

Role of Macrophage-Mediated Toll-like receptor 4–Interleukin-1R Signaling in Ectopic Tongue Pain Associated With Tooth Pulp Inflammation

Kohei Kanno

Nihon Daigaku Shigakubu Daigakuin Shigaku Kenkyuka

Kohei Shimizu (✉ shimizu.kouhei01@nihon-u.ac.jp)

Nihon University School of Dentistry <https://orcid.org/0000-0001-6339-1094>

Masamichi Shinoda

Nihon Daigaku Shigakubu Daigakuin Shigaku Kenkyuka

Makoto Hayashi

Nihon Daigaku Shigakubu Daigakuin Shigaku Kenkyuka

Osamu Takeichi

Nihon Daigaku Shigakubu Daigakuin Shigaku Kenkyuka

Koichi Iwata

Nihon Daigaku Shigakubu Daigakuin Shigaku Kenkyuka

Research

Keywords: Intercellular signaling, Ectopic tongue pain, Toll-like receptors, Interleukin-1 β , Transient receptor potential vanilloid 1, Cytokine receptors, Chemokine, Hyperalgesia, Trigeminal ganglion

DOI: <https://doi.org/10.21203/rs.3.rs-28935/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background Ectopic orofacial pain is frequently caused by tooth pulp inflammation. However, the detailed mechanism underlying such pain remains poorly understood.

Methods To better understand this phenomenon, ectopic pain mechanism was studied in a rat model of mandibular first molar tooth pulp exposure (M1-TPE).

Results One day after M1-TPE, obvious pulpal inflammation was observed in M1 pulp. The head withdrawal threshold to mechanical and heat stimulation of the tongue was significantly reduced in M1-TPE rats on day 1 after TPE. In addition, the production of interleukin-1 β (IL-1 β) in activated macrophages and expression levels of Toll-like receptors (TLRs) and IL-1 type I receptor (IL-1RI) were significantly increased in the trigeminal ganglion (TG) neurons innervating the tongue following M1-TPE. Injection of the selective macrophage depletion compound liposomal clodronate Clophosome-A into the TG significantly suppressed tongue hypersensitivity; however, expression levels of TLR4 and IL-1RI in TG neurons innervating the tongue were not significantly altered. Injection of lipopolysaccharide from *Rhodobacter sphaeroides*, a TLR4 antagonist, into the TG following TPE significantly suppressed tongue hypersensitivity and reduced IL-1RI expression in TG neurons innervating the tongue. Moreover, an intra-TG injection of recombinant heat shock protein 70, a selective TLR4 agonist, significantly promoted the development of tongue-hypersensitivity and increased the production of IL-1RI in TG neurons innervating the tongue in naive rats. Furthermore, an intra-TG injection of recombinant IL-1 β led to the development of tongue hypersensitivity in naive rats and enhanced the expression of transient receptor potential vanilloid 1 in the TG neurons innervating the tongue.

Conclusions The present findings suggest that the neuron-macrophage interaction mediated by TLR4 and IL-1RI activation in TG neurons affects the pathogenesis of abnormal tongue pain following tooth pulp inflammation via TLR4-ILR and TRPV1 signaling in the TG.

Background

Ectopic pain frequently occurs in the orofacial regions following tooth pulp inflammation [1]. This pain is likely to be misdiagnosed and leads to inappropriate treatment [2, 3]. Therefore, it is imperative to unravel the mechanism of ectopic orofacial pain linked to tooth pulp inflammation, which would facilitate the development of appropriate prophylactics.

Macrophages constitute a major source of pro-inflammatory cytokines, including interleukin-1 β (IL-1 β) [4–7], a prototypic pro-inflammatory cytokine that induces cellular signal transduction via the IL-1 type I receptor (IL-1RI) and regulates the sensation of pain and inflammatory condition [8, 9]. Following peripheral tissue inflammation, the accumulated macrophages release IL-1 β in the dorsal root ganglion, leading to secondary hyperalgesia [4]. However, despite evidence suggesting the involvement of microglia-neuron interaction in this process via pro-inflammatory cytokines, the detailed mechanism

underlying the observed effects in the trigeminal ganglion (TG) mediated by the accumulated macrophages associated with tooth pulp inflammation is not fully understood.

The toll-like receptors (TLRs) mediate signaling in response to various pathogen-associated molecular patterns [10, 11]. Following tissue injury, inflammation, or cellular stress, TLRs recognize danger- and pathogen-associated molecular patterns that act as endogenous ligands [11]. TLRs expressed in primary sensory neurons detect danger- and pathogen-associated molecular patterns released following tissue damage or cellular stress and assist in modulating neuronal excitation [12–14]. Peripheral orofacial inflammation leads to enhanced TLR4 expression in the TG, in turn inducing ectopic orofacial pain [13–15]. Additionally, the occurrence of peripheral inflammation or nerve injury leads to enhanced IL-1RI expression in the TG, which has a pivotal role in facilitating TG neuronal excitability, and thereby contributes to orofacial hyperalgesia development [8, 16, 17]. We therefore hypothesized that TLR4 expression is increased in the TG during tooth pulp inflammatory disorder and involved in the enhancement of neuronal excitability in TG.

Transient receptor potential vanilloid 1 (TRPV1), which is activated by noxious heat (> 43 °C), low extracellular pH (< 6.5), and some irritants, is also considered to contribute to mechanical nociception [18]. TRPV1 expression in sensory neurons increases following the up-regulation of IL-1 β production by activated satellite glial cells [19]. Hence, it is likely that the expression of danger-associated molecular patterns induced by the inflammation of the orofacial organ, such as tooth pulp inflammation induced by mandibular first molar tooth pulp exposure (M1-TPE), also induces TRPV1 expression as well as increases IL-1RI expression following TLR4 activation in TG neurons.

Accordingly, we hypothesized that TPE induces tongue ectopic pain as a symptom of orofacial ectopic pain. We further hypothesized that cytokines produced by macrophages are involved in this process and that the macrophage-neuron interaction contributes to the tongue hyperalgesia following TPE. To verify this hypothesis, we investigated TPE-induced mechanical and heat-evoked nocifensive reflex of the tongue in a rat model of M1 TPE and then performed immunohistochemical staining for IBA1, a marker of active macrophages, IL-1 β , IL-1RI, and TLR4 in the TG. In addition, we investigated the effects of injections of liposomal clodronate Clophosome-A (LCCA), a selective macrophage depletion agent, lipopolysaccharide from *Rhodobacter sphaeroides* (LPS-RS, a TLR4 antagonist), IL-1 β , or heat shock protein 70 (HSP70, a selective agonist of TLR4) to verify the change in tongue hypersensitivity and in the regulation of IL-1RI, TLR4, and TRPV1 biosynthesis.

Methods

Animals

We utilized male Sprague-Dawley rats ($n = 260$, Japan SLC, Shizuoka, Japan) weighing 250–350 g. Rats were housed at a stable temperature (23 °C) with a 12 h light:dark cycle (7:00–19:00:19:00–7:00). Rats were raised under aseptic conditions with free intake to food and water. The study was approved by the

Nihon University Animal Experiment Committee (protocol numbers AP17D021 and AP19DEN009-1). The study was conducted according to the National Institutes of Health guidelines on laboratory animal management and use and based the previous pain study reports [20]. The statistical design of these experiments minimized the number of rats used. The study complied with ARRIVE guidelines.

Histochemical analysis of TPE pulp in M1-TPE rats

We performed light inhalant anesthesia using a mixture of isoflurane (2%, Mylan, Canonsburg, PA) and oxygen. Rats were deeply anesthetized by an intraperitoneal (i.p.) injection of butorphanol (2.5 mg/kg, Meiji Seika Pharma, Tokyo, Japan), medetomidine (0.375 mg/kg, Zenocq, Fukushima, Japan), and midazolam (2.0 mg/kg, Sandoz, Tokyo, Japan) dissolved in saline solution (three-mixed anesthetic). The M1-TPE procedure was performed with reference to a previous report [14]. One day after TPE, rats ($n = 3$) were deeply anesthetized with the three-mixed anesthetic and transcardially perfused as previously described [21]. The mandibular jaw was removed and decalcified with EDTA for 7 days, 4-mm tooth sections were cut from TPE, and sham teeth were stained with hematoxylin and eosin.

Head withdrawal threshold (HWT) measurements

The HWT was measured under light anesthesia with 2% isoflurane and oxygen for mechanical and heat stimulation of the tongue ipsilateral to TPE (3 mm behind the tip of the tongue). HWT measurements were performed on days 1, 2, 3, 5, and 9 after TPE as previously described [13]. Briefly, the lower jaw was gently pulled with plastic strings to hold the mouth open and then mechanical stimulation (0 to 130 g; 10 g/s; cutoff, 130 g) or heat stimulation (35–60 °C; 1 °C/s; cutoff, 60 °C) was applied to the lateral tongue edge. Flat-tip forceps (4 mm²; Panlab s.l., Barcelona, Spain) or a contact heat probe (9 mm²; Intercross, Tokyo, Japan) were used for mechanical or heat stimulation, respectively. The changes in mean HWT values over time were measured in TPE or sham rats. Baseline HWT levels were measured before TPE or sham treatment.

Immunohistochemistry

TG neurons innervating the tongue were visualized using fluorogold (FG, Fluorochrome, Denver, CO) which was injected into the tongue. In rats anesthetized with 2% isoflurane, 10.0 µL of 4% FG dissolved in saline was injected into the tongue ipsilateral to TPE. TPE was performed on the second day after FG injection. The next day (day 1 after TPE), the rats were deeply anesthetized with the three-mixed anesthetic and transcardially perfused as previously described [21]. TG sections were prepared (15-µm-thick), mounted on microscope slides (Matsunami, Tokyo, Japan), and blocked to avoid non-specific binding of antibodies. Sections were then incubated in a solution of rabbit anti-TLR4 (1:1000, ab13556; Abcam, Cambridge, UK), rabbit anti-IL1RI (1:500, SC-689; Santa Cruz Biotechnology, Dallas, TX), mouse anti-IL-1β (1:800, ab8320; Abcam), rabbit anti-IBA1 (1:2000, 019-19741; Wako, Shiga, Japan), mouse anti-IL1RI (1:500, ab40101; Abcam), or guinea-pig anti-TRPV1 (1:500, AB10295; Abcam) polyclonal antibody. The sections were then incubated in the secondary antibody solution of Alexa Fluor 568-conjugated goat anti-rabbit IgG, Alexa Fluor 568-conjugated goat anti-mouse IgG, Alexa Fluor 488-conjugated goat anti-mouse IgG, and/or Alexa Fluor 568-conjugated goat anti-guinea pig IgG (all 1:200 in 0.01 M phosphate-

buffered saline; Thermo Fisher Scientific, Waltham, MA). Following the application of mounting medium, TG sections were cover-slipped, and the immunoreactive (IR) cells were observed by fluorescence microscopy using a BZ-9000 system (Keyence, Osaka, Japan). Double- or triple-labeled cells (FG and TLR4; FG and IL-1RI; IBA1 and IL-1 β ; FG, TLR4, and IL-1RI; FG and TRPV1) were recognized as those exhibiting co-expression of respective markers. Then, we precisely counted the number of cells and performed analysis using a computer-assisted imaging analysis system (BZ-X analyzer, Keyence). Cells displaying the signal over twice the saturation level compared to the mean background signal were identified as positive. FG-labeled IR cells in the V3 branch region of the TG were counted. The equations used to calculate and analyze IR cells in the TG of the third branch region are given in the legends to Figs. 2–4.

Drug injection into the TG

Under anesthesia, a midline skin incision (2-cm-long) was made from head to neck and a small hole (1 mm in diameter) was made in the skull above the TG. A guide cannula was inserted into the brain through the hole to reach the TG; the cannula tip was placed 9 mm below the skull surface. Next, as previously reported, the cannulation procedure was performed on the skull surface [22]. Phosphate-buffered saline (1 μ L/day), LPS-RS (0.1 mM, 1 μ L/day; InvivoGen, San Diego, CA), LCCA (7 mg/mL, 1 μ L/day; F70101CA, FormuMax Scientific, Sunnyvale, CA), or plain control liposomes for LCCA (Cont-LCCA; 20 mM, 1 μ L/day; F70101-A, FormuMax Scientific) was injected into the TG once daily for two successive days. Then, we measured HWT and performed immunohistochemistry to investigate each drug's effect on tongue hypersensitivity following M1-TPE.

Injection of HSP70 or IL-1 β into the TG

Rats were anesthetized with the three-mixed anesthetic, then recombinant human HSP70/HSPA1A (1 mL, 1 mg/mL, AP-100-100, R&D Systems, Minneapolis, MN) or recombinant mouse IL-1 β protein (1 mL, 1 mg/mL, AB9723, Abcam) was injected into the left TG using a guide cannula on day 2 following FG injection. On day 1 after injection, HWT to mechanical and heat stimulation of the tongue was measured, then rats were transcardially perfused as previously described [13, 21, 22]. Then, we prepared TG sections, mounted them on microscopic slides, and incubated them with rabbit anti-IL1RI or guinea pig anti-TRPV1 polyclonal antibody solutions. Saline was also injected as vehicle control.

Statistical analysis

Data are presented as the mean \pm standard error of the mean (SEM). HWTs between the groups were compared using two-way repeated-measures analysis of variance (ANOVA) followed by Tukey's *post hoc* tests. The percentages of positive cells between the groups were compared using the Student's *t*-test. All statistical analyses were conducted with a significance level of $\alpha = 0.05$ ($P < 0.05$).

Results

Tooth pulp inflammation and nocifensive reflexes against mechanical or thermal stimulation of the tongue

Numerous inflammatory cells were observed in the coronal but not apical pulp in TPE rats on day 1 after TPE (Fig. 1a). The HWT to mechanical or thermal stimulation of the tongue ipsilateral to the TPE was significantly reduced on days 1–3 after TPE compared to that in sham-treated or pre-TPE rats (Fig. 1b–d). No changes in HWT were observed in sham rats (Fig. 1c, d).

Expression of TLR4, IL-1RI, IBA1, and IL-1 β in TG after TPE

We observed an increased number of FG-labeled TLR4-IR and FG-labeled IL-1RI-IR cells in TPE rats on day 1 after TPE. Furthermore, the average percentages of FG-labeled TLR4-IR and IL-1RI-IR cells were significantly greater in TPE rats than those in sham-rats (Fig. 2a–c). More IBA1-IR and IBA1-IL-1 β -IR cells were also observed in TPE-rats (Fig. 2d–f). In addition, the areas occupied by IBA1 and IBA1-IL-1 β immuno-products were significantly larger in TPE-rats than those in sham-rats (Fig. 2e, f).

Effect of LCCA injection into the TG on HWT and IBA1 expression

LCCA injection into the TG markedly reduced macrophage accumulation on day 1 after TPE (Fig. 3a, b). The decrease in HWT in response to mechanical or heat stimulation of the tongue was significantly reversed on day 1 after TPE in rats that were administered daily injections of LCCA into the TG compared to TPE in rats that received vehicle (Fig. 3c, d). No significant differences were observed in the mean percentages of FG-labeled TLR4-IR and IL-1RI-IR cells between vehicle- and LCCA-injected rats (Fig. 3e, f). The mean percentages of FG-labeled TLR4-IR, FG-labeled IL-1RI-IR, and FG-labeled TLR4-IR + IL-1RI-IR cells were 42.1%, 9.9%, and 48.0%, respectively (Fig. 3g).

Effect of LPS-RS injection into the TG on HWT and IL-1RI expression in TG neurons

The decreased HWTs to mechanical or heat stimulation of the tongue were significantly reversed on day 1 following TPE in the rats receiving daily LPS-RS relative to those in rats receiving vehicle into the TG (Fig. 3h–j). FG-labeled IL-1RI-IR cell number was decreased in the TG in rats treated with LPS-RS (Fig. 3K). The averaged percentage of FG-labeled IL-1RI-IR cells was significantly reduced in LPS-RS-treated compared to vehicle-treated rats (Fig. 3L).

Effect of HSP70 injection into the TG on HWT and IL-1RI expression in TG neurons

The mechanical or heat HWTs were significantly decreased on day 1 following HSP70 injection in the TG relative to those in naive rats injected with vehicle on day 1 after TPE (Fig. 4a–c). The number of FG-labeled IL-1RI-IR cells in the TG was increased in HSP70-injected naive rats (Fig. 4d). The mean

percentage of FG-labeled IL-1RI-IR cells was significantly higher in HSP70-injected rats relative to that in vehicle-injected naive rats (Fig. 4E).

Effect of IL-1 β injection into the TG on HWT and TRPV1 expression in TG neurons

The mechanical and heat HWTs were significantly decreased on day 1 following IL-1 β injection into the TG relative to those measured in the naive rats following vehicle injection (Fig. 4f–h). The number of FG-labeled TRPV1-IR cells in the TG was increased in naive rats treated with IL-1 β . Additionally, IL-1 β treatment significantly increased the average percentage of FG-labeled TRPV1-IR cells relative to that in vehicle-injected naive rats (Fig. 4i, j).

Discussion

In our experiments, we reliably observed tongue mechanical and heat hypersensitivity following TPE. IL-1 β production was observed in accumulated macrophages, with higher expression levels of TLR4 and IL-1RI in TG neurons innervating the tongue in TPE rats. LCCA injection into the TG significantly suppressed tongue hypersensitivity. However, no changes were observed in TLR4 and IL-1RI expression levels in TG neurons innervating the tongue. LPS-RS injection into the TG after TPE significantly suppressed tongue hypersensitivity and decreased IL-1R expression in TG neurons, whereas intra-TG injection of recombinant HSP70 enhanced tongue hyperalgesia and promoted IL-1RI expression in TG neurons innervating the tongue in naive rats. Furthermore, intra-TG injection of recombinant IL-1 β also enhanced tongue hyperalgesia and promoted TRPV1 expression in TG neurons innervating the tongue in naive rats. These results suggested that the neuron-macrophage interaction mediated through TLR4 and IL-1RI activation in the TG neurons is important for the pathogenesis of ectopic and abnormal tongue pain sensation via TRPV1 following tooth pulp inflammation.

Macrophage activation and its interaction with TLR4 and IL-1RI in the tongue ectopic pain following TPE

Tooth pulp inflammation frequently causes persistent pain in the orofacial regions, which is termed ectopic orofacial pain, along with tooth pain [1, 23]. Tooth pulp inflammation is accompanied by the enhancement of TLR4 and IL-1 β expression levels in TG neurons [14]. In turn, IL-1 β up-regulates the expression of its own receptor IL-1RI [24]. In acute lung injury, LPS-TLR4 signaling potentiates IL-1 β release and subsequently up-regulates IL-1RI expression on the surface of macrophages through MyD88 and NF- κ B-dependent signaling [25]. Taken together, these results suggest that TLR4 activation and subsequent up-regulation of IL-1RI likely play an important role in ectopic orofacial pain associated with IL-1 β expression in macrophages following tooth pulp inflammation.

In addition, orofacial inflammation or trigeminal nerve injury causes macrophage accumulation in the TG [6, 21, 26]. Accumulated macrophages release various substances, including IL-1 β , that enhance neuronal excitability [4, 5, 7, 27]. Conversely, macrophage depletion by LCCA significantly suppresses macrophage accumulation, which reduces TNF α release, finally resulting in the recovery of the mechanical

hypersensitivity of whisker pad skin following inferior alveolar nerve transection [21]. We observed a significant increase in the heat and mechanical tongue-hypersensitivity following TPE. TPE enhanced the expression of TLR4 and IL-1RI in TG neurons and accelerated the release of IL-1 β subsequent to macrophage accumulation in the TG. Notably, although the depletion of activated macrophages attenuated the TPE-induced tongue hyperalgesia, TLR4 and IL-1RI expression levels were not altered, indicating macrophages are not involved in the up-regulation of TLR4 and IL-1RI expression. Furthermore, intra-TG injection of LPS-RS decreased IL-1RI expression following TPE. Together, these results indicated that TLR4-IL-1RI signaling mediated by IL-1 β released from accumulated macrophages likely plays an important role in the development of ectopic pain in the tongue following TPE.

HSP70 involvement in the enhancement of IL-1RI expression in TG neurons

HSP70 is expressed in the tooth pulp following pulpal trauma, heat stress, or inflammation [28–30]. Recombinant-HSP70 applied to the tooth pulp is axonally transported to the TG and released from TG neurons into the extracellular space [13]. The secreted HSP70 binds to TLR4 of the TG neurons innervating the tongue, leading to tongue hypersensitivity [13]. In the present study, intra-TG injection of recombinant HSP70 induced tongue-mechanical and heat hypersensitivity accompanied by increases IL-1RI expression. Together, these data suggested that HSP70 expression in the tooth pulp following TPE likely contributes to IL-1RI up-regulation through TLR4 activation in the TG, resulting in the pathogenesis of tongue hypersensitivity. Moreover, the present results demonstrated that the elimination of IL-1 β through LCCA-mediated macrophage depletion did not affect TLR4 and IL-1RI expression. On the other hand, although LPS-RS down-regulated IL-1RI expression, HSP70, conversely, up-regulated it. These results also suggested that the regulation of IL-1RI expression occurs downstream of TLR4 signaling.

IL-1 β involvement in the up-regulation of TRPV1 expression in TG neurons

TRPV1 expression is potentiated in TG neurons following peripheral inflammation or nerve injury, resulting in the development of mechanical and/or thermal hypersensitivity [31, 32]. The activation of IL-1RI by IL-1 β facilitates TRPV1 expression and promotes neuronal hyperexcitability [17, 19, 33]. In the present study, intra-TG injection of IL-1 β promoted tongue hypersensitivity to mechanical and heat stimuli and augmented TRPV1 expression. Thus, these results suggested that TRPV1 up-regulation is involved in the occurrence of tongue hypersensitivity subsequent to the activation of IL-1RI by the IL-1 β expressed in the TG.

Conclusions

Our experiments suggest the following mechanism underlying tongue hypersensitivity after TPE (Fig. 5). M1 TPE induces HSP70 expression in the pulpal tissue, which is axonally transported from the lower first molar to the TG and released into the intercellular space in the TG. Subsequent HSP70 binding to TLR4 of

the TG neurons innervating the tongue promotes IL-1RI expression. Simultaneously, accumulated macrophages generate and release IL-1 β in the TG intercellular space following TPE, which binds to the overexpressed IL-1RI of TG neurons innervating the tongue. The IL-1 β -IL-1RI signaling, in turn, facilitates TRPV1 expression in the tongue-innervating TG neurons. Finally, augmented levels of TRPV1 enhance TG neuronal excitability, resulting in mechanical and heat hypersensitivity of the tongue. Our results indicate that the development of ectopic tongue hypersensitivity induced by M1 pulp inflammation is associated with the accumulation of macrophages in the third branch region in the TG, IL-1 β production by macrophages, and increased expression levels of TLR4 and IL-1RI. These findings suggest that the TLR4/IL-1 β /IL-1RI signaling enhances TRPV1 expression in the TG, which promotes the development of tongue hypersensitivity. Thus, our study provides clues regarding the neural mechanisms of abnormal pain development and may lead to the development of new treatments and therapeutics.

Abbreviations

ANOVA

analysis of variance; FG:fluorogold; HSP70:heat shock protein 70; HWT:head withdrawal threshold; IL-1 β :interleukin-1 β ; IL-1RI:interleukin-1 type I receptor; IR:immunoreactive; LCCA, liposomal clodronate Clophosome-A; LPS-RS:lipopolysaccharide from *Rhodobacter sphaeroides*; M1-TPE:first molar tooth pulp exposure; SEM:standard error of the mean; TG:trigeminal ganglion; TLR:toll-like receptor; TPE:tooth pulp exposure; TRPV1:transient receptor potential vanilloid 1

Declarations

Authors' contributions

K.K., K.S., M.H., O.T., and K.I. contributed to the conception of the study. All authors contributed to the design of the study. K.K. and K.S. acquired and interpreted experimental data and drafted the manuscript. M.S. performed statistical analysis. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Acknowledgement

Not applicable.

Funding

This work was supported by KAKENHI (Grants-in-Aid for Scientific Research [C] 16K11566 and 19K10160).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The study was approved by the Nihon University Animal Experiment Committee (protocol numbers AP17D021 and AP19DEN009-1). The study was conducted according to the National Institutes of Health guidelines on laboratory animal management and use and based the previous pain study reports [20]. The statistical design of these experiments minimized the number of rats used. The study complied with ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Author details

¹Department of Endodontics, Nihon University School of Dentistry, Tokyo, Japan. ²Division of Advanced Dental Treatment, Dental Research Center, Nihon University School of Dentistry, Tokyo, Japan.

³Department of Physiology, Nihon University School of Dentistry, Tokyo, Japan. ⁴Division of Functional Morphology, Dental Research Center, Nihon University School of Dentistry, Tokyo, Japan.

References

1. Glick DH. Locating referred pulpal pains. *Oral Surg Oral Med Oral Pathol.* 1962;15:613–23.
2. Grushka M, Sessle BJ. Applicability of the McGill pain questionnaire to the differentiation of 'toothache' pain. *Pain.* 1984;19(1):49–57.
3. Owatz CB, Khan AA, Schindler WG, Schwartz SA, Keiser K, Hargreaves KM. The incidence of mechanical allodynia in patients with irreversible pulpitis. *J Endod.* 2007;33(5):552–6.
4. Scholz J, Woolf CJ. The neuropathic pain triad: Neurons, immune cells and glia. *Nat Neurosci.* 2007;10(11):1361–8.
5. Thacker MA, Clark AK, Marchand F, McMahon SB. Pathophysiology of peripheral neuropathic pain: Immune cells and molecules. *Anesth Analg.* 2007;105(3):838–47.
6. Ji RR, Chamesian A, Zhang YQ. Pain regulation by non-neuronal cells and inflammation. *Science.* 2016;354(6312):572–7.
7. Ji RR, Xu ZZ, Gao YJ. Emerging targets in neuroinflammation-driven chronic pain. *Nat Rev Drug Discov.* 2014;13(7):533–48.
8. Takeda M, Tanimoto T, Kadoi J, Nasu M, Takahashi M, Kitagawa J, et al. Enhanced excitability of nociceptive trigeminal ganglion neurons by satellite glial cytokine following peripheral inflammation.

- Pain. 2007;129(1–2):155–66.
9. Souza GR, Talbot J, Lotufo CM, Cunha FQ, Cunha TM, Ferreira SH. Fractalkine mediates inflammatory pain through activation of satellite glial cells. *Proc Natl Acad Sci U S A*. 2013;110(27):11193–8.
 10. Kawai T, Akira S. TLR signaling. *Semin Immunol*. 2007;19(1):24–32.
 11. Okun E, Griffioen KJ, Mattson MP. Toll-like receptor signaling in neural plasticity and disease. *Trends Neurosci*. 2011;34(5):269–81.
 12. Liu T, Gao YJ, Ji RR. Emerging role of Toll-like receptors in the control of pain and itch. *Neurosci Bull*. 2012;28(2):131–44.
 13. Ohara K, Shimizu K, Matsuura S, Ogiso B, Omagari D, Asano M, et al. Toll-like receptor 4 signaling in trigeminal ganglion neurons contributes tongue-referred pain associated with tooth pulp inflammation. *J Neuroinflammation*. 2013;10:139.
 14. Lin JJ, Du Y, Cai WK, Kuang R, Chang T, Zhang Z, et al. Toll-like receptor 4 signaling in neurons of trigeminal ganglion contributes to nociception induced by acute pulpitis in rats. *Sci Rep*. 2015;5:12549.
 15. Diogenes A, Ferraz CC, Akopian AN, Henry MA, Hargreaves KM. LPS sensitizes TRPV1 via activation of TLR4 in trigeminal sensory neurons. *J Dent Res*. 2011;90(6):759–64.
 16. Takeda M, Takahashi M, Matsumoto S. Contribution of the activation of satellite glia in sensory ganglia to pathological pain. *Neurosci Biobehav Rev*. 2009;33(6):784–92.
 17. Ren K, Torres R. Role of interleukin-1beta during pain and inflammation. *Brain Res Rev*. 2009;60(1):57–64.
 18. Nozadze I, Tsiklauri N, Gurtskaia G, Tsagareli MG. Role of thermo TRPA1 and TRPV1 channels in heat, cold, and mechanical nociception of rats. *Behav Pharmacol*. 2016;27(1):29–36.
 19. Komiya H, Shimizu K, Noma N, Tsuboi Y, Honda K, Kanno K, et al. Role of neuron-glia interaction mediated by il-1beta in ectopic tooth pain. *J Dent Res*. 2018;97(4):467–75.
 20. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain*. 1983;16(2):109–10.
 21. Batbold D, Shinoda M, Honda K, Furukawa A, Koizumi M, Akasaka R, et al. Macrophages in trigeminal ganglion contribute to ectopic mechanical hypersensitivity following inferior alveolar nerve injury in rats. *J Neuroinflammation*. 2017;14(1):249.
 22. Katagiri A, Shinoda M, Honda K, Toyofuku A, Sessle BJ, Iwata K. Satellite glial cell P2Y12 receptor in the trigeminal ganglion is involved in lingual neuropathic pain mechanisms in rats. *Mol Pain*. 2012;8:23.
 23. Bender IB. Pulpal pain diagnosis—a review. *J Endod*. 2000;26(3):175–9.
 24. Alheim K, Bartfai T. The interleukin-1 system: Receptors, ligands, and ice in the brain and their involvement in the fever response. *Ann N Y Acad Sci*. 1998;840:51–8.

25. He X, Qian Y, Li Z, Fan EK, Li Y, Wu L, et al. TLR4-upregulated IL-1beta and IL-1Ri promote alveolar macrophage pyroptosis and lung inflammation through an autocrine mechanism. *Sci Rep.* 2016;6:31663.
26. Iwata K, Shinoda M. Role of neuron and non-neuronal cell communication in persistent orofacial pain. *J Dent Anesth Pain Med.* 2019;19(2):77–82.
27. Kiguchi N, Kobayashi D, Saika F, Matsuzaki S, Kishioka S. Pharmacological regulation of neuropathic pain driven by inflammatory macrophages. *Int J Mol Sci.* 2017;18(11):E2296.
28. Amano T, Muramatsu T, Amemiya K, Kubo K, Shimono M. Responses of rat pulp cells to heat stress in vitro. *J Dent Res.* 2006;85(5):432–5.
29. Pileggi R, Holland GR. The expression of heat shock protein 70 in the dental pulp following trauma. *Dent Traumatol.* 2009;25(4):426–8.
30. Turturici G, Sconzo G, Geraci F. Hsp70 and its molecular role in nervous system diseases. *Biochem Res Int.* 2011;2011:618127.
31. Urata K, Shinoda M, Honda K, Lee J, Maruno M, Ito R, et al. Involvement of TRPV1 and TRPA1 in incisional intraoral and extraoral pain. *J Dent Res.* 2015;94(3):446–54.
32. Honda K, Shinoda M, Kondo M, Shimizu K, Yonemoto H, Otsuki K, et al. Sensitization of TRPV1 and TRPA1 via peripheral mGLUR5 signaling contributes to thermal and mechanical hypersensitivity. *Pain.* 2017;158(9):1754–64.
33. Ebbinghaus M, Uhlig B, Richter F, von Banchet GS, Gajda M, Bräuer R, et al. The role of interleukin-1beta in arthritic pain: Main involvement in thermal, but not mechanical, hyperalgesia in rat antigen-induced arthritis. *Arthritis Rheum.* 2012;64(12):3897–907.

Figures

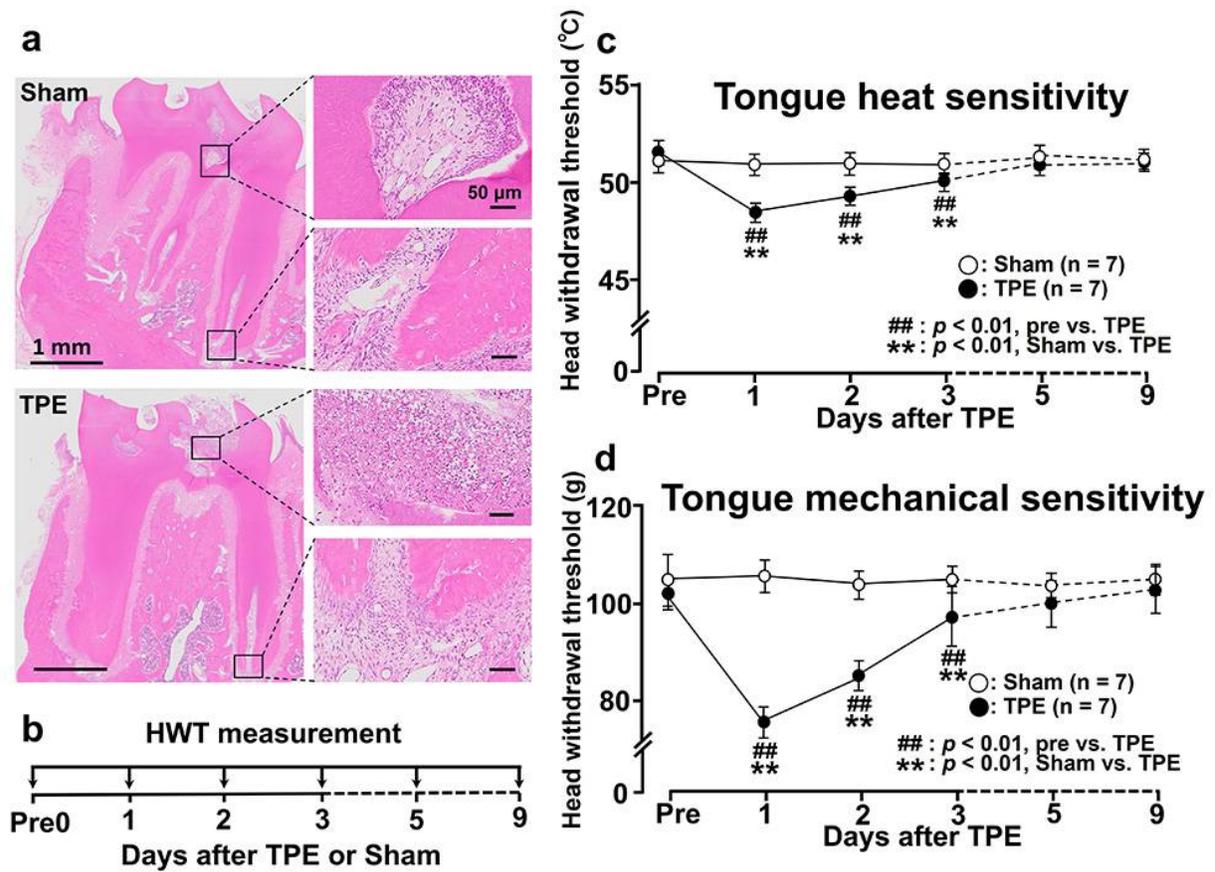


Figure 1

Nocifensive reflex to mechanical or heat stimulation of the tongue following tooth pulp exposure. a Photomicrographs of histological sections of the M1 tooth in 1 day after tooth pulp exposure (TPE). b The time course of behavioral tests. c, d Head withdrawal threshold (HWT) to heat stimulation (c) or mechanical stimulation (d) of the ipsilateral tongue following TPE. Group comparisons were performed by using two-way repeated-measures analysis of variance (ANOVA) followed by Tukey's post hoc tests. Data are expressed as the mean \pm SEM.

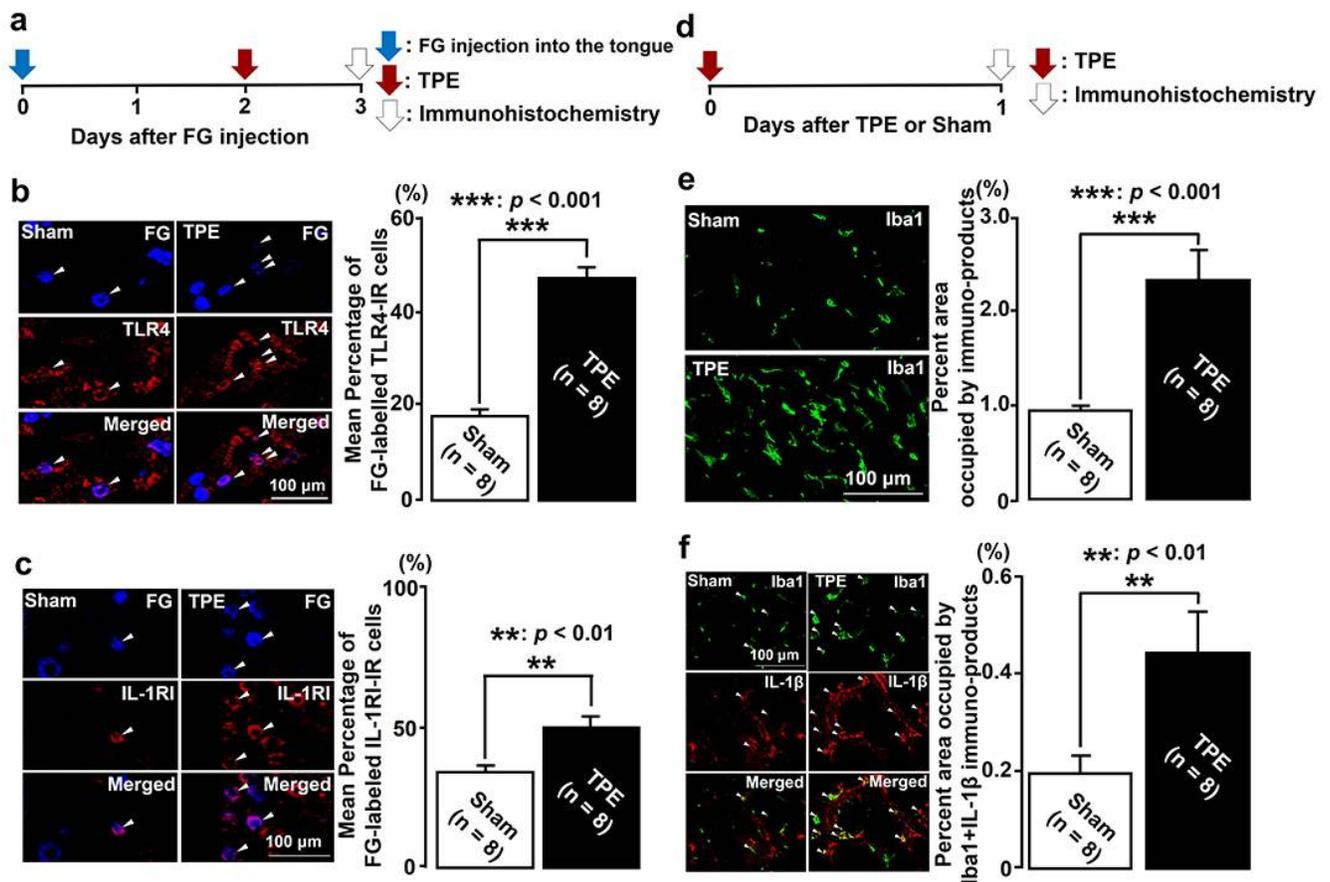


Figure 2

TLR4, IL-1RI, IBA1, and IL-1 β expression in the trigeminal ganglion following tooth pulp exposure. a Time course of the experimental procedure. b Fluorogold (FG)-labeling of TLR4-IR cells in the trigeminal ganglion (TG) following tooth pulp exposure (TPE). The arrowheads indicate FG-labeled TLR4-IR cells. Scale bar: 100 μ m. The number of each immunoreactive (IR) cell type in the TG of the third branch region was calculated by using the following formula: FG-labeled TLR4-IR cells / FG-labeled cells \times 100%. c FG-labeled IL-1RI-IR cells in the TG following TPE. The arrowheads indicate FG-labeled IL-1RI-IR cells. Scale bar: 100 μ m. The number of each IR cell type in the TG of the third branch region was calculated by using the following formula: FG-labeled IL-1RI-IR cells / FG-labeled cells \times 100%. The Student's t-test was employed for the comparison of the percentages of positive cells between groups. d Time course of the experimental procedure. e IBA1-IR cells in the TG following TPE. Scale bar: 100 μ m. The percentage of the area occupied by immuno-products was calculated. f IBA1-IR cells expressing IL-1 β in the TG following TPE. The arrowheads indicate IBA1-IR cells expressing IL-1 β . Scale bar: 100 μ m. The number of each IR cell type in the TG of the third branch region was calculated by using the following formula: immuno-products of IBA1+IL-1 β / IBA1 \times 100%. The Student's t-test was employed for the comparison of the percentages of positive cells between groups. Data are expressed as the mean \pm SEM.

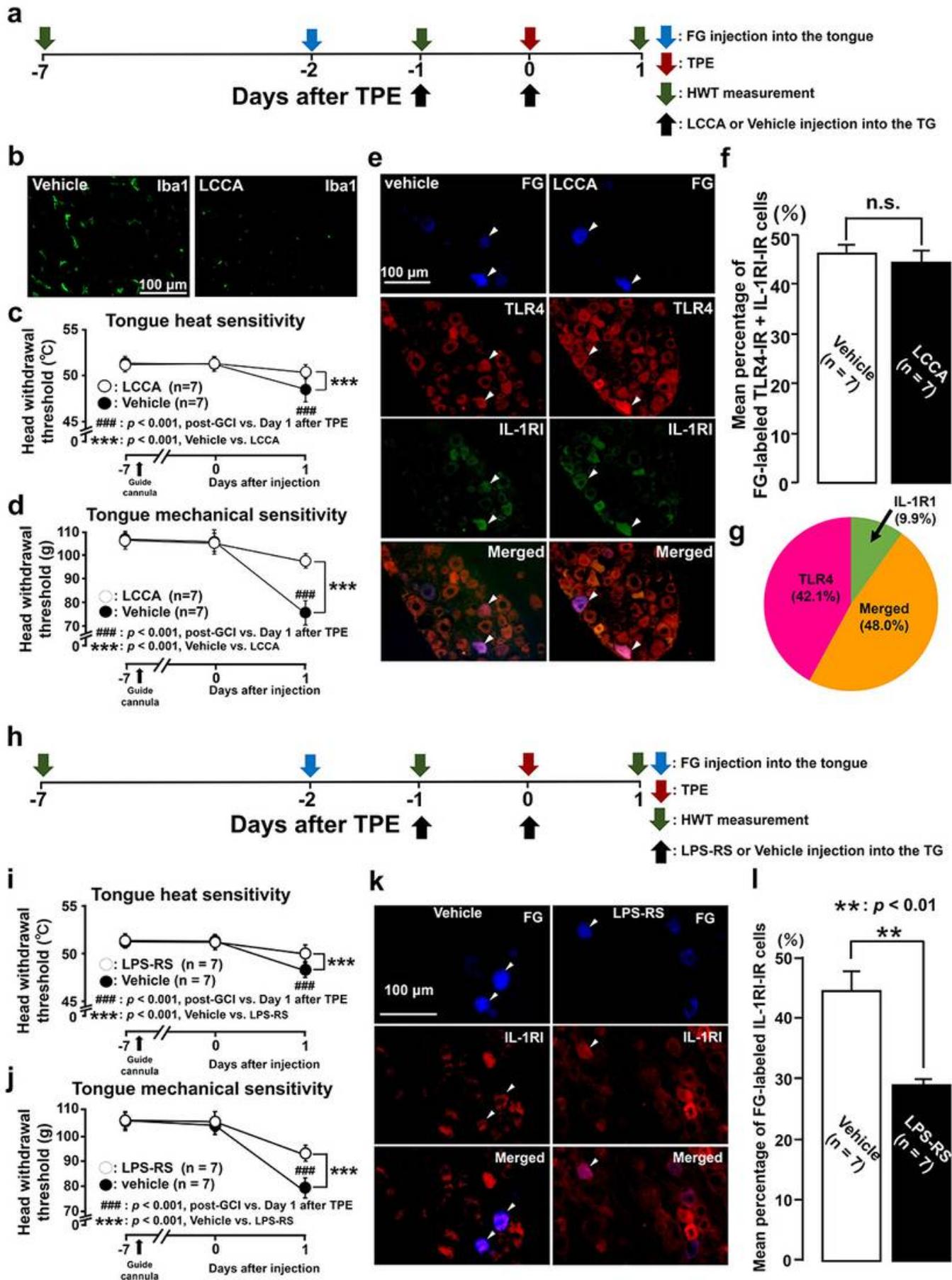


Figure 3

Effect of liposomal clodronate Clophosome-A (LCCA) or LPS-RS injection. a Time course of the experimental procedure. b Change in IBA1 expression in the trigeminal ganglion (TG) following LCCA injection. Scale bar: 100 μm . c, d Head withdrawal threshold (HWT) to heat stimulation (c) or mechanical stimulation (d) of the ipsilateral tongue following LCCA injection into the TG following tooth pulp exposure (TPE). Two-way repeated-measures ANOVA followed by Tukey's post hoc tests were used to compare HWTs between groups. e, f Fluorogold (FG)-labeled TLR4-IR and IL-1RI-IR cells in the TG following LCCA injection after TPE. The arrowheads indicate FG-labeled TLR4-IR and IL-1RI-IR cells. Scale bar: 100 μm . The number of each immunoreactive (IR) cell type in the TG of the third branch region was calculated by using the following formula: $\text{FG-labeled TLR4-IR and IL-1RI-IR cells} / \text{FG-labeled cells} \times 100\%$. The Student's t-test was employed for the comparison of the percentages of positive cells between groups. g Percentages of TLR4-IR, IL-1RI-IR, and merged cells in the TG. h Time course of the experimental procedure. i, j HWT to heat stimulation (i) or mechanical stimulation (j) of the ipsilateral tongue following LPS-RS injection into the TG after TPE. Two-way repeated-measures ANOVA followed by Tukey's post hoc tests were used to compare HWTs between groups. k, l FG-labeled IL-1RI-IR cells in the TG following LPS-RS injection after TPE. The arrowheads indicate FG-labeled IL-1RI-IR cells. Scale bar: 100 μm . The number of each IR cell type in the TG of the third branch region was calculated by using the following formula: $\text{FG-labeled IL-1RI-IR cells} / \text{FG-labeled cells} \times 100\%$. The Student's t-test was employed for the comparison of the percentages of positive cells between groups. Data are expressed as the mean \pm SEM.

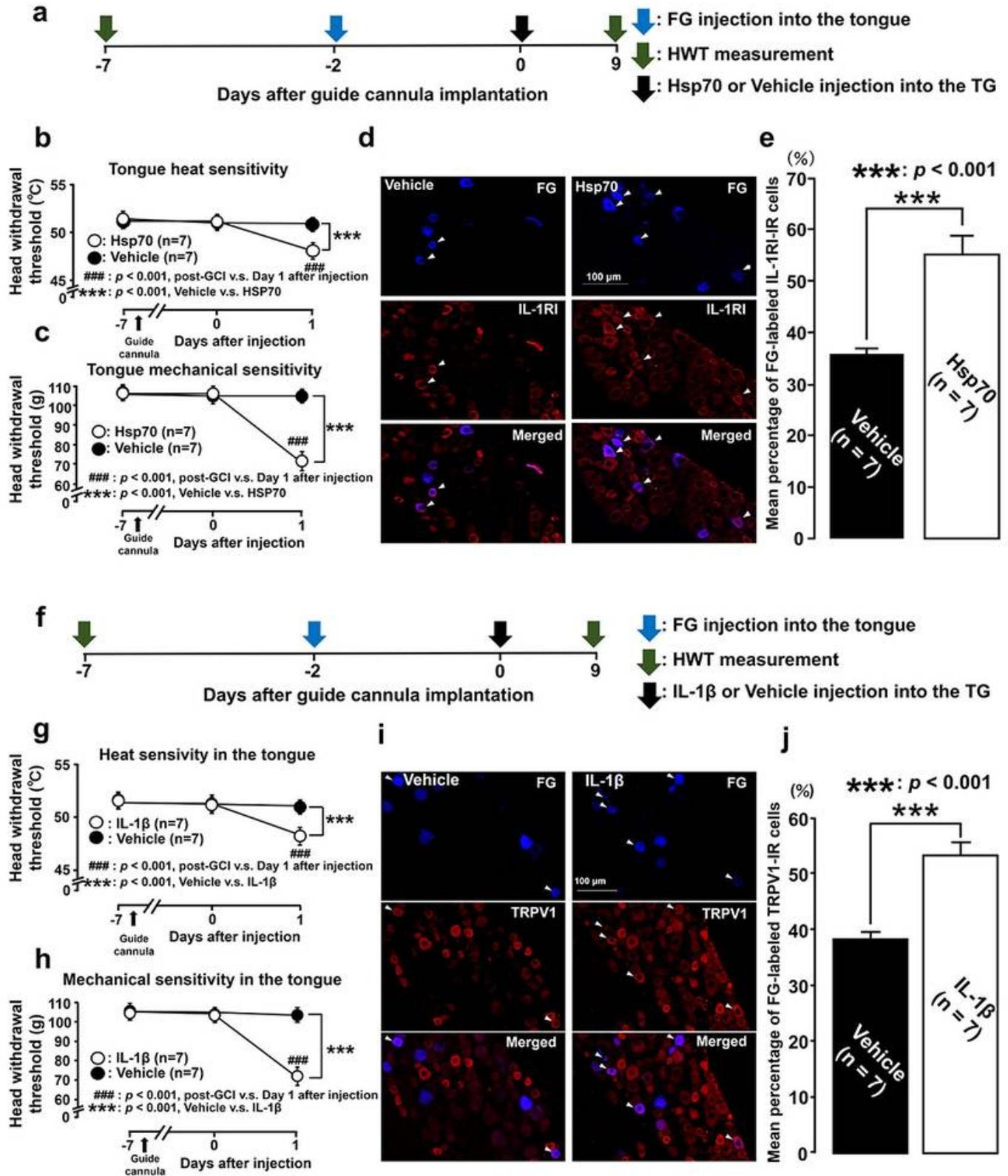


Figure 4

Effect of HSP70 or IL-1 β injection in the trigeminal ganglion. a Time course of the experimental procedure. b, c Head withdrawal threshold (HWT) to heat stimulation (b) or mechanical stimulation (c) of the ipsilateral tongue following HSP70 injection in the trigeminal ganglion (TG). Two-way repeated-measures ANOVA followed by Tukey's post hoc tests were used to compare HWTs between groups. d, e Fluorogold (FG)-labeled IL-1RI-IR cells following HSP70 injection into the TG. The arrowheads indicate FG-labeled IL-1RI-IR cells. Scale bar: 100 μ m. The number of each immunoreactive (IR) cell type in the TG of the third branch region was calculated by using the following formula: FG-labeled IL-1RI-IR cells / FG-labeled cells \times 100%. The Student's t-test was employed for the comparison of percentages of positive cell between groups. f Time course of the experimental procedure. g, h HWT to heat stimulation (g) or mechanical stimulation (h) of the ipsilateral tongue following IL-1 β injection into the TG. Two-way repeated-measures ANOVA followed by Tukey's post hoc tests were used to compare HWTs between groups. i, j FG-labeled TRPV1-IR cells following IL-1 β injection into the TG. The arrowheads indicate FG-labeled TRPV1-IR cells. Scale bar: 100 μ m. The number of each IR cell type in the TG of the third branch region was calculated by using the following formula: FG-labeled TRPV1-IR cells / FG-labeled cells \times 100%. The Student's t-test was employed for the comparison of percentages of positive cells between groups. Data are expressed as the mean \pm SEM.

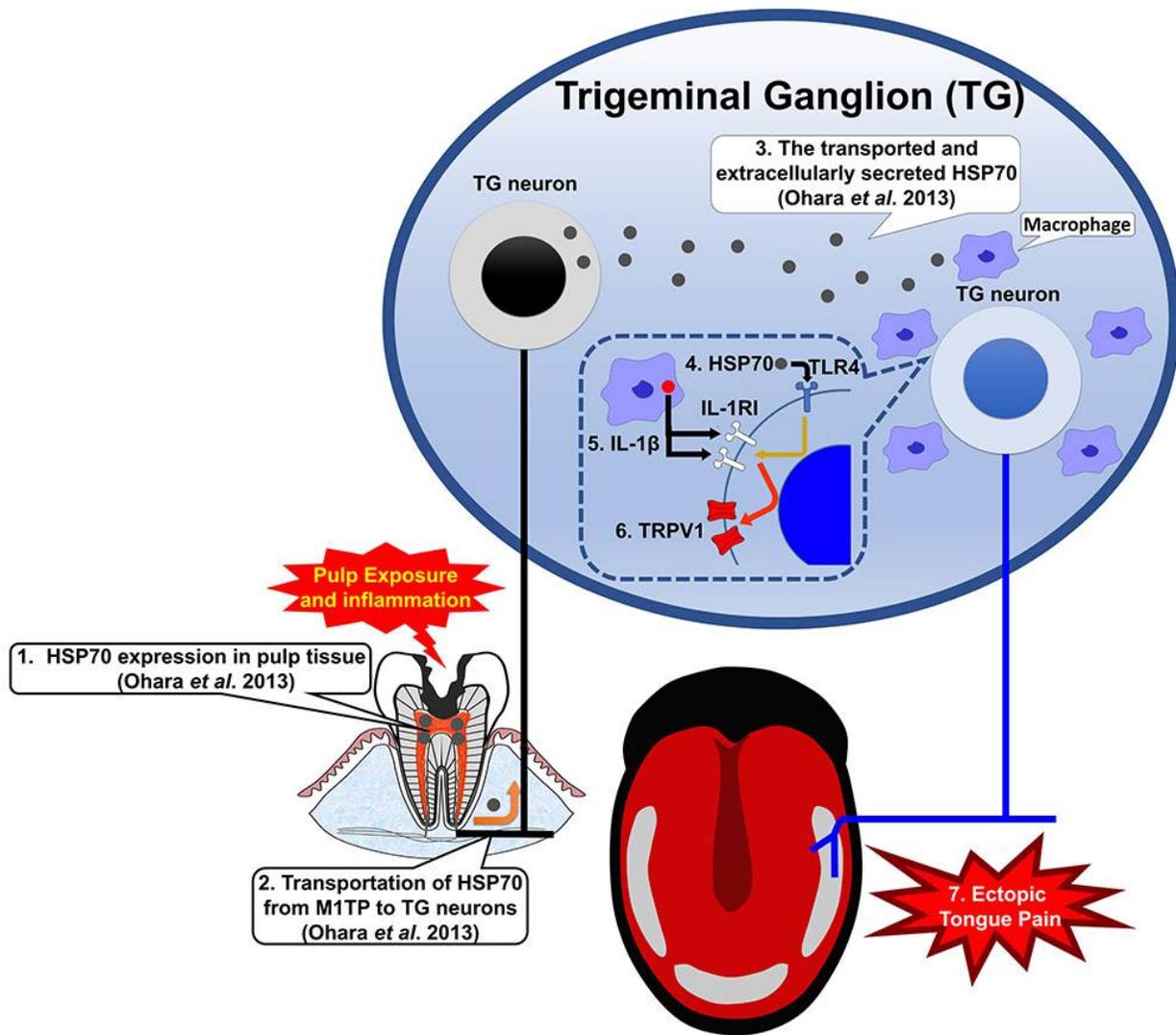


Figure 5

Schematic results of the current study. 1) Heat shock protein 70 (HSP70) is expressed in the pulpal tissue following tooth pulp exposure (TPE). 2) HSP70 is axonally transported from the inflamed tooth pulp of the lower first molar to the TG. 3) The transported HSP70 is released into the intercellular space in the TG. 4) HSP70 binds to the TLR4 of TG neurons innervating the tongue and promotes IL-1RI expression. 5) Simultaneously, the accumulated macrophages following tooth pulp inflammation generate and release IL-1β in the extracellular space in the TG. Larger amounts of IL-1β bind to the larger number of IL-1RI of TG neurons innervating the tongue. 6) The IL-1β/IL-1RI signaling facilitates TRPV1 expression. 7) Enhanced expression of TRPV1 on the tongue-innervating TG neurons promotes neuronal excitability and results in mechanical and heat hypersensitivity of the tongue.