amplitude of the optical signal at the center of excitation. The excitation outline was traced with black. (**C**) The temporal profiles of optical signals at the center of excitation marked with white circles in (**A**). Train pulses of electrical stimulation (5 arrows) are shown in the bottom trace. The dotted lines indicate the MCA and RF.

In 1-d and 3-d ETM, the excitatory area expanded in comparison to controls, but the center region of excitation in S2/IOR was similar to controls (Fig. 3E, F). In addition, 1-d and 3-d ETM showed distinct excitation in the rostral part of S1. These characteristic features of excitatory propagation were also found in 1-d and 3-d sham (Fig. 3B, C).

In contrast to 1-d and 3-d sham and ETM models, 7-d sham and 7-d ETM showed a substantially similar pattern to controls (Fig. 3D, G). The intensity-dependent shift of the center region of excitation was also observed in both sham and ETM models.

Excitatory Propagation Expands following ETM

The area of the initial response of ETM models showed little difference in comparison to those of controls and sham groups except for the responses to 6 to 7 V stimulation (Fig. 3H-J). In contrast, the maximum response evoked by larger stimulation intensities (≥ 3 V) showed a significant increase in 1-d ETM compared with controls ($n = 9$, $P < 0.0003$–0.016, Student's t test with Bonferroni correction), even though the difference between sham and ETM groups was insignificant (Fig. 3H).

Similar to 1-d ETM, 3-d ETM showed a larger maximum excitation area than controls at a 6 V stimulation intensity ($n = 7$, $P < 0.003$, Student's t test with Bonferroni correction). However, there were no significant differences between controls and 3-d ETM at other stimulation intensities ($P > 0.07$, Student's t test with Bonferroni correction). Furthermore, no significant difference in the maximum excitation area was observed between 3-d sham and 3-d ETM.

The excitatory area of the maximum response in 7-d ETM was substantially the same as those in controls ($n = 9$, $P > 0.27$, Student's t test with Bonferroni correction) and in 7-d sham ($n = 4$, $P > 0.11$, Student's t test with Bonferroni correction).

Enhancement of the Peak Amplitude following ETM

The peak amplitude in the center of the initial response in S2/IOR following the maxillary first molar stimulation was analyzed (Fig. 3H-J). The peak amplitude was significantly increased at larger stimulation intensities (≥ 3 V) in 1-d ETM compared with those in controls ($n = 9$, $P < 0.0003$–0.016, Student's t test with Bonferroni correction). In comparison to those in 1-d sham ($n = 7$), 1-d ETM showed the larger peak amplitude in response to 4 and 8 V ($P < 0.016$, Student's t test with Bonferroni correction). The peak amplitude of 3-d ETM partially recovered to control values, and there was no significant difference among controls ($n = 12$), 3-d sham ($n = 7$), and 3-d ETM ($n = 7$, $P > 0.07$, Student's t test with Bonferroni correction). The curve of the peak amplitude against the stimulation intensity of 7-d ETM was similar to those of controls ($n = 9$, $P > 0.26$, Student's t test with Bonferroni correction) and 7-d sham ($n = 4$, $P > 0.16$, Student's t test with Bonferroni correction), suggesting that ETM-induced facilitation of excitation in S2/IOR recovers to control levels within a week.

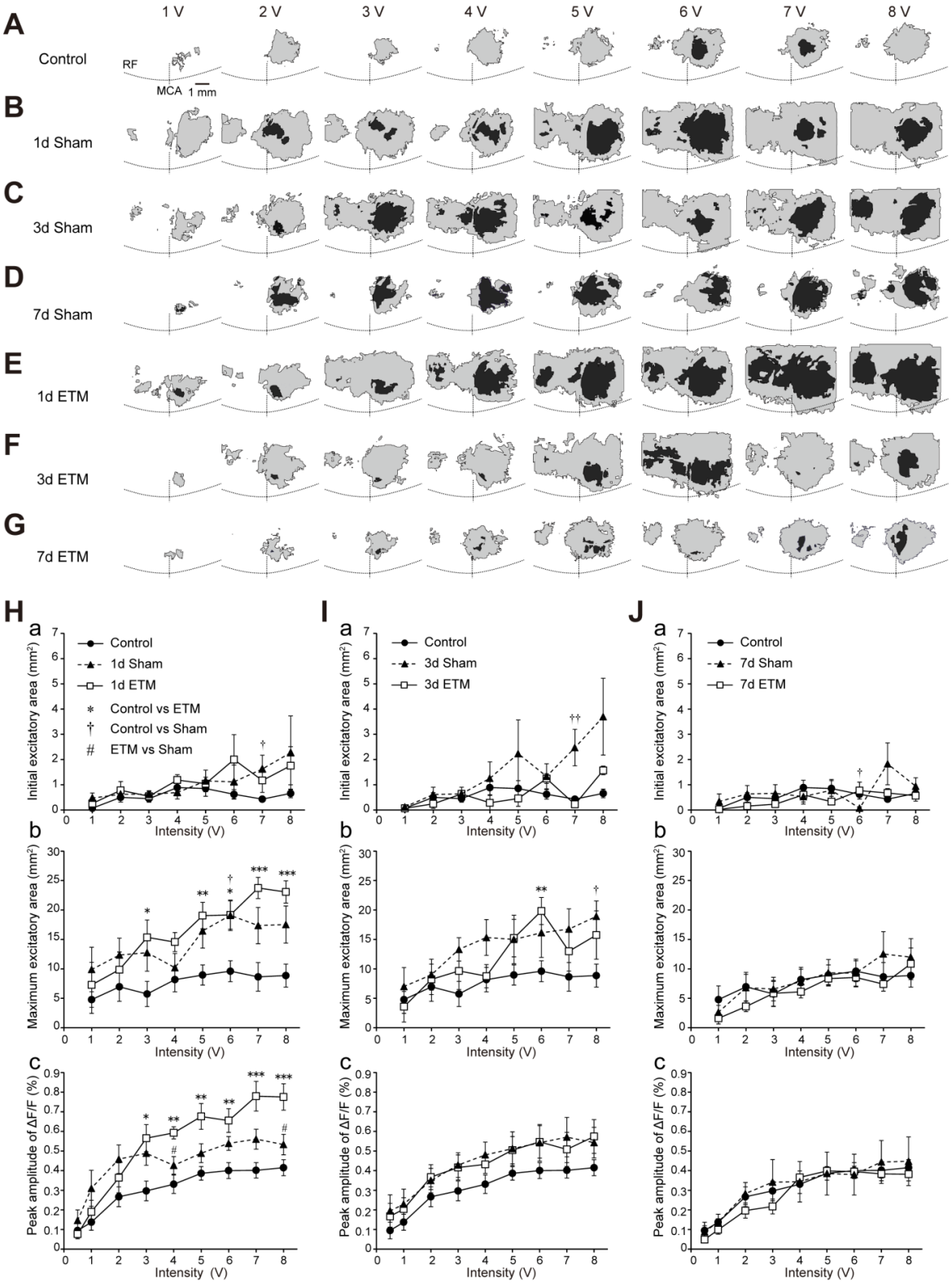

Figure 3. Intensity-dependent changes in optical responses evoked by the stimulation of the maxillary first molar PDL. (**A**) Overlapped excitation areas in 50% rats (gray) and all rats (black) in response to 1 to 8 V stimulation intensity in controls (n = 12). (**B–D**) Overlapped areas in 1-d (**B**, n = 7), 3-d (**C**, n = 7), and 7-d (**D**, n = 4) sham rats. (**E–G**) Overlapped areas in 1-d (**E**, n = 9), 3-d (**F**, n = 7), and 7-d (**G**, n = 9) ETM models. (**H**) The area of the initial (**a**) and maximum responses (**b**) and the peak amplitude obtained from the center of the initial responses (**c**) in controls, 1-d sham, and 1-d ETM models are plotted against stimulus intensity. (**I**) Those in controls, 3-d sham, and 3-d ETM models. (**J**) Those in controls, 7-d sham, and 7-d ETM models. *P < 0.016, **P < 0.003, ***P < 0.0003 between controls and ETM models. †P < 0.016, ††P < 0.003 between controls and sham groups. #P < 0.016 between sham and ETM models. The dotted lines indicate the MCA and RF.

ETM-Induced Expression of IL-1β and TNF-α

Previous studies have demonstrated that PDL inflammation caused by ETM plays a role in the facilitation of nociception (Ngan et al., 1989; Vandevska-Radunovic, 1999; Krishnan, 2007; Luppanapornlarp et al., 2010; Horinuki et al., 2015). To examine this hypothesis, the correlation between cortical activity and expression of inflammation-associated cytokines in PDL was examined by immunohistochemistry (Fig. 4).

In controls, both IL-1β and TNF-α immunopositive cells were barely detected in the PDL. In contrast, 1-d ETM showed a significant increase in the number of IL-1β and TNF-α immunopositive cells principally at the border between the PDL and alveolar bone ($n = 5$, $P < 0.016$, Student's t test with Bonferroni correction). The expression of IL-1β and TNF-α immunopositive cells was also increased in 1-d sham, indicating that the sham treatment itself induces inflammation in PDL. The 1-d ETM tended to exhibit more expression of IL-1β than did 1-d sham, although the difference in IL-1β expression between 1-d ETM and 1-d sham fell below the statistical criterion.

Three days after ETM, IL-1β and TNF-α expressions in PDL were not different from those in controls. On the other hand, 3-d sham showed a significant increase in IL-1β but not TNF-α expression in comparison with controls. The expression of IL-1β and TNF-α immunopositive cells in 7-d ETM and 7-d sham essentially recovered to the control level.

Figure 5 shows the relationship between the peak amplitude of optical signals evoked by 3 V and 7 V stimulation and the number of IL-1β and TNF-α immunopositive cells in PDL. In both stimulation intensities, the peak amplitude of optical signals tended to be increased as the number of IL-1β and TNF-α immunopositive cells increased: a linear correlation between the peak amplitude of cortical responses and expression of IL-1β and TNF-α (coefficient of determination $R^2 = 0.55$-0.85). However, several points such as 7-d ETM were out of the linear correlation.

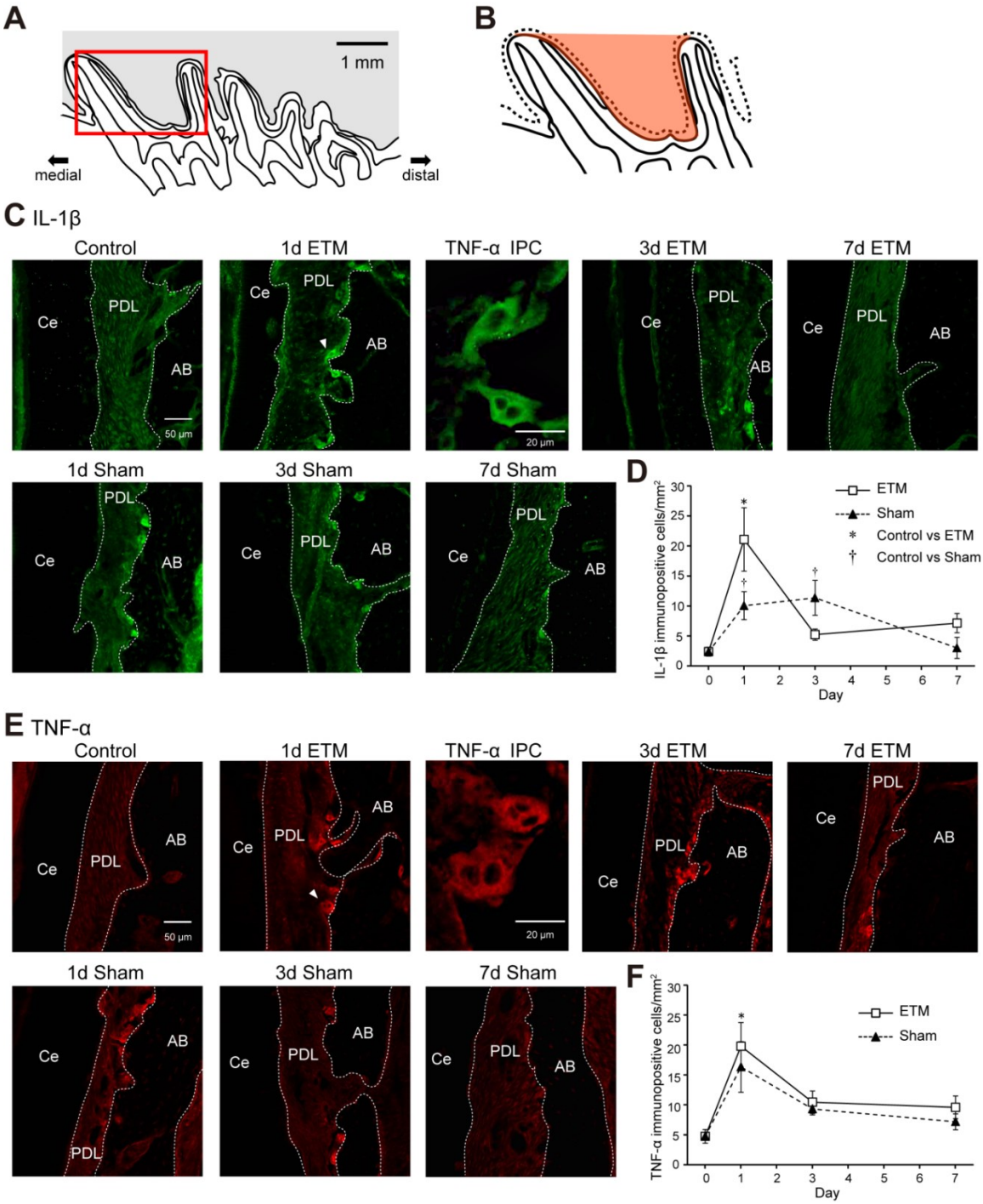

Figure 4. Immunofluorescent confocal images of IL-β and TNF-α immunopositive cells. (**A**) A schematic showing the sagittal section of the maxillary molars. (**B**) A magnification of the investigated area in (**A**). The dotted lines indicate the margin between the alveolar bone and PDL, and immunopositive cells in the colored area were counted. (**C**) Immunohistochemistry of IL-1β in PDL of control, sham, and ETM models. IL-1β immunopositive cells (IPCs) indicated by an arrowhead are expanded in the middle column. (**D**) The density of IL-1β immunopositive cells in controls (n = 6), sham (n = 5), and ETM models (n = 5). (**E**) Immunohistochemistry of TNF-α in PDL of control, sham, and ETM models. (**F**) The density of TNF-α immunopositive cells in control (n = 5), sham (n = 4), and ETM models (n = 5). AB, alveolar bone; Ce, cementum. *P < 0.016 between controls and ETM models. †P < 0.016 between controls and sham groups.

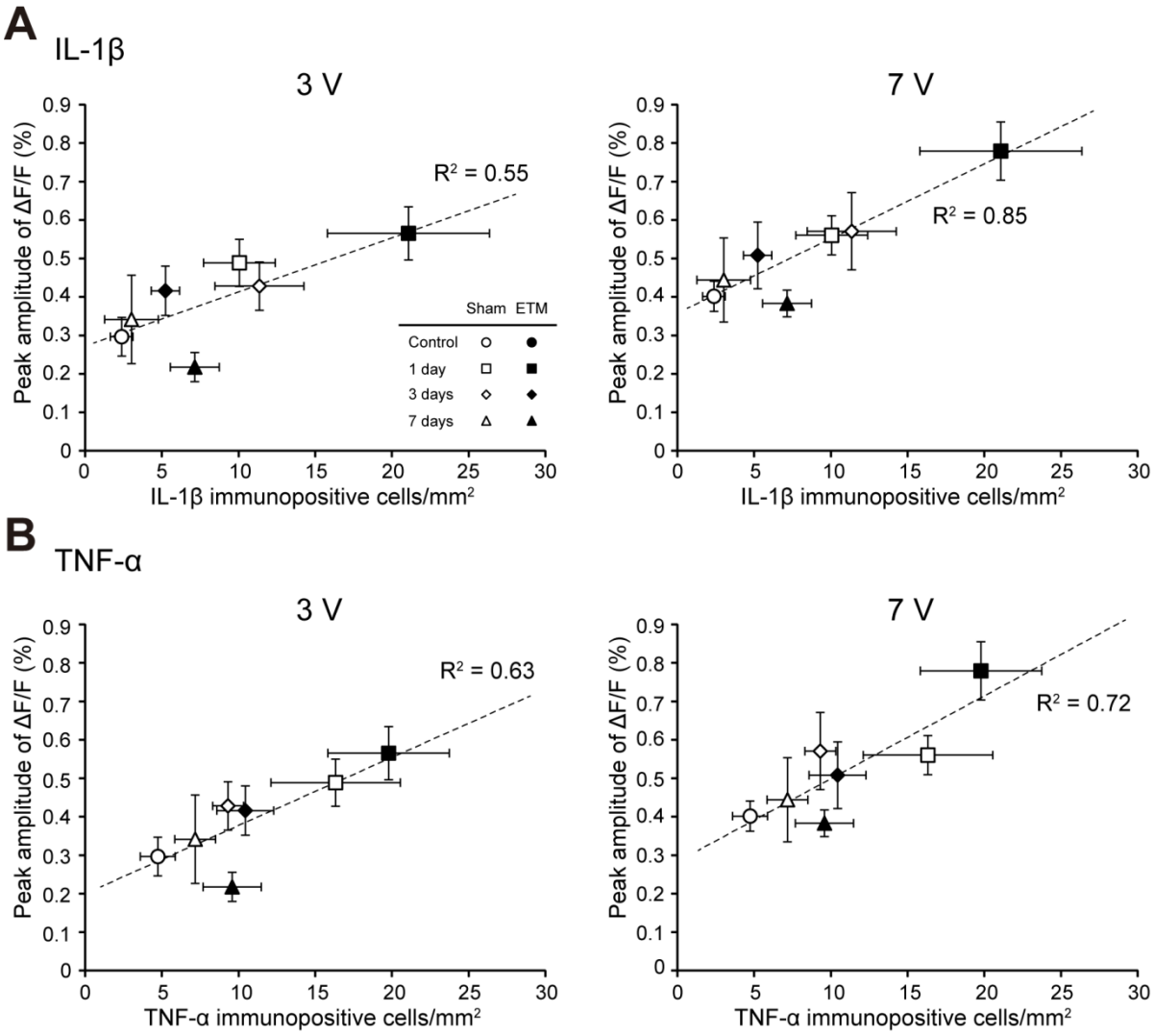

Figure 5. Relationship between the peak amplitude of cortical excitation and the number of cells expressing inflammatory cytokines. The peak amplitude evoked by PDL stimulation at 3 V (**a**) or 7 V (**b**) was plotted against the number of IL-1β immunopositive (**A**) and TNF-α immunopositive cells in control, sham, and ETM models (**B**). Dotted lines indicate regression lines.

Discussion

Facilitation of cortical responses declined in 3-d ETM, and 7-d ETM showed almost recovered responses to controls. This temporal profile suggests that ETM-induced cortical facilitation does not induce a long-lasting plastic change in S2/IOR. This finding is supported by the anatomical studies that the neuronal activity markers, Fos expression and phosphorylation of ERK, in the nociception-related brain regions are upregulated by ETM (Kato et al., 1994; Yamashiro et al., 1997; Magdalena et al., 2004; Hasegawa et al., 2012). The expression of these neuronal activity markers starts to be upregulated several hours to one day after ETM (Kato et al., 1994; Yamashiro et al., 1998; Hiroshima et al., 2001; Joviliano et al., 2008; Hasegawa et al., 2012) but returns to the control level within a few days (Fujiyoshi et al., 2000). The ETM-induced temporal profile of cortical excitation looks to contradict the

previous reports that demonstrate long-term potentiation of nociceptive responses in the peripheral nerve endings and the dorsal horn as well as cortical areas, including the somatosensory and cingulate cortices (Ikeda et al., 2006; Zhuo, 2008; Thibault et al., 2014; Sood et al., 2015a). This discrepancy might be due to less invasion with shorter duration of PDL inflammation by ETM in comparison to the previous studies described above.

Clinically, pain and discomfort in patients tend to appear one day after the application of orthodontic force, last for a few days, and disappear approximately 7 days after treatment (Ngan et al., 1989; Krishnan, 2007). This clinical observation is supported by several behavioral studies using rat models of orthodontic tooth movement (Ren et al., 2004; Liao et al., 2014; Sood et al., 2015b). For these reasons, I consider that facilitation and recovery of the cortical responses shown in this study are likely to reflect the temporal changes of pain sensation during orthodontic treatment. The age correlation between laboratory rats and humans is still an open issue—that is, a month in rats is comparable to 3 years in humans (Sengupta, 2013). Even though the time course of orthodontic pain is not necessarily parallel to the correlation above, I consider that human study is needed in the future to apply the present findings to humans.

The present study showed that not only ETM but also sham treatment increased cortical excitatory propagation between controls and 1-d ETM. This finding suggests that the sham treatment itself facilitates cortical responses to somatosensory stimulation of PDL, but its impact is smaller than ETM. The most likely mechanism for the sham treatment-induced facilitation of cortical excitation is inflammation in PDL as previously suggested (Horinuki et al., 2015).

ETM promotes production of proinflammatory cytokines such as IL-1β and TNF-α, which cause bone remodeling through activation of osteoclasts. In addition, these cytokines decrease the pain threshold (Cunha et al., 1992). IL-1β stimulates the expression of cyclooxygenase-2 and subsequently promotes release of prostaglandins (Crofford et al., 1994; Cunha et al., 2005). It has been reported that prostaglandins sensitize the nociceptor (Ferreira et al., 1978), and therefore, IL-1β is considered to play a key role in inflammatory hyperalgesia (Ferreira et al., 1988). Similarly, TNF-α is known as one of the mediators that induce mechanical hyperalgesia by producing IL-1β and CINC-1 (cytokine-induced neutrophil chemoattractant-1) (Cunha et al., 2005; Verri et al., 2006). The present study shows the roughly linear relationship between the peak amplitude of optical signals evoked by PDL stimulation and the density of IL-1β and TNF-α immunopositive cells. Therefore, it is rational to consider that the facilitation of cortical excitatory propagation is, at least in part, induced by inflammation in PDL.

Conclusions

To elucidate how orthodontic treatment modulates higher brain functions, I focused on the sequential changes in cortical excitation evoked by PDL stimulation during ETM. Noxious and nonnoxious information from PDL was processed in S1 and S2/IOR, and the projection pattern from maxillary and mandibular PDL is somatotopically organized. One day after ETM, cortical responses induced by stimulation of maxillary molar PDL were significantly enhanced. This finding demonstrates that orthodontic force facilitates the activities in nociception related cortical regions. However, the ETM-induced facilitation of cortical responses were recovered to the control level within a week, suggesting that orthodontic treatment does not induce a long-lasting plastic change in the rat somatosensory and insular cortices. In addition, the numbers of IL-1β and TNF-α immunopositive cells in PDL significantly correlate with the optical signals in S2/IOR. Therefore, it is likely that inflammation in PDL causes the facilitation of cortical excitatory propagation.

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