Phosphorylation of p38 in Trigeminal Ganglion Neurons Contributes to Tongue Heat Hypersensitivity in Mice

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Aims: To develop a tongue pain model with no mucosal pathologic changes and to examine whether phosphorylation of p38 in trigeminal ganglion (TG) neurons innervating the tongue is associated with tongue heat hypersensitivity in mice. **Methods:** Tongue heat sensitivity in mice was assessed following application of the irritant 2,4,6-trinitrobenzene sulfonic acid (TNBS) to the tongue. After TNBS application, the expressions of p38, phosphorylated p38 (pp38), and transient receptor potential vanilloid 1 (TRPV1) were examined in TG neurons innervating the tongue. To further assess changes in tongue heat sensitivity and TRPV1 expression, a specific inhibitor of p38 phosphorylation (SB203580) was also administered into the TG. Student *t* test or two-way repeated-measures analysis of variance followed by Sidak multiple comparison test were used for statistical analysis, and $P < .05$ was considered statistically significant. **Results:** TNBS application to the tongue induced noninflammatory heat hypersensitivity accompanied by the enhancement of p38 phosphorylation in TG neurons innervating the tongue and by an increase in the number of TRPV1 and pp38–immunoreactive (IR) TG neurons innervating the tongue. Intra-TG administration of SB203580 suppressed the increase in the TRPV1 and pp38–IR TG neurons and alleviated the noninflammatory tongue heat hypersensitivity induced by TNBS. **Conclusion:** p38 signaling cascades are involved in tongue heat hyperalgesia in association with TRPV1 upregulation in TG neurons innervating the TNBS-treated tongue. *J Oral Facial Pain Headache 2017;31:372–380. doi: 10.11607/ofph.1849*

Keywords: *burning mouth syndrome, heat hyperalgesia, mitogen-activated protein kinase, transient receptor potential vanilloid 1, trigeminal ganglion*

Burning mouth syndrome (BMS) is a dysesthesia that manifests as an oral mucosal burning sensation without intraoral pathologic changes or pathogenic observations in laboratory testing. This intraoral burning sensation lasts for several months or years in BMS patients.[1–4] It is well known that lingual nerve injury or mucosal inflammation of the mouth induces tongue hypersensitivity in rodents due to changes in the neuronal excitability and/or expression of ion channels closely related to nociception in trigeminal ganglion (TG) neurons.[5,6] Nevertheless, the exact mechanisms underlying tongue hypersensitivity without any apparent pathologic changes in the tongue are not known, although it is suspected that sensitization of TG neurons is caused by a deficit of trigeminal nociceptors in humans.[7,8]

The mitogen-activated protein kinases (MAPKs) that belong to the serine/threonine protein kinase family are known to peripherally regulate intracellular signaling or gene expression associated with the modulation of sensory neuronal excitability.[9,10] The MAPK family is composed of three major members: extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38. Each has been shown to play an important role in the pathologic transduction of pain signaling,[11] and each MAPK is unique in its intracellular signal transduction pathways.[12] Noxious stimuli, such as heat and pinching, are known to result in phosphorylation of p38 in dorsal root ganglion (DRG) neurons, and the p38 phosphorylation in turn modulates nociceptive neuronal excitability via downstream signaling.[13] Activation of p38 in DRG neurons following peripheral inflammation leads to the phosphorylation of the voltage-gated

sodium channel 1.8 (Nav1.8), which enhances membrane trafficking of this channel, resulting in nociceptive neuronal hyperexcitability.[14] Furthermore, activated p38 may be increased in association with Nav1.8, which is expressed in painful neuromas caused by peripheral nerve injury in humans.[15]

The chemical 2,4,6-trinitrobenzene sulfonic acid (TNBS) is a well-known irritant that causes chronic visceral hypersensitivity related to irritable bowel syndrome, which, like BMS, shows no clear features of pathologic changes, including inflammation.[16] Recently, it has been reported that TNBS application to the tongue surface induces tongue heat hypersensitivity without any lingual mucosal pathologic changes, indicating that this may be a useful animal model for the study of lingual hypersensitivity associated with BMS.[17]

The aims of this study were to develop a tongue pain model with no mucosal pathologic changes and to examine whether p38 phosphorylation in TG neurons is associated with tongue heat hypersensitivity in mice. Therefore, the following experiments were performed: Following application of TNBS to the tongue, *(1)* changes in the immunoreactivity of transient receptor potential vanilloid 1 (TRPV1) in TG neurons innervating the tongue were examined, and *(2)* the effects on heat hypersensitivity of p38 phosphorylation and the inhibition of p38 phosphorylation in TG neurons innervating the tongue were assessed.

Materials and Methods

Animals

Male C57BL/6NCr mice (n = 70, 20 to 30 g, Japan SLC) were used in this study. Mice were bred under laboratory conditions (23˚C, 12/12–hour light/dark cycle) with free access to tap water and food. This study was performed with the approval of the local animal ethics committee in Nihon University (AP14D002) and complied with the ethical guidelines published by the International Association for the Study of Pain.[18] In all experiments, the intensity of pain or discomfort was reduced to as low as possible, and the number of mice was kept to a minimum.

Tongue Treatment

Under deep anesthesia produced by an intraperitoneal (ip) injection of sodium pentobarbital (50 mg/kg, Schering-Plough), TNBS (10 mg/mL; diluted in 50% ethanol, Sigma-Aldrich) suspension or isotonic saline (as a vehicle control) was applied on the tongue dorsum over a period of 60 minutes in 12 mice (6 TNBS and 6 vehicle). Five days after treatment, these mice were perfused transcardially with isotonic saline under deep sodium pentobarbital anesthesia (ip injection, 50 mg/kg). The tongue was removed and embedded in Tissue Tek (Sakura Finetechnical). Tongue sections were produced by a cryostat and placed on slides (–20°C). Sections were moved to room temperature and fixed with 4% paraformaldehyde (PFA) for 1 hour. The sections were stained with hematoxylin and eosin to assess any pathologic changes in the tongue.

Assessment of Thermal Sensitivity on the Tongue

A total of 12 mice (6 TNBS and 6 vehicle) were anesthetized by using 2% isoflurane (Mylan). Heat stimulation was applied to the tongue after terminating the supply of isoflurane once noxious pinch stimulation (150 g) applied to the hindpaw induced an identical weak flexion reflex of the hindlimb to ensure that an adequate level of anesthesia was maintained. Under the adjusted depth of anesthesia, heat stimulation (35°C to 55°C, 1°C/second) to the left dorsum of the tongue was applied by using a contact thermal probe. The application of the heat stimulation was performed three times at 5-minute intervals, and the mean temperature at which a head withdrawal reflex occurred was defined as the head withdrawal reflex threshold (HWT). The HWTs were determined once daily on alternate days for 17 days. The HWT behavioral testing was conducted under blind conditions.

Immunohistochemistry

To identify the TG neurons innervating the tongue, 3% hydroxystilbamidine (2 µL, FluoroGold [FG]; Fluorochrome) was subcutaneously injected into the tongue dorsum by using a 30-gauge needle in parallel with TNBS or vehicle treatment in 20 mice (10 TNBS and 10 vehicle). Five days after treatment, these mice were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PFA, pH 7.4) following isotonic saline and under the deep anesthesia produced by sodium pentobarbital (ip injection, 50 mg/kg). The TG ipsilateral to the treatment was dissected and soaked in 4% PFA (4 hours, 4°C) for postfixation. For cryoprotection, the TGs were exposed to 0.1 M phosphate-buffered saline (PBS) containing 20% sucrose for 12 hours. Then, the TGs were embedded in Tissue Tek (Sakura Finetechnical). The TGs were cut horizontally by using a cryostat (thickness of 15 µm) in parallel with the long axis. The sections were mounted and desiccated onto microscope slides (MAS-coated Superfrost plus, Matsunami). The analysis (see below) was performed in 4 sections (every 10th section) per TG. The sections were then incubated for 3 days at 4°C with anti-glial fibrillary acidic protein (GFAP) polyclonal rabbit antibody (1:500; Abcam), anti-p38 mitogen-activated protein kinase (MAPK) polyclonal rabbit antibody (1:1,000; #9212L, Cell Signaling),

anti-phospho-p38 (pp38) MAPK polyclonal rabbit antibody (1:1,000; #9211S, Cell Signaling), and/or anti-TRPV1 polyclonal guinea pig antibody (1:1,000; AB5566, Merck Millipore) diluted with 0.01 M PBS, which contained 0.3% Triton X-100 (Sigma-Aldrich) and 4% normal goat serum. GFAP and TRPV1 were used because GFAP is a marker of satellite glial cells and TRPV1 is a cation channel that possesses the ability to detect noxious heat stimuli expressed in nociceptive neurons. Next, the sections were exposed to Alexa Fluor 488–conjugated goat anti-rabbit immunoglobulin G (IgG) (1:100; A11008, Thermo Fisher Scientific), alone or together with Alexa Fluor 568–conjugated goat anti-guinea pig IgG (1:100; A11075, Thermo Fisher Scientific), and diluted with 0.01 M PBS (2 hours, 23°C). In turn, the TG sections were coverslipped in mounting medium (Thermo Fisher Scientific).

The analysis of identified FG-labeled, FG-labeled p38-immunoreactive (IR), FG-labeled TRPV1- and pp38-IR, and GFAP-IR TG neurons was carried out by the use of a BZ-9000 system equipped with appropriate filters (Keyence). No specific labeling was observed in the absence of primary antibodies under the same conditions. Cells that showed a fluorescence intensity more than twice as large compared to the level of background average were defined as IR cells. The ratios of p38-IR neurons and pp38-IR neurons were evaluated by using the following formula per the four sections of each TG (assessed in TGs from 5 TNBS-treated mice and 5 vehicle-treated mice): 100 × number of FG-labeled p38-IR or pp38-IR cells/total number of FG-labeled cells. The ratio of FG-labeled TRPV1 and pp38–IR neurons was evaluated by using the following formula per the four sections of each TG (assessed in TGs from 5 TNBS-treated mice and 5 vehicle-treated mice): 100 × total number of FG-labeled pp38 and TRPV1–IR cells/total number of FG-labeled pp38-positive cells. Counting of cells was conducted under blinded conditions.

Selective Inhibition of p38 MAPK in the TG

Under deep anesthesia produced by sodium pentobarbital (ip injection, 50 mg/kg), 26 mice were mounted into a stereotaxic frame, and the cortical surface was exposed. A small hole of 1-mm diameter was made 4.5 to 5.5 mm below the cortical surface, 2.5 to 3.0 mm anterior from the posterior fontanelle, and 0.5 to 1.2 mm lateral to the sagittal suture. A guide cannula was inserted through the small hole into the TG and anchored to the cortical bone by using three stainless steel screws implanted with dental cement. TNBS or isotonic saline treatment was conducted in these mice 1 week after the hole was made.

A specific inhibitor of p38 phosphorylation, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580) (100 μM; Merck Millipore), was dissolved in 4% dimethyl sulfoxide (DMSO) in isotonic saline. Following TNBS treatment, administration of vehicle (4% DMSO) or SB203580 (13 mice each) into the TG was performed by using a 30-gauge needle inserted through the guide cannula under light anesthesia with 2% isoflurane. SB203580 or vehicle (1 μL) was administered daily once per day for 17 days.

The HWTs in 16 mice treated with SB203580 or vehicle (8 in each group) were measured on day 3 before starting treatment and for 17 days after treatment, as described above. In 10 mice (5 SB203580 and 5 vehicle), the ratio of FG-labeled TRPV1 and pp38–IR neurons in the TG was calculated as described above.

Statistical Analyses

Data were presented as mean ± standard deviation (SD) or standard error of the mean (SEM). Student *t* test or two-way repeated-measures analysis of variance (ANOVA) followed by Sidak multiple comparison test were used. P values < .05 were considered statistically significant.

Results

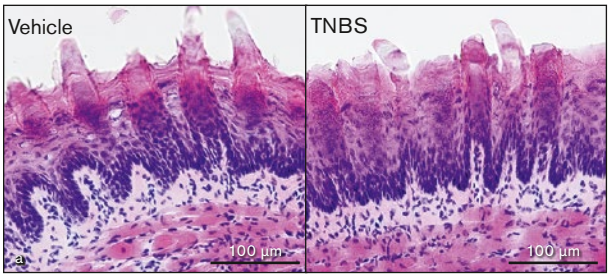
Histology and Tongue Heat Sensitivity Following TNBS Application

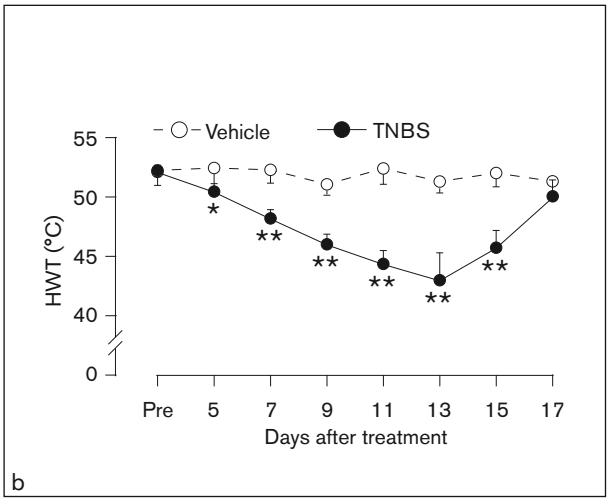
No histologic changes such as mucosal hypertrophy, inflammatory cell infiltration, or scar formation were observed in the tongues of mice 5 days after treatment with TNBS (Fig 1a), but the HWT in these mice (50.5°C ± 0.3°C) was significantly decreased compared to that of vehicle-treated controls (52.5°C ± 0.6°C) at this time point. This decreased HWT lasted until day 15 of the experimental period (Fig 1b). There was no change in HWT at any time in the vehicle-treated group. Despite the tongue heat hypersensitivity of the TNBS-treated group, these mice gained weight normally and did not display any motor disturbance during the experimental period (data not shown).

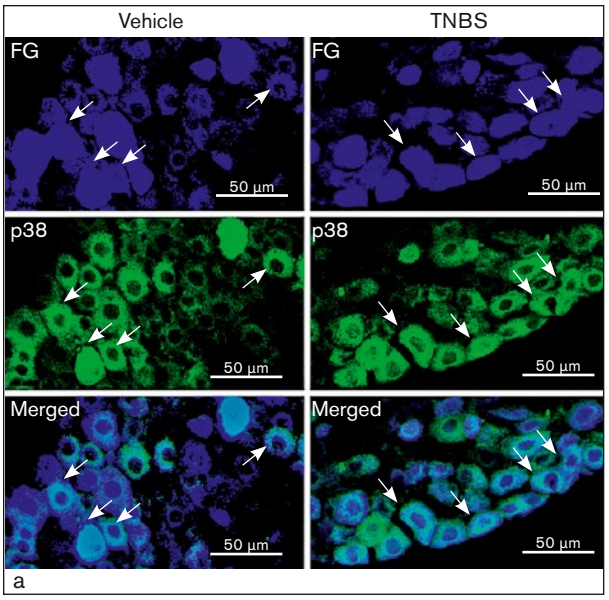
Phosphorylation of p38 in TG Neurons

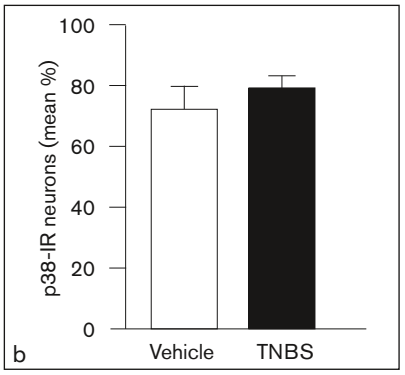
FG-labeled neurons expressed p38, and no changes in the mean proportion of FG-labeled p38-IR neurons were observed on day 5 following treatment with TNBS (78.8% ± 3.4%) or vehicle (71.6% ± 6.9%) (Fig 2a); however, the mean proportion of p38-IR neurons was significantly increased in TNBS-treated mice (72.5% ± 8.2%) compared to those treated with vehicle (51.1% ± 3.7%) (Fig 2b). There were no significant differences in the number of the FG-labeled p38-IR neurons between TNBS-treated and vehicle-treated mice (data not shown).

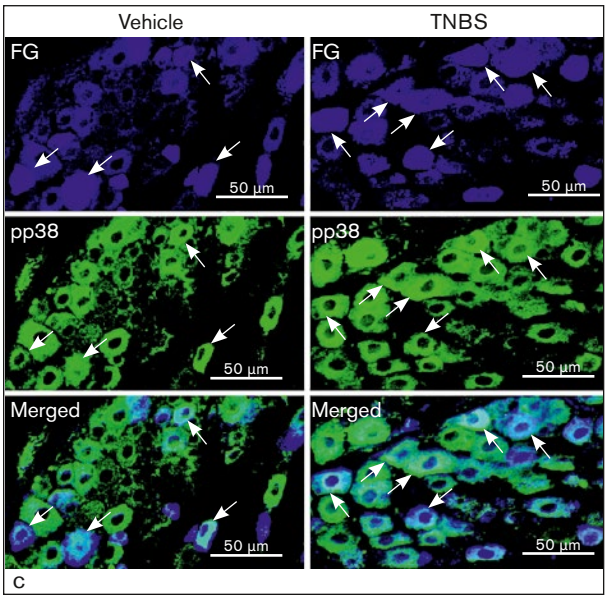
Fig 1 Morphologic and heat sensitivity analyses of the tongue in mice following treatment with TNBS or vehicle. **(a)** Histologic appearance of the tongue mucosa 5 days after treatment. **(b)** Changes in head withdrawal threshold (HWT). Data represent mean ± standard error of the mean (SEM). *P < .05, **P < .01 for TNBS- vs vehicle-treated mice (n = 6 in each group). Two-way repeated-measures ANOVA followed by Bonferroni multiple-comparison test.











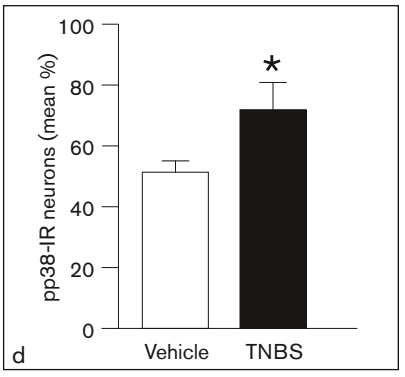
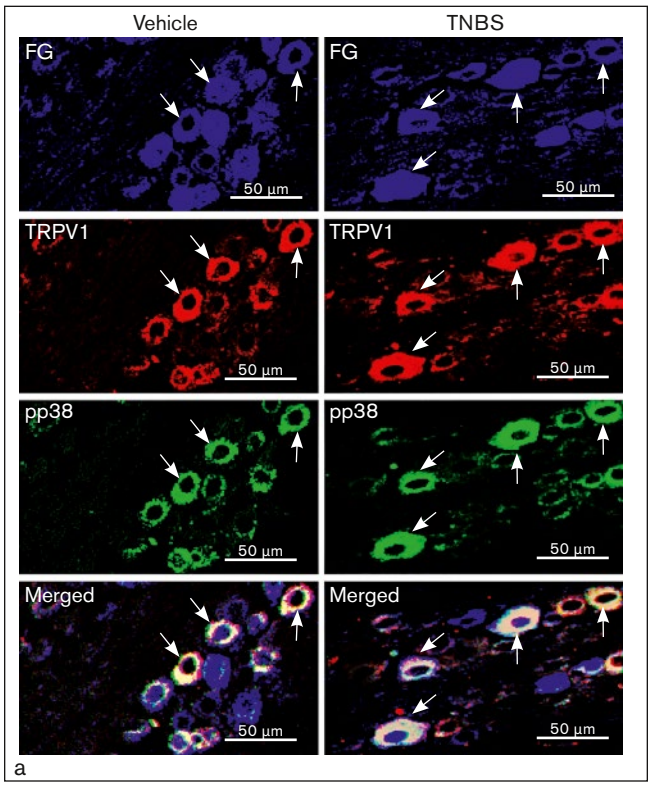


Fig 2 Phoshorylation of p38 in TG neurons innervating the tongue following treatment with TNBS or vehicle. **(a)** FG-labeled p38-IR TG neurons *(arrows)* and **(b)** the mean proportion in the tongue 5 days after treatment. **(c)** FG-labeled pp38-IR TG neurons *(arrows)* and **(d)** the mean proportion in the tongue 5 days after treatment. Data represent mean ± standard error of the mean (SEM). *P < .05 (n = 5 in each group). Student t test.



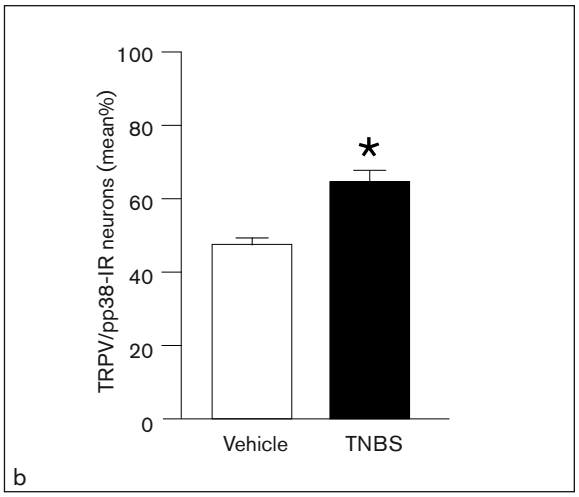


Fig 3 Changes in TRPV1 expression in pp38-IR TG neurons innervating the tongue. **(a)** FG-labeled TRPV1 and pp38–IR TG neurons *(arrows)* and **(b)** the mean proportion in the tongue 5 days after treatment. Data represent mean ± standard error of the mean (SEM). *$P < .05$ (n = 5 in each group). Student *t* test.

TRPV1 Expression in pp38-IR TG Neurons

TG neurons innervating the tongue expressed both TRPV1 and pp38, and the mean proportion of TRPV1 and pp38–IR neurons was significantly increased on day 5 in mice treated with TNBS (65.1% ± 2.6%) compared to those treated with vehicle (48.1% ± 1.3%) (Figs 3a and 3b).

Effect of p38 Inhibitor on TRPV1 Expression and Tongue Heat Hypersensitivity

TG neurons innervating the tongue expressed pp38 and TRPV1, and the mean proportion of TRPV1 and pp38–IR TG neurons was significantly decreased on day 5 in mice treated with TNBS and SB203580 (32.1% ± 3.5%) compared to mice treated with TNBS and vehicle (57.6% ± 4.6%) (Figs 4a and 4b). The administration of SB203580 in the TG resulted in a marked reversal of the TNBS-induced tongue heat hyperalgesia from days 5 to 15 when compared to TNBS-treated mice administered vehicle (Fig 4c). Mice did not show any motility or sensory disturbances during the experimental period (data not shown).

GFAP Expression in TG Following TNBS Treatment

Five days after treatment, GFAP-IR cells in the TG were not identified in the TNBS-treated or vehicle-treated groups (Fig 5).

Discussion

BMS patients complain of aberrant burning sensations in the tongue but have no evidence of pathologic changes.[3,19] Animal models that mimic the characteristics of the actual disease are important for studies of its mechanisms, and several models of tongue pain have been established—for example, injection of Complete Freund's Adjuvant (CFA) or formalin into the tongue induces the development of tongue hypersensitivity.[20,21] However, since the CFA and formalin models produce lingual pathologic changes, these tongue pain models are markedly different from BMS and are thus not appropriate for examining tongue pain in BMS patients. Therefore, the present study tested whether TNBS application to the tongue could cause noninflammatory tongue hypersensitivity, which is a distinctive sign of BMS. The study revealed a persistent tongue heat hyperalgesia lasting from days 5 to 15 after application of TNBS. Moreover, the induced heat hyperalgesia persisted in the noninflamed tongue despite no apparent pathologic changes, such as inflammation or hypertrophy. However, while BMS in humans is characterized as a chronic pain state with a continuous burning sensation that may last for years, the TNBS-induced tongue heat hypersensitivity lasted from days 5 to 15 in this model.[22,23] Therefore, this new model of BMS does not fully reflect BMS in humans, but is likely to be helpful in elucidating tongue pain mechanisms related to BMS.

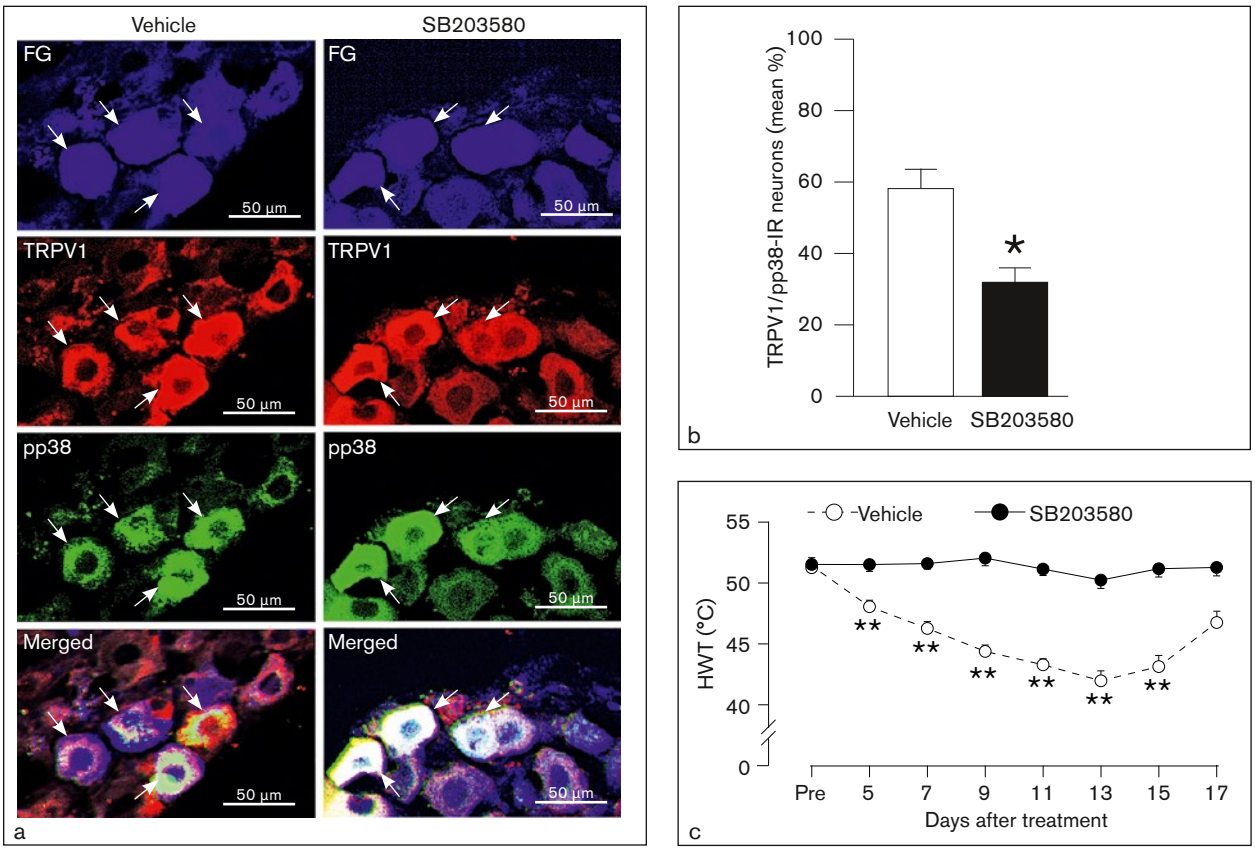


Fig 4 Effect of p38 phosphorylation on TRPV1 expression in TG neurons innervating the tongue and on tongue heat sensitivity. **(a)** FG-labeled TRPV1 and pp38–IR TG neurons *(arrows)* and their mean proportion in the tongue **(b)** 5 days after intra-TG administration of vehicle or p38 phosphorylation inhibitor (SB203580). Data represent mean ± standard error of the mean (SEM). *$P < .05$ (n = 5 in each group). Student *t* test. **(c)** Changes in HWT after administration of vehicle or SB203580 in TNBS-treated mice over the course of the experiment. Data represent mean ± SEM. **$P < .01$ vs vehicle-treated mice (n = 8 in each). Two-way repeated-measures ANOVA followed by Bonferroni multiple comparison test.

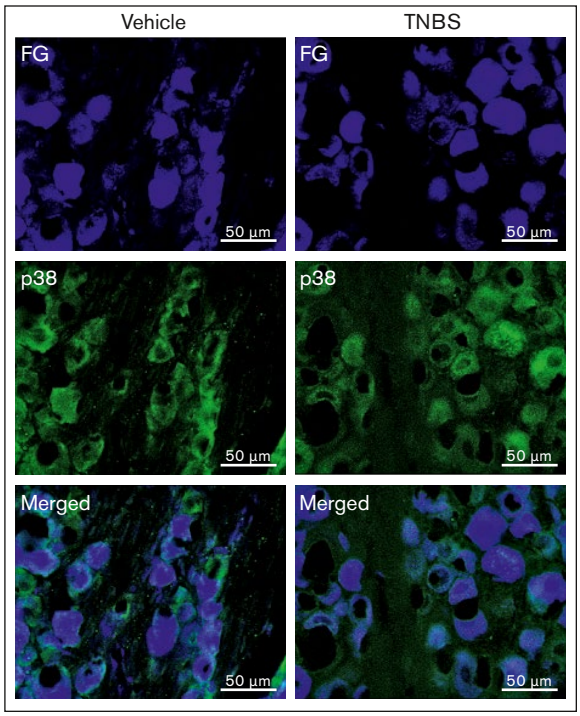


Fig 5 Satellite glial cell activity in the TG. FG-labeled neurons and GFAP immunoreactivity in the TG 5 days after vehicle or TNBS treatment.

A variety of extracellular stimuli cause the phosphorylation of intracellular p38, which is a member of the MAPK family and modulates the activity of intracellular cytoplasmic molecules driving different downstream signaling pathways capable of modulating gene expression.[13,24] In a previous study, expressions of the proteins glial cell line–derived neurotrophic factor (GDNF), nerve growth factor (NGF), interleukin 1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α) in the tongue mucosa were significantly increased on days 1 and 5 following TNBS treatment.[17] Upregulated NGF in peripheral tissues has been reported to be retrogradely transported to the DRG neuronal soma.[25] Intrathecal NGF administration induces hypersensitivity and enhances phosphorylation of p38 in small- to medium-sized DRG neurons that express TrkA, and peripheral anti-NGF treatment suppresses phosphorylation of p38 in DRG neurons following CFA injection.[26] Likewise, treatment with anti-NGF neutralizing antibody significantly inhibited phosphorylation of p38 in DRG neurons in a diabetic neuropathic pain model in mice.[27] In cultured neural cells, GDNF signaling mediated through the RET signaling receptor induced p38 activation via apolipoprotein B expression.[28] Moreover, recombinant IL-1β injection into the sciatic nerve increased the number of pp38-positive L4 DRG neurons and induced plantar thermal and mechanical hypersensitivity in rats.[29] In addition, intraplantar IL-1β injection caused a dose-dependent enhancement of thermal sensitivity, and this enhancement was significantly attenuated by intraplantar administration of a p38 inhibitor.[30,31] TNF-α signaling leading to the activation of TNF receptor 1 induced phosphorylation of p38 in DRG neurons and enhanced tetrodotoxin-resistant currents in a p38-dependent manner in mice,[32] resulting in nociceptive neuronal hyperexcitability.[33] In the present study, pp38 expression in TG neurons innervating the tongue was significantly increased at 5 days following TNBS treatment, although p38 expression did not change. These results therefore suggest that TNBS application to the tongue is involved in the phosphorylation of p38 in TG neurons innervating the tongue, resulting in tongue heat hyperalgesia via enhancement of nociceptive neuronal excitability in the TG. However, further studies are necessary to identify the TNBS-induced molecules that are produced in peripheral tissues and activate p38 in TG neurons.

TRPV1 is a TRPV subfamily member that contributes to normal and pathologic nociceptive processes and is expressed on a subclass of nociceptive afferent neurons.[34,35] Noxious heat (> 43°C), several other exogenous agents, and endogenous agents such as camphor, N-arachidonoyl dopamine, and anandamide can induce activation of TRPV1.[36–38] Recent studies have demonstrated that TRPV1 upregulation in DRG or TG neurons contributes to the enhancement of sensitivity to heat stimulation, resulting in chronic heat hypersensitivity after peripheral inflammation or nerve injury.[39,40]

The phosphorylation of p38 resulting from various stimuli to primary sensory neurons is translocated to the neuronal nuclei, resulting in an increase in pain signaling from the peripheral nociceptors via modification of the translation of proteins and regulation of the transcription of critical genes that play a significant role in pain modulation in primary nociceptors.[11] In response to the stimulation of lipopolysaccharides, adenylate-uridylate–binding proteins are phosphorylated in a p38 MAPK, resulting in the TNF-α and IL-1β translation and secretion from monocytes.[41,42] Phosphorylation of p38 in the DRG neurons by peripheral NGF administration increases expression of TRPV1 in peripheral nociceptor terminals, contributing to the maintenance of the pain hypersensitivity induced by local inflammation.[26] Moreover, intraplantar artemin administration causes upregulation of pp38 in TRPV1-positive neurons in small- and medium-sized L4-5 DRG in vivo, and inhibition of p38 phosphorylation significantly suppresses TRPV1 upregulation in DRG neurons induced by artemin in vitro.[43] In the present study, the number of pp38-IR TG neurons innervating the tongue was significantly increased 5 days after TNBS treatment. The number of TRPV1-IR TG neurons that expressed pp38 also increased on day 5 following TNBS treatment, and this increase was significantly suppressed by the inhibition of p38 phosphorylation in the TG. The continuous inhibition of p38 phosphorylation in the TG produced a significant decrease in the TNBS-induced tongue heat hypersensitivity. Together, these findings suggest that in TG neurons innervating the tongue, upregulation of TRPV1 caused by TNBS treatment is mediated through the p38 signaling cascade, resulting in tongue hypersensitivity.

Recent studies have also reported that activation of satellite glial cells in the TG is involved in orofacial hypersensitivity following inferior alveolar nerve transection, lingual nerve crush, and tooth pulp inflammation.[5,44,45] GFAP immunoreactivity indicates the activation of satellite glial cells in the TG, but was not clearly identified following TNBS treatment in the present study, suggesting that satellite glial cell activation in the TG has little importance for TNBS-induced tongue heat hypersensitivity.

Although the findings of the present study suggest that peripheral TG changes may be more prominent in this BMS model, it remains possible that central nervous system changes, such as central sensitization in the trigeminal spinal subnucleus caudalis, may also accompany or make even greater contributions to the irritant-induced tongue heat hyperalgesia. For example, phosphorylation of glutamate receptor

subunits 1, which is a component of a-amino-3-hydrozy-5methylisoxazole-4-propionic acid receptor via extracellular signal-regulated kinase phosphorylation, is involved in central sensitization of nociceptive neurons, resulting in tongue mechanical hyperalgesia following tongue drying.[46] Metabotropic glutamate receptor 5 signaling in the subnucleus caudalis enhanced by tongue inflammation is also involved in tongue mechanical and heat hypersensitivity.[20]

A limitation of this study was the use of male mice to elucidate the possible peripheral mechanisms of the TNBS-induced tongue heat hyperalgesia, as most BMS patients are female.[4,23] Therefore, further studies using female animals are needed to clarify the mechanisms of tongue pain relevant to BMS.

Conclusions

This study has established a mouse model that manifests tongue heat hypersensitivity produced by TNBS application to the tongue but that shows no tongue pathologic changes. It has been demonstrated that TNBS treatment of the tongue can provide a useful model to clarify the mechanisms of BMS-related tongue heat hypersensitivity. The data suggest that tongue heat hypersensitivity can be induced by upregulation of TRPV1 in TG neurons innervating the tongue by intracellular signaling cascades via p38 signaling. The blockade of p38 signaling might be a potential therapeutic target for tongue heat hypersensitivity relevant to BMS.

Acknowledgments

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