# Involvement of butyric acid in periodontal nociception in *Porphyromonas gingivalis*-induced periodontitis

Naoki Murakami

Nihon University Graduate School of Dentistry
Major in Periodontology
(Directors: Profs. Shuichi Sato and Masamichi Shinoda and
Assist. Prof. Suzuro Hitomi)

# Index



This thesis is composed of the following article and additional results in terms of the involvement of $P2X_3$ receptor in the periodontal mechanical nociception in *P. gingivalis*-induced periodontitis (Fig. 4).

Murakami N, Yoshikawa K, Tsukada K, Kamio N, Hayashi Y, Hitomi S, Kimura Y, Shibuta I, Osada A, Sato S, Iwata K, Shinoda M (2021) Butyric acid modulates periodontal nociception in *Porphyromonas gingivalis*-induced periodontitis. J Oral Sci. in press.

# Abstract

**Purpose:** Periodontitis progresses with chronic inflammation, without periodontal pain. However, the underlying mechanisms are not well known. Here, I examined the involvement of butyric acid (BA) in periodontal pain sensitivity in *Porphyromonas gingivalis* (*P. gingivalis*)-induced periodontitis.

**Methods:** *P. gingivalis* was inoculated into the ligature which was tied around the molar (*P. gingivalis*-L) and the gingival mechanical head withdrawal threshold (MHWT) was measured. Following *P. gingivalis*-L, the expressions of orphan G protein-coupled receptor 41 (GPR41) and $P2X_3$ receptor in trigeminal ganglion (TG) neurons were examined by immunohistochemical staining. The amount of gingival BA following the *P. gingivalis*-L was measured. The influence of BA or GPR41 antagonist (HA) on the MHWT induced by *P. gingivalis*-L or complete Freund's adjuvant (CFA) were examined.

**Results:** The *P. gingivalis*-L did not change the MHWT. GPR41-immunoreactive TG neurons were increased and $P2X_3$ receptor-immunoreactive TG neurons were decreased following *P. gingivalis*-L. The gingival BA amount increased following *P. gingivalis*-L. CFA-induced MHWT was reduced by BA injection to gingiva. Moreover, *P. gingivalis*-L-induced MHWT was decreased by HA injection.

**Conclusion:** Gingival BA modulates $P2X_3$ receptor-regulated periodontal mechanical nociception via GPR41 signaling in *P. gingivalis*-L-induced periodontitis.

# Introduction

Periodontal disease which is well known to be a chronic inflammatory disease caused by various bacterial infections originating from dental plaque causes progressive destruction of periodontal tissues such as alveolar bone [1,2]. It is well known that many inflammatory diseases generally progress with pain symptoms, although interestingly, periodontal disease often progresses without evident pain awareness [3]. Hence, many patients with periodontitis are oblivious to the progression of periodontitis, leading to severe periodontal tissue destruction, starting with delayed diagnosis and treatment of periodontitis [4]. However, it remains unclear why periodontitis progresses without pain symptoms in the periodontal tissue.

The leading pathogenic bacteria that causes periodontitis in humans is *Porphyromonas gingivalis* (*P. gingivalis*), and the releasing factors from *P. gingivalis* in dental plaque trigger chronic inflammation of the periodontal tissue [5]. Short chain fatty acids (SCFAs) including butyric acid (BA) which are metabolites of bacteria are known to be released from *P. gingivalis* in subgingival plaques and produced in large quantities during periodontal disease progression [6]. BA produced in the periodontal tissue of periodontal disease promotes the progression of periodontal disease by causing overproduction of matrix metalloproteinases or consistent damage to periodontal immunomodulatory cells [7-9]. It has been reported that orphan G protein-coupled receptor 41 (GPR41), which is the receptor of SCFAs, is expressed in primary sensory neurons [10]. This report suggests that BA signaling via GPR41 in the sensory nerve endings of the periodontal tissue may change the somatosensory characteristics of the periodontal tissue in a chronic inflammation state. Nevertheless, it is completely unknown whether BA signaling in sensory nerve endings is involved in the painless progression of periodontal tissue in periodontitis.

$P2X_3$ receptors that are opened by adenosine 5′-triphosphate (ATP) binding are expressed in primary nociceptive neurons, and ATP signaling via the $P2X_3$ receptor may be responsible for the ascending transmission of noxious mechanical stimuli [11]. Previous studies have strongly suggested the involvement of the $P2X_3$ receptor in TG neurons that innervate the orofacial region in orofacial mechanical pain hypersensitivity [12,13].

This study aimed to elucidate the mechanism by which periodontal pain is not induced in the periodontal tissue in a chronic inflammation state by examining the involvement of BA signaling and $P2X_3$ expression in primary afferent nerve endings in periodontal mechanical nociception associated with periodontitis using a *P. gingivalis*-induced periodontitis mouse model.

# Materials and Methods

## Animals

Male C57BL/6 mice (*n* = 99, 20–30 g; Japan SLC, Hamamatsu, Japan) were used for all experiments in this study. Mice were reared in a well-controlled laboratory with free intake to water and food. (light–dark cycle: 12 h/12 h, humidity: 55 ± 5%, ambient temperature: 23 ± 2°C). The Animal Experimentation Committee at Nihon University approved this study (AP18DEN016-1), and the experiments were performed in compliance with the International Association for the Study of Pain guidelines [14]. Every effort has been made to minimize distress in mice and the number of mice used.

## Establishment of periodontitis

The mouth of the mouse lying on a temperature-controlled mat (37°C) was opened by mouth opener under the anesthesia with intraperitoneal (i.p.) administration of midazolam (2.0 mg/kg; Sandoz, Tokyo, Japan), medetomidine (0.375 mg/kg; Zenoaq, Koriyama, Japan), and butorphanol (2.5 mg/kg, Meiji Seika Pharma, Tokyo, Japan). The neck of the right maxillary second molar was coiled with a 5-0 silk ligature while avoiding damage to the surrounding tissues. On days 0 to 2 after ligation, the mice were inoculated *P. gingivalis* (FDC381, $10^{10}$ colony-forming units/mL) into the silk ligature under the anesthesia (1.5% isoflurane, Mylan, Canonsburg, PA, USA) [15]. Mice inoculated with *P. gingivalis* on the silk ligature around the neck of the maxillary second molar (*P. gingivalis*-L) were defined as *P. gingivalis*-L group. Mice were separately injected Complete Freund's adjuvant (CFA; Sigma-Aldrich, St. Louis, MO, USA) into the gingival tissue with the mouth opening under deep anesthesia, as described above (CFA group) [15]. Mice that underwent the same treatment with the *P. gingivalis*-L group

except for the *P. gingivalis* inoculation to the ligature were defined as the control group.

**Measurement of mechanical nociceptive sensitivity**

Under light anesthesia (2% isoflurane, Mylan), the mouth of the mice was kept open by the mouth opener. After interruption of isoflurane inhalation, it was confirmed that the appropriate level of anesthesia was maintained by inducing the same withdrawal reflex with the same noxious stimuli applied to the hind paw. Immediately after confirming the appropriate level of anesthesia, the gingival tissue was applied graded mechanical stimulation by an electronic von Frey anesthesiometer (Bioseb, Vitrolles, France) and the lowest mechanical intensity required the induction of a head withdrawal reflex was determined as the mechanical head withdrawal threshold (MHWT) in a manner similar to previous study [15]. The mice were free to escape the mechanical stimulation. Each graded mechanical stimulus was performed at 1-minute intervals. The MHWT measurement was performed three times, and the average MHWT was determined as the MHWT for each. All MHWT measurements were completed under blinded conditions.

**High performance liquid chromatography**

Under the above-described deep anesthesia, mice were transcardially perfused with saline on day 4 following the *P. gingivalis*-L and control treatment, and the gingival tissue in each was removed. Following the homogenization of gingival tissue in distilled water, the supernatant was collected. Then, the BA amount was quantified by high-performance liquid chromatography (HPLC) as follows.

Briefly, the HPLC instrument comprised an LC-20AT pump (Shimadzu. Kyoto, Japan), a CTO-10A column oven (Shimadzu), a UV-2075 UV detector (Jasco, Tokyo, Japan), and an LC-Net II/ADC recorder (Jasco). ChromNAV Lite (Jasco) was used for data acquisition

and handling. A V-630 spectrophotometer (Jasco) was used to measure the absorbance of all sample solutions. First-grade sulfuric acid (FUJIFILM Wako, Osaka, Japan) was used as the mobile phase. To prepare the standard solution, reagent grade sodium acetate, first-grade sodium propionate, and sodium butylate were purchased from FUJIFILM Wako. Reagent grade sodium isobutylate and sodium isovalerate were purchased from Kanto Chemical. Standard solutions (1,000 mg/L) were prepared by diluting the certified standards for several short-chain fatty acids. Ethanol and absolute ethanol (reagent or HPLC grade, FUJIFILM Wako) were used as extraction reagents.

To remove proteins from the real sample solution, 300 μL of a real sample solution was centrifuged at 3,000 rpm for 60 min. After standing, the sample solution was transferred to a 5 mL of volumetric flask, and the volume was fixed at 5 mL by adding ultrapure water. Before measuring butyric acid, the absorbance of each sample solution was measured to confirm the effect of deproteinization. Only the sample solution with protein-derived absorbance below 0.2 was measured using HPLC.

**Drug administration**

Four microliters of 3-hydroxybutyric acid (HA, 50 μg/mL dissolved in 0.01 M PBS; #085-03571, FUJIFILM Wako), which is a GPR41 antagonist or 4 μL of BA (50 μg/mL) was administered once daily for 15 days to the gingival tissue after *P. gingivalis*-L or CFA treatment under the above-described light anesthesia. On the day of MHWT measurement, drug administration into the gingival tissue was performed immediately after MHWT measurement.

**Immunohistochemistry**

On day 2 before *P. gingivalis*-L or CFA, 4% hydroxystilbamidine (Fluoro-Gold [FG], Fluorochrome, Denver, CO, USA) dissolved in saline was injected to the gingival tissue to

identify the trigeminal ganglion (TG) neurons innervating the gingival tissue under the deep anesthesia described above. On day 8 following the *P. gingivalis*-L, CFA, or control treatment, the expression of GPR41, a short-chain fatty acid receptor in TG neurons innervating the gingival tissue, was immunohistochemically examined. Briefly, mice were perfused with 4% paraformaldehyde (PFA) under the deep anesthesia. After removing the TG and the gingival tissue, these samples were immersed in 4% PFA for 4 h. For cryoprotection, these samples were placed in 20% sucrose and embedded at −20°C in Tissue Tek (Sakura Finetek, Tokyo, Japan). These samples were sliced to a thickness of 15 μm, and the section was pasted onto microscope slides. The sections of the TG were rinsed and reacted with anti-GPR41 polyclonal rabbit antibody (1:100, #G250, AssayBiotech, Fremont, CA, USA) or anti-$P2X_3$ polyclonal rabbit antibody (1:1000, GTX80786, GeneTex, Irvine, CA, USA) diluted in 0.01 M PBS containing 4% normal goat serum (Sigma-Aldrich) with 0.3% TritonX-100 at 4 °C overnight. After rinsing, the sections were reacted in Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:200, #ab175471, Thermo Fisher Scientific, Waltham, MA, USA) for 2 h. After washing, the sections were cover-slipped. FG-labeled GPR41 and $P2X_3$ immunoreactive neurons in the TG were detected using a BZ-9000 system (Keyence, Osaka, Japan). Twice as strong as the average intensity of background was considered immunoreactive. Without the primary antibodies, no specific immunoreactivity was detected. The percentage of FG-labeled neurons expressing GPR41 and $P2X_3$ in five sections of TG was defined as FG-labeled GPR41 and $P2X_3$ immunoreactive neurons in each mouse, respectively. The mean percentage of FG-labeled GPR41 and $P2X_3$ immunoreactive neurons was calculated in each group.

**Statistical Analysis**

It was confirmed that all data were normally distributed and satisfy the homoscedasticity by the Shapiro-Wilk normality test and the Brown-Forsythe test, respectively. Therefore, all

data were presented as mean ± standard error (SEM). For immunohistochemistry and HPLC, dot plots were added to show individual sample sizes. Two-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni's multiple comparison test was conducted to examine the differences in MHWT. One-way ANOVA followed by Tukey's multiple comparison test was conducted to examine the difference in the number of FG-labeled GPR41 and $P2X_3$ immunoreactive neurons. For the statistical analysis of the amount of BA in the gingival tissue, Student's *t*-test was used. A $p$-value $< 0.05$ was considered significant.

# Results

**Changes in gingival mechanical sensitivity**

In the *P. gingivalis*-L group, the MHWT did not change and there was no significant difference compared to MHWT in the control group during the experimental period (Fig. 1). On day 2 after the gingival CFA injection, the MHWT in the CFA group was considerably decreased compared with the *P. gingivalis*-L or control group, and the decrease in the MHWT continued until day 14. During the experimental period, there was no motor deficits or sedation (data not shown).

**Amount of BA in the gingival tissue following the *P. gingivalis*-L treatment**

On day 4 following *P. gingivalis*-L or control treatment, chromatograms were obtained from the gingiva under optimal HPLC conditions (Fig. 2a). Prior to measuring the sample solutions of the *P. gingivalis*-L group and the control group, a standard solution containing only BA was analyzed. It was confirmed in advance that BA was detected at 16 min. Therefore, the peak indicated by the arrow is confirmed to be the peak derived from BA. Amount of BA in the *P. gingivalis*-L group (69.4 ± 15.7 mg/L) was larger than that of the control group (17.9 ± 6.9 mg/L) on day 4 after these treatments.

**Effect of BA on the inflamed gingival mechanical sensitivity**

To assess the effect of BA on the inflamed gingival mechanical sensitivity, the MHWTs were measured in the BA-injected-CFA group and the BA-injected-*P. gingivalis*-L group. In the *P. gingivalis*-L group, BA injection into the gingival tissue did not alter the MHWT during the experimental period (Fig. 2b). Although vehicle administration into the gingival tissue

considerably decreased the MHWT after CFA injection, the gingival BA recovered the decrease in the MHWT in the CFA group from day 6 onward. Motor deficits and sedation were not observed during the experimental period (data not shown).

**Expression of GPR41 in TG neurons after the *P. gingivalis*-L treatment**

The expression of GPR41 immunoreactive neurons in the TG was examined after *P. gingivalis*-L, CFA or control treatment. FG-labeled GPR41 immunoreactive neurons were detected in the TG on day 2 following *P. gingivalis*-L (30.1 ± 3.1%), CFA (20.7 ± 1.7%), or control treatment (19.2 ± 0.9%), which indicates that GPR41 immunoreactive neurons innervating the gingival tissue exist in the TG (Fig. 3a). On day 2 after *P. gingivalis*-L, the number of FG-labeled GPR41 immunoreactive TG neurons was larger than that of CFA or control treatments.

**Effect of GPR41 antagonism on the inflamed gingival mechanical sensitivity**

To assess the effect of GPR41 antagonism on the inflamed gingival mechanical sensitivity, the MHWTs were measured in the HA-injected-CFA group and the HA-injected-*P. gingivalis*-L group. HA administration into the gingival tissue in *P. gingivalis*-L group decreased the gingival MHWT from the day 8 after *P. gingivalis*-L, while vehicle administration did not change the gingival MHWT (Fig. 3b). In contrast, CFA administration decreased the gingival MHWT from the second day. Daily HA administration into the gingival tissue had no effect on the decrease in MHWT in the gingival tissue in the CFA group. Motor deficits and sedation were not observed during the experimental period (data not shown).

**$P2X_3$ expression in TG and gingival tissue after the *P. gingivalis*-L treatment**

Next, the change in $P2X_3$ expression in TG and gingival tissue after the *P. gingivalis*-L

treatment was examined. FG-labeled $P2X_3$ immunoreactive neurons were detected in the TG on day 8 after *P. gingivalis*-L or control treatment (Fig. 4a). On day 8, *P. gingivalis*-L treatment deceased the number of FG-labeled $P2X_3$ immunoreactive neurons in TG (*P. gingivalis*-L: 36.9 ± 0.7%; Control: 49.2 ± 1.7%) (Fig. 4b).

# Discussion

Various chemical mediators released from peripheral tissues associated with local inflammation act on nociceptive nerve endings, which induce an enhancement of peripheral nociceptive nerve excitability. Thus, nerve afferent firings were increased, leading to the development of pain hypersensitivity [16]. In previous studies, local inflammation increased glutamate or calcitonin gene-related peptides at the site of inflammation, and enhancement of their signals induced increased expression of $P2X_3$ receptors, resulting in orofacial pain hypersensitivity dependent on primary afferent neuronal sensitization [17,12]. However, patients with periodontitis, which are chronic inflammations of the periodontal tissue, complain of little periodontal pain [3]. Here, there was little or no change in *P. gingivalis*-L-inflamed gingival mechanical sensitivity, while CFA-induced gingival inflammation caused mechanical pain hypersensitivity. These results indicate that periodontitis progresses without periodontal mechanical pain hypersensitivity in the *P. gingivalis*-L model. Periodontitis in humans progresses without overt periodontal mechanical pain hypersensitivity; thus, this model can be used to assess the modulation of periodontal nociception in periodontitis patients. The *P. gingivalis*-L model which is different from the model of inflammation by administration of pathogenic substances is useful in elucidating the regulatory mechanism of periodontal nociception in periodontitis patients because it resembles human periodontitis characterized by progression without overt periodontal pain.

SCFAs, such as BA, are found in sites of bacterial infections like periodontal diseases and play important roles in their progress [18]. Small amounts of BA produced by *P. gingivalis* were detected in the gingiva in chronic periodontitis patients and disrupted the tight attachment between epithelial cells, resulting in bacterial invasion into the periodontal tissue and its

destruction [19,20]. In previous study, significant inflammatory cellular infiltration in the periodontal tissue were generated by *P. gingivalis*-L treatment with resorption of alveolar bone [15]. It is also reported that BA produced by *P. gingivalis* suppresses cell proliferation and cell cycling progression in gingival fibroblasts by inducing apoptosis in monocytes and macrophages in humans [21]. Therefore, BA is likely to be involved in the pathogenesis of periodontal disease [22,7]. Inflammation induced by CFA causes pain hypersensitivity by increasing the nociceptive sensitivity and causing plastic changes in sensory and spinal cord neurons that innervate inflamed tissues [23-25]. These plastic changes are initiated by a complex pattern of chemical signals that interact with nociceptive neurons at the site of inflammation. Here, the amount of BA in the periodontal tissue in the *P. gingivalis*-L group was larger than that in the control group on day 4 after these treatments. Interestingly, BA administration into the gingival tissue suppressed periodontal inflammatory pain hypersensitivity induced by periodontal CFA, although periodontal CFA significantly enhanced the periodontal mechanical pain sensitivity. BA gingival administration did not alter the periodontal mechanical pain sensitivity in the *P. gingivalis*-L group during the experimental period. Therefore, the suppression of inflammatory pain hypersensitivity by the signal of BA released from *P. gingivalis* may be the reason for the absence of periodontal pain despite tissue inflammation in periodontal disease.

SCFAs, which are essential nutrients, are involved in various cell function processes as signal transduction molecules [26]. It should be noted that SCFAs can activate GPR41 and GPR43 [27,28]. GPR41 is a G-protein-coupled receptor in the Gα (i/o) family, and activation of GPR41 by SCFAs suppresses extracellular signal-regulated kinase (ERK) cascade signaling activity and the production of intracellular cyclic adenosine monophosphate (cAMP) [29,30]. GPR41 is expressed in fatty tissues and its signaling induces leptin secretion [31]. At this point, GPR41 is reportedly expressed in primary sensory neurons, although little is known on GPR41

neurophysiological functions [10]. Here, it was defined as TG neurons which innervate inflamed gingival tissues in the *P. gingivalis*-L group expressing GPR41. Successive GPR41 antagonism in the inflamed gingival tissue induced periodontal mechanical pain hypersensitivity in the *P. gingivalis*-L group. In contrast, periodontal CFA administration induced periodontal mechanical pain hypersensitivity, which was not suppressed by successive GPR41 antagonism in the inflamed gingival tissue. Altogether, these findings suggest that the BA signal via GPR41 in inflamed gingival tissue suppresses the periodontal inflammatory pain caused by *P. gingivalis*-L. Contrary to expectations, *P. gingivalis*-L treatment increased the number of GPR41 immunoreactive neurons which innervate the inflamed gingival tissue, although no changes were observed in the CFA and control group. The kind of signal in the *P. gingivalis*-L-inflamed gingival tissue that regulates the expression of GPR41 in TG neurons remains unclear and requires further studies.

The increase of $P2X_3$ receptor immunoreactive TG neurons plays an important role in orofacial inflammation-induced mechanical pain hypersensitivity [32]. An earlier study has demonstrated that the enhancement of membrane expression of $P2X_3$ receptors in sensory ganglionic neurons was highly dependent on the intracellular cAMP signaling cascade via protein kinase C phosphorylation in peripheral inflammatory conditions [33]. ERK phosphorylation also facilitates the expression and activity of $P2X_3$ receptors in sensory ganglionic neurons, resulting in inflammatory pain hypersensitivity [34,35]. *P. gingivalis*-L treatment decreased the number of $P2X_3$ immunoreactive TG neurons innervating the *P. gingivalis*-L-inflamed gingival tissue. Together with previous studies indicating that GPR41 activation suppresses intracellular cAMP production and ERK cascade signaling activity, these results suggest that GPR41 signaling by *P. gingivalis*-L-induced BA suppresses the increase of $P2X_3$ receptor- immunoreactive TG neurons associated with gingival tissue inflammation via cAMP or ERK activation, resulting in the progression of *P. gingivalis*-L-induced periodontitis

without periodontal inflammatory pain hypersensitivity.

# Conclusion

*P. gingivalis*-L-induced periodontitis, which progresses unaccompanied by periodontal inflammatory pain hypersensitivity, resembles the clinical condition of periodontitis in humans. This study suggests that *P. gingivalis*-L-induced BA-GPR41 signaling inhibits the augmentation of $P2X_3$ receptor expression in TG neurons via the suppression of cAMP or ERK signaling, resulting in the periodontitis progression without periodontal inflammatory pain. Therefore, *P. gingivalis*-L-induced BA-GPR41 signaling may be a key cascade in the regulation of periodontal nociception in periodontal disease.

# Acknowledgments

I am grateful to Prof. Sato for giving the opportunity to this study, Prof. Shinoda and Assist. Prof. Hitomi for their experimental guidance of this study. I also thank colleagues in the Department of Physiology for their technical advice and assistance.

# References


1. Mariotti A (1999) Dental plaque-induced gingival diseases. Ann Periodontol 4, 7-19.

2. Lakhssassi N, Elhajoui N, Lodter JP, Pineill JL, Sixou M (2005) Antimicrobial susceptibility variation of 50 anaerobic periopathogens in aggressive periodontitis: an interindividual variability study. Oral Microbiol Immunol 20, 244-252.

3. Gaurilcikaite E, Renton T, Grant AD (2017) The paradox of painless periodontal disease. Oral Dis 23, 451-463.

4. Shaddox LM, Walker CB (2010) Treating chronic periodontitis: current status, challenges, and future directions. Clin Cosmet Investig Dent 2, 79-91.

5. Pihlstrom BL, Michalowicz BS, Johnson NW (2005) Periodontal diseases. Lancet 366, 1809-1820.

6. Niederman R, Buyle-Bodin Y, Lu BY, Robinson P, Naleway C (1997) Short-chain carboxylic acid concentration in human gingival crevicular fluid. J Dent Res 76, 575-579.

7. Abe K (2012) Butyric acid induces apoptosis in both human monocytes and lymphocytes equivalently. J Oral Sci 54, 7-14.

8. Shirasugi M, Nakagawa M, Nishioka K, Yamamoto T, Nakaya T, Kanamura N (2018) Relationship between periodontal disease and butyric acid produced by periodontopathic bacteria. Inflamm Regen 38, 23.

9. Nakagawa M, Shirasugi M, Yamamoto T, Nakaya T, Kanamura N (2021) Long-term exposure to butyric acid induces excessive production of matrix metalloproteases in human gingival fibroblasts. Arch Oral Biol 123, 105035.

10. Nøhr MK, Egerod KL, Christiansen SH, Gille A, Offermanns S, Schwartz TW et al. (2015) Expression of the short chain fatty acid receptor GPR41/FFAR3 in autonomic and somatic sensory ganglia. Neuroscience 290, 126-137.

11. Burnstock G (2013) Purinergic mechanisms and pain--an update. Eur J Pharmacol 716, 24-

40.

12. Yasuda M, Shinoda M, Kiyomoto M, Honda K, Suzuki A, Tamagawa T et al. (2012) P2X3 receptor mediates ectopic mechanical allodynia with inflamed lower lip in mice. Neurosci Lett 528, 67-72.

13. Koizumi M, Asano S, Furukawa A, Hayashi Y, Hitomi S, Shibuta I et al. (2021) $P2X_3$ receptor upregulation in trigeminal ganglion neurons through TNF-α production in macrophages contributes to trigeminal neuropathic pain in rats. J Headache Pain 22, 31.

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

15. Nagashima H, Shinoda M, Honda K, Kamio N, Watanabe M, Suzuki T et al. (2017) CXCR4 signaling in macrophages contributes to periodontal mechanical hypersensitivity in *Porphyromonas gingivalis*-induced periodontitis in mice. Mol Pain 13, 1744806916689269.

16. Muley MM, Krustev E, McDougall JJ (2016) Preclinical assessment of inflammatory pain. CNS Neurosci Ther 22, 88-101.

17. Honda K, Noma N, Shinoda M, Miyamoto M, Katagiri A, Kita D et al. (2011) Involvement of peripheral ionotropic glutamate receptors in orofacial thermal hyperalgesia in rats. Mol Pain 7, 75.

18. Tsuda H, Ochiai K, Suzuki N, Otsuka K (2010) Butyrate, a bacterial metabolite, induces apoptosis and autophagic cell death in gingival epithelial cells. J Periodontal Res 45, 626-634.

19. Takahashi N, Saito K, Schachtele CF, Yamada T (1997) Acid tolerance and acid-neutralizing activity of Porphyromonas gingivalis, Prevotella intermedia and Fusobacterium nucleatum. Oral Microbiol Immunol 12, 323-328.

20. Takigawa S, Sugano N, Nishihara R, Koshi R, Murai M, Yoshinuma N et al. (2008) The effect of butyric acid on adhesion molecule expression by human gingival epithelial cells. J Periodontal Res 43, 386-390.

21. Shirasugi M, Nishioka K, Yamamoto T, Nakaya T, Kanamura N (2017) Normal human gingival fibroblasts undergo cytostasis and apoptosis after long-term exposure to butyric acid. Biochem Biophys Res Commun 482, 1122-1128.

22. Kurita-Ochiai T, Seto S, Suzuki N, Yamamoto M, Otsuka K, Abe K et al. (2008) Butyric acid induces apoptosis in inflamed fibroblasts. J Dent Res 87, 51-55.

23. Neumann S, Doubell TP, Leslie T, Woolf CJ (1996) Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. Nature 384, 360-364.

24. Ji RR, Woolf CJ (2001) Neuronal plasticity and signal transduction in nociceptive neurons: implications for the initiation and maintenance of pathological pain. Neurobiol Dis 8, 1-10.

25. Kidd BL, Urban LA (2001) Mechanisms of inflammatory pain. Br J Anaesth 87, 3-11.

26. Kimura I, Ichimura A, Ohue-Kitano R, Igarashi M (2020) Free fatty acid receptors in health and disease. Physiol Rev 100, 171-210.

27. Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D et al. (2003) The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem 278, 11312-11319.

28. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D et al. (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 461, 1282-1286.

29. Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME et al. (2003) Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J Biol Chem 278, 25481-25489.

30. Stoddart LA, Smith NJ, Jenkins L, Brown AJ, Milligan G (2008) Conserved polar residues in transmembrane domains V, VI, and VII of free fatty acid receptor 2 and free fatty acid receptor 3 are required for the binding and function of short chain fatty acids. J Biol Chem 283, 32913-32924.

31. Xiong Y, Miyamoto N, Shibata K, Valasek MA, Motoike T, Kedzierski RM et al. (2004)

Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proc Natl Acad Sci U S A 101, 1045-1050.

32. Shinoda M, Ozaki N, Asai H, Nagamine K, Sugiura Y (2005) Changes in P2X3 receptor expression in the trigeminal ganglion following monoarthritis of the temporomandibular joint in rats. Pain 116, 42-51.

33. Gu Y, Wang C, Li G, Huang LY (2016) F-actin links Epac-PKC signaling to purinergic P2X3 receptors sensitization in dorsal root ganglia following inflammation. Mol Pain 12, 1744806916660557.

34. Dai Y, Fukuoka T, Wang H, Yamanaka H, Obata K, Tokunaga A et al. (2004) Contribution of sensitized P2X receptors in inflamed tissue to the mechanical hypersensitivity revealed by phosphorylated ERK in DRG neurons. Pain 108, 258-266.

35. Jiang Q, Li WX, Sun JR, Zhu TT, Fan J, Yu LH et al. (2017) Inhibitory effect of estrogen receptor beta on P2X3 receptors during inflammation in rats. Purinergic Signal 13, 105-117.

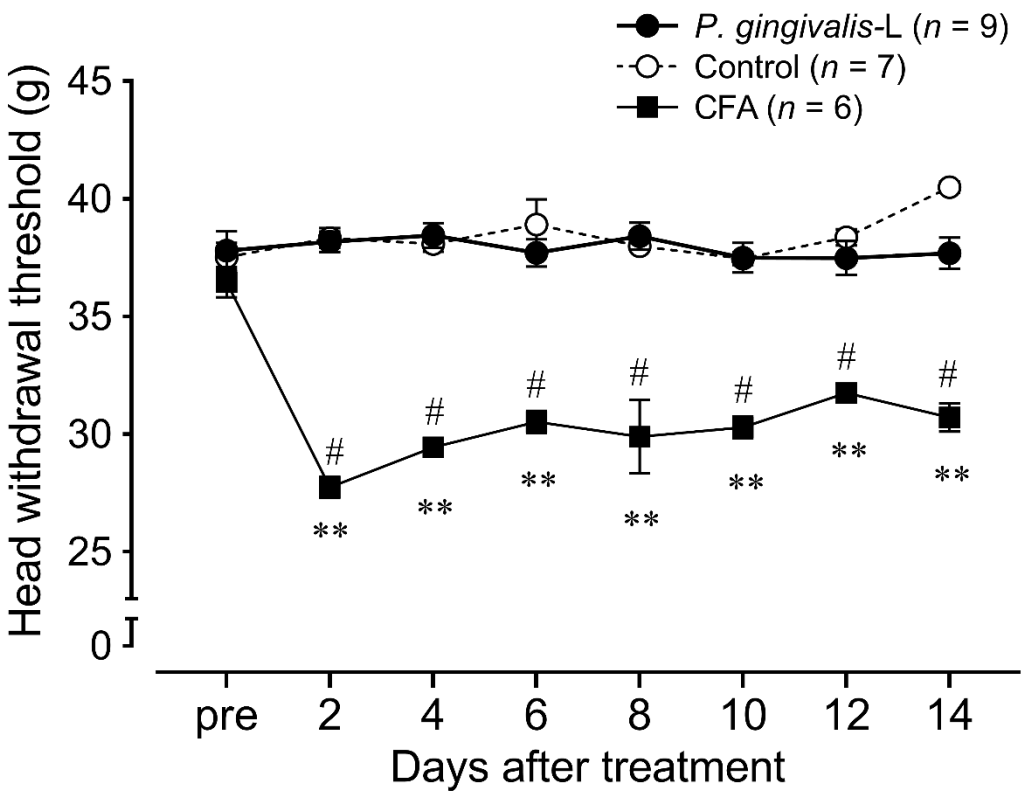


Figure 1. Changes in the MHWT in the gingival tissue for 14 days after gingival *P. gingivalis*-L, CFA or control treatment. ***P* < 0.01, CFA vs. control group; #, *P* < 0.0001, vs. pre in CFA group (two-way ANOVA followed by Bonferroni's multiple comparison test).

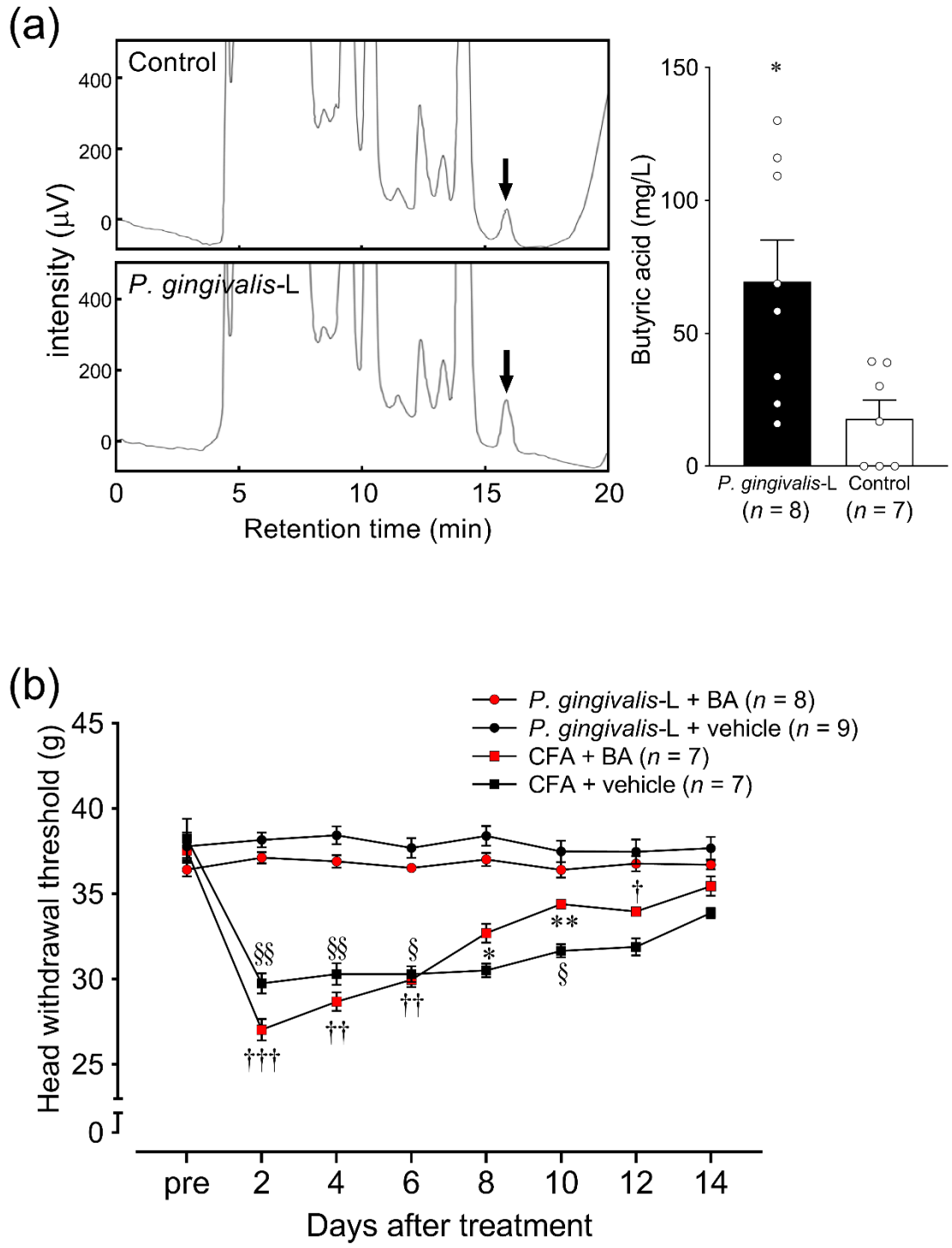


Figure 2. Effect of butyric acid (BA) in the gingival tissue on the gingival mechanical sensitivity after gingival *P. gingivalis*-L or control treatment. (a) Amount of BA in the gingival tissue on day 4 after *P. gingivalis*-L or control treatment. The vertical axis is the peak intensity, which indicates the value according to the type of detector used. Meanwhile, the horizontal

axis is the retention time, which can be regarded as the time for each component to pass through the detector. Arrows indicate the peaks derived from BA. Data represent mean ± SEM. *$P$ < 0.05 (Student's $t$-test). (b) Effect of BA on the changes in the MHWT in the gingival tissue for 14 days after gingival *P. gingivalis*-L or control treatment. Data represent mean ± SEM. *$P$ < 0.05, **$P$ < 0.01, CFA + BA vs. CFA + vehicle group; §, $P$ < 0.05, §§, $P$ < 0.01, vs. pre in CFA + vehicle group; †, $P$ < 0.05, ††, $P$ < 0.01, †††, $P$ < 0.001, vs. pre in CFA + BA group (two-way repeated-measures ANOVA followed by Bonferroni's multiple comparison test).

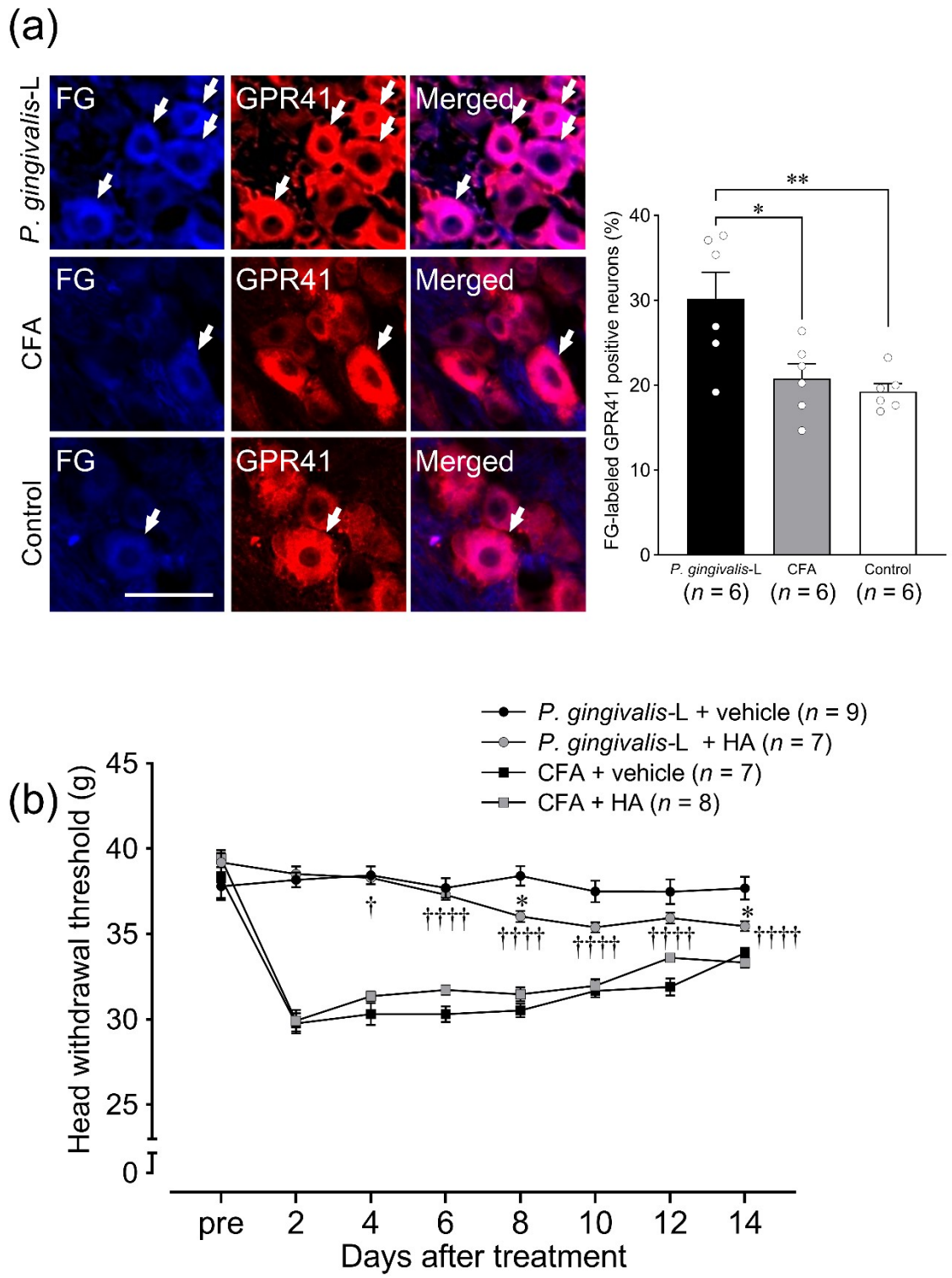


Figure 3. Effect of GPR41 signaling on the gingival mechanical sensitivity after gingival *P. gingivalis*-L or CFA treatment. (a) GPR41 expression in TG neuron innervating into the gingival tissue on day 8 after gingival *P. gingivalis*-L, CFA or control treatment. Arrows

indicate GPR41 immunoreactive TG neurons innervating into the gingival tissue. Scale bar: 50 μm. Data represent mean ± SEM. *$P < 0.05$, **$P < 0.01$ (one-way ANOVA followed by Tukey's multiple comparison test). (b) Effect of HA on the MHWT in the gingival tissue for 14 days after gingival *P. gingivalis*-L or CFA treatment. Data represent mean ± SEM. *$P < 0.05$ vs. *P. gingivalis*-L + vehicle group; †, $P < 0.05$, ††††, $P < 0.0001$, vs. pre in CFA + HA group (two-way ANOVA followed by Bonferroni's multiple comparison test).

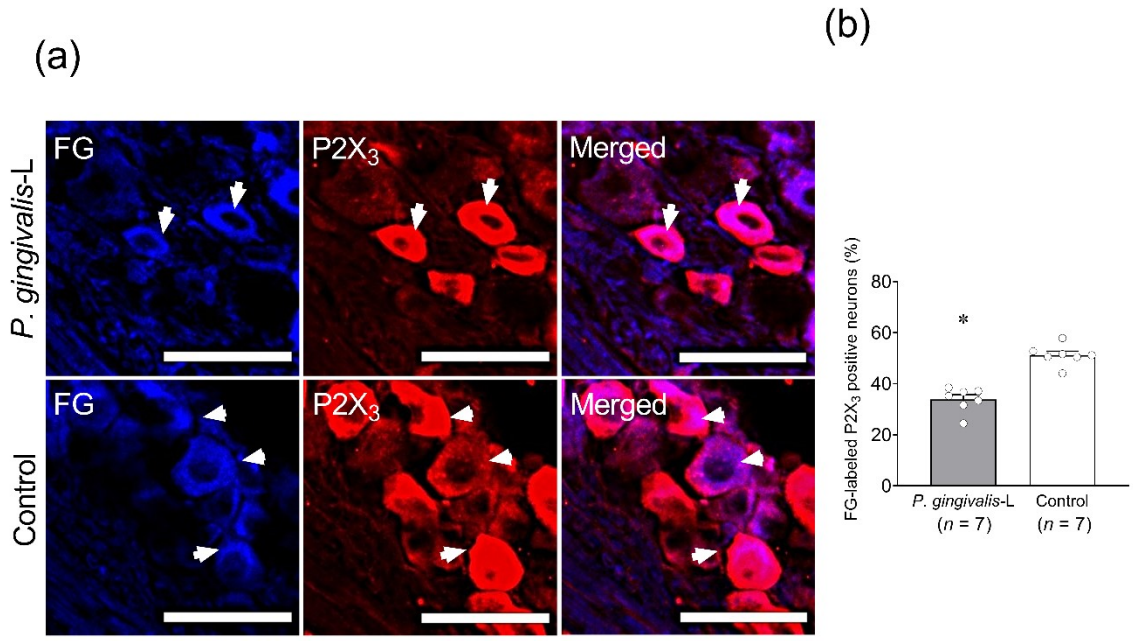


Figure 4. $P2X_3$ expression in TG and the gingival tissue after gingival *P. gingivalis*-L or control treatment. (a) $P2X_3$ expression in TG neurons innervating into the gingival tissue on day 8 after gingival *P. gingivalis*-L or control treatment. Arrows indicate $P2X_3$ immunoreactive TG neurons innervating into the gingival tissue. Scale bar: 50 μm. (b) The number of $P2X_3$ immunoreactive TG neurons innervating into the gingival tissue on day 8 after *P. gingivalis*-L or control treatment. Data represent mean ± SEM. $^*P < 0.05$ (Student's t-test).