Integrative Medicine Research 12 (2023) 100999

Contents lists available at ScienceDirect

Integrative Medicine Research

journal homepage: www.elsevier.com/locate/imr

Original Article

GV16 acupoint stimulation with bee venom reduces peripheral hypersensitivity via activation of α2 adrenoceptors in a nitroglycerin-induced migraine mouse model

Sol-Ji Kim [a], Ji-Hee Yeo [a], Seo-Yeon Yoon [b,*], Dae-Hyun Roh [a,*]

[a] Department of Oral Physiology, College of Dentistry, Kyung Hee University, Seoul 02447, Republic of Korea
[b] Department of Companion Animals, Yuhan University, Bucheon-si, Gyeonggi-do 14780, Republic of Korea

ARTICLE INFO

Keywords:
Bee venom
Nitroglycerin
Allodynia
Fos
Alpha-2 adrenoceptors

ABSTRACT

Background: Peripheral hypersensitivities develop in the face and hindpaws of mice with nitroglycerin (NTG)-induced migraine. We evaluated whether diluted bee venom (DBV) injections at acupoints prevented these peripheral hypersensitivities and c-Fos expression in the trigeminal nucleus caudalis (TNC).

Methods: NTG (10 mg/kg, intraperitoneal, i.p.) was administered every other day for nine days. DBV (0.1 mg/kg) was subcutaneously injected into the ST36 (Zusanli), LI4 (Hegu), or GV16 (Fengfu) acupoints 75 min after each NTG injection. Mice were pretreated with naloxone (5 mg/kg, i.p.) or yohimbine (5 mg/kg, i.p.) 30 min before the DBV injections.

Results: NTG injection caused facial cold allodynia, hindpaw mechanical allodynia, and increased c-Fos-immunoreactive (ir) cells in the TNC. Repetitive DBV injections at GV16, but not the ST36, or LI4 acupoints, suppressed NTG-induced hindpaw mechanical allodynia and facial cold allodynia. The number of c-Fos-ir cells also decreased in response to DBV injections at the GV16 acupoint. Remarkably, pretreatment with yohimbine reversed the anti-allodynic effects of DBV injections and attenuated the decreased c-Fos expression in response to GV16 DBV treatment. Naloxone did not block the effects of GV16 DBV stimulation.

Conclusion: These findings demonstrate that repetitive DBV treatment at the GV16 acupoint relieves NTG-induced facial and hindpaw hypersensitivities and decreases in c-Fos expression in the TNC via activation of the alpha-2 adrenoceptors, but not the opioid receptors.

1. Introduction

Migraine, a multifactorial primary headache disorder, is characterized by unilateral, severe, and pulsatile headaches.[1] Migraine disease occurs in episodes separated by headache-free days; however, 3 % of patients experience a chronic migraine pattern, consisting of 15, or more headache days per month.[2,3] Normally harmless stimuli, such as ambient light, or sounds, can be unpleasant during migraine headaches. Most patients with migraines experience increased cutaneous sensitivity to innocuous mechanical, cold, and thermal stimuli of the skin in the cephalic or non-cephalic regions of the body.[4]

We recently developed a mouse model of migraine associated with the development of peripheral hypersensitivities, facial cold allodynia, and mechanical allodynia in the hindpaw, using repetitive injections of nitroglycerin (NTG).[5,6] NTG, a nitric oxide donor, and vasodilator, is commonly used to treat people with angina pectoris. A major side effect of NTG is migraine-like headaches, which occur minutes to hours after administration.[7] A single injection of NTG in mice induces acute mechanical, cold, and thermal hypersensitivity lasting up to 4 h.[8,9] Several recent papers, including our studies, demonstrated chronic hypersensitivity to mechanical and cold stimuli in mice following repeated systemic administration of NTG, which mimics the chronic migraine headache pattern observed in patients.[5,6,10-12]

Chemical stimulation with diluted bee venom (DBV) at acupuncture points (acupoints), termed *apipuncture*, has been used in traditional oriental medicine. DBV has significant anti-inflammatory and anti-nociceptive effects in human patients with arthritis.[13] In experimental studies using mouse or rat models of acute and chronic pain, DBV acupoint injection elicits potent anti-nociceptive effects.[14-16] Our previous studies demonstrated that DBV injection at the ST36 (Zusanli)

* Corresponding authors at: Department of Oral Physiology, School of Dentistry, Kyung Hee University, 26 Kyungheedae-ro, Dongdaemun-gu, Seoul 02447, Republic of Korea (D.-H. Roh); Department of Pet Total Care, Division of Business and Fine Art, Daejeon Health Institute of Technology, 21 Chungjeong-ro, Dong-gu, Daejeon 34504, Republic of Korea (S.-Y. Yoon).

E-mail addresses: osoon@hanmail.net (S.-Y. Yoon), dhroh@khu.ac.kr (D.-H. Roh).

https://doi.org/10.1016/j.imr.2023.100999
Received 5 March 2023; Received in revised form 23 July 2023; Accepted 18 October 2023
Available online 20 October 2023

acupoint, but not non-acupoints, alleviates thermal hyperalgesia and/or mechanical allodynia in rat neuropathic pain models, including sciatic nerve injury[17,18] and chemotherapy-induced neuropathy.[19,20] Despite the anti-nociceptive effects of DBV injections, no studies have investigated the potential effects and underlying mechanisms of DBV stimulation of acupuncture points in migraine-associated peripheral hypersensitivity.

The objective of this study was to determine if repetitive DBV injections at the ST36 (Zusanli), LI4 (Hegu), or GV16 (Fengfu) acupoints suppress facial cold allodynia and hindpaw mechanical allodynia and reduce c-Fos expression in the trigeminal nucleus caudalis (TNC) in mice after repetitive injections of NTG. In addition, we investigated the effects of pretreatment with naloxone (5 mg/kg, i.p.), an opioid receptor antagonist, and yohimbine (5 mg/kg, i.p.), an alpha-2 adrenoceptor antagonist, on the anti-allodynic effects of acupoint DBV injections in a mouse migraine model.

2. Methods

2.1. Animals

Male C57BL/6 mice (25–30 g; DBL Animal Inc., Seoul, Korea) were housed in colony cages with free access to food and water, and maintained in temperature- and light-controlled rooms (24 ˚C ± 2 ˚C, 12/12-hour light/dark cycle with lights on at 07:00) for at least 1 week before the experiment. The experimental protocols for animal usage were reviewed and approved by the Kyung Hee University Institutional Animal Care and Use Committee (KHUASP[SE]−16–058) and conformed to the National Institutes of Health guidelines (NIH publication No. 86–23, revised 1985) and ARRIVE guidelines.

2.2. Repetitive nitroglycerin administration

Facial and peripheral hypersensitivity was induced by repetitive NTG injections in this mouse chronic migraine model. A stock solution of NTG (5.0 mg/mL NTG in 30 % alcohol, 30 % propylene glycol, and water) was diluted with 0.9 % saline to 10 mg/kg. NTG was intraperitoneally administrated every other day for 9 days. The pre-injection basal response was measured before every NTG injection (Pre-injection Basal Response), while post-injection responses were obtained 2 h after each NTG injection (Post-injection Response) .[5,6] All experiments were performed with researchers blinded to the pharmacological treatment of the mice.

2.3. DBV and pharmacological antagonist treatments

Bee venom from Apis mellifera (Sigma, St Louis, Mo) was dissolved in physiologic saline (20 μl) at a dose of 0.1 mg/kg (DBV).[19,21] DBV was subcutaneously injected at the ST36 (Zusanli), LI4 (Hegu), or GV16 (Fengfu) acupoints 75 min after each NTG injection. Animals in the control group received an injection of saline (20 μl) at each acupoint (VEH). The ST36 acupoint is a frequently used and extensively studied acupoint in pain medicine as a complementary and alternative approach to pain management.[22] The ST36 acupoint is located on the lateral side of the stifle joint adjacent to the anterior tubercle of the tibia, as previously described.[21] The LI4 acupoint is located on the dorsum of the hand, radial to the midpoint of the forepaw's second metacarpal bone.[23] This acupoint is also used for several pain conditions, including inflammatory pain, and lower back pain.[24,25] The GV16 acupoint is located at the posterior occipital ridge atlas joint. Recently, Cheng et al. reported that the GV23 (Shangxing) and GV16 acupoints are associated with a reduction in stress-induced depressive behavior.[26]

The roles of the opioid and alpha-2 adrenergic systems in the anti-allodynic effects of repeated DBV stimulation were examined by pretreating separate groups of mice with the non-selective opioid receptor antagonist, naloxone (NAL, Tocris; 5 mg/kg dissolved in saline), or the alpha2-adrenergic antagonist, yohimbine (YOH, Sigma; 5 mg/kg dissolved in distilled water, D.W.).[27-29] Naloxone and yohimbine were injected intraperitoneally 30 min before each DBV (NAL+DBV, YOH+DBV) or vehicle treatment (NAL+VEH, YOH-VEH).

2.4. Mechanical and cold allodynia measurements in the hindpaws and facial region

All behavioral experiments were carried out in a quiet room. Paw withdrawal and facial pain responses were used to quantify mechanical sensitivity. Mice were acclimated for 30 min to an acrylic cylinder (6.5 cm in diameter, 17 cm in height) on a metal mesh grid before testing for mechanical allodynia. An ascending series (0.008, 0.02, 0.04, 0.07, 0.16, 0.4, and 0.6 g) of von-Frey filaments (North Coast Medical, Morgan Hill, CA, USA) were applied to the mid-plantar region of the hindpaw or the whisker pad. Each monofilament was applied six times before the next higher force monofilament was tested. After consistent withdrawal was noted (i.e., the filament that evoked a response in three out of the six trials was defined as the 50 % mechanical withdrawal threshold), the test was complete (Hindpaw Withdrawal Threshold). The monofilament that caused face wiping or rubbing with the forelimb in three out of the six applications was also defined as the 50 % mechanical withdrawal threshold in the facial region (Facial Withdrawal Threshold) .[5,6]

To test cold allodynia, 0.02 mL of acetone solution was applied to the hindpaw or the whisker pad with a 1 cc syringe connected to PE-10 tubing.[5,6] The animals were returned to the acrylic cylinder immediately after the acetone application, and nociceptive behavior was recorded for one minute. The duration (*sec*) of withdrawal response (e.g., lifting, shaking, or licking the hindpaws) was measured (Hindpaw Response to Acetone). Facial nociceptive behavior was measured as asymmetric orofacial wiping or rubbing on the whisker pad using the forelimb (facial response to acetone) .[6] Scratching the face with the hindlimb was considered an itch response and was excluded from the data.[30]

2.5. Immunohistochemistry for c-Fos

To measure changes in c-Fos protein expression in the TNC region of NTG-treated animals, another set of mice was sacrificed 2 h after the last injection of NTG (9 days after the first NTG injection) .[6] Animals were deeply anesthetized with 5 % isoflurane and perfused transcardially with 0.1 mol/L phosphate buffer saline (PBS, pH 7.4) followed by a fixative containing 4 % paraformaldehyde in PBS (50 mL). The TNC was removed immediately after perfusion, postfixed in the fixative for 4 h, and cryoprotected in 30 % sucrose in PBS for 48 h (pH 7.4). A cryostat (Leica Microsystems, Wetzlar, Germany) was used to cut 30-uM-thick transverse frozen sections through the TNC. After elimination of endogenous peroxidase activity with 3 % hydrogen peroxide in PBS and blocking with 3 % normal goat serum and 0.3 % Triton X-100 in PBS for 1 h at room temperature, the sections were incubated in polyclonal rabbit anti-c-Fos antibody (1:1000, Santa Cruz Biotechnology Inc., Dallas, TX) overnight at 4 ˚C. After several PBS washes, the TNC tissue sections were incubated with a secondary biotinylated anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The sections were processed using the avidin-biotin method (Elite ABC; Vector Laboratories, USA). Fos-immunoreactive (ir) cells were visualized using a 3–3-diaminobenzidine reaction intensified with 0.2 % nickel chloride.

2.6. Image analysis

TNC sections were scanned using a brightfield and fluorescent microscope ECLIPSE 80i (Nikon Corp., Kanagawa, Japan) and digitized using a cooled CCD camera (Cool Snap ES model, Nihon Roper, Tokyo, Japan). Six nonadjacent tissue sections of TNC per mouse were randomly selected and quantitatively analyzed using a computer-assisted image analysis system (MetaMorph version 7.7.2.0, Westchester, PA,

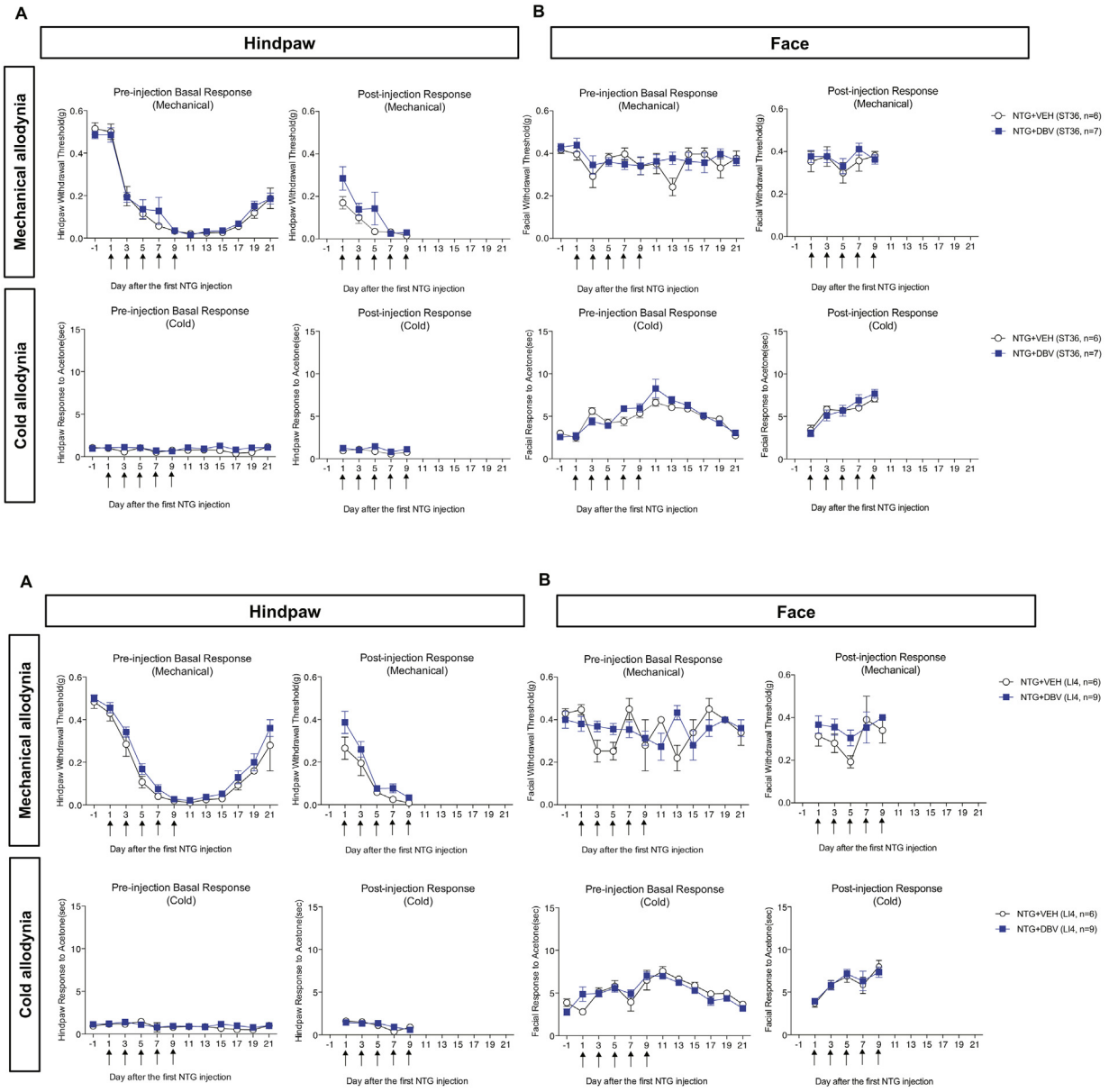

Fig. 1. The effects of diluted bee venom (DBV) stimulation at ST36 (A, B) or LI4 (C, D) on pain responses to mechanical and cold stimulation in the hindpaw (A, C) and facial region (B, D), respectively, after repetitive NTG injections. NTG injections decreased the mechanical withdrawal threshold in the hindpaw (cold allodynia not shown) (A, C). On the other hand, NTG injection did not affect the mechanical withdrawal threshold in the facial region, while cold allodynia gradually occurred in the basal and post-treatment responses (B, D). DBV injection at ST36 or LI4 did not produce an anti-allodynic effect against hindpaw mechanical allodynia or facial cold allodynia (A-D).

USA). The shape factor was set to a range of 0.5 to 1.0, and c-Fos-ir cells were counted only if they were at least 30 % darker than the average gray level of each image.[6,31] The average number of c-Fos-ir cells per section was determined for each animal. These values were averaged across each group. All analytical procedures described above were performed without advanced knowledge of the experimental conditions.

2.7. Statistical analysis

All values are expressed as means ± standard errors of the mean. Mechanical and cold allodynia measurements were compared using a two-way repeated-measures analysis of variance followed by posthoc Bonferroni tests. Unpaired *t*-tests were used to compare the number of c-Fos-ir cells in the TNC. All statistical analyses were performed using GraphPad Prism (Version 6.0, GraphPad Software, San Diego, CA, USA), and p values <0.05 were considered statistically significant.

3. Results

3.1. Acupoint-dependent effects of DBV on NTG-induced acute and chronic hypersensitivity

Mechanical threshold and cold allodynic responses to acetone were evaluated in the hindpaw (Fig. 1A) and whisker pad (Fig. 1B) of NTG-injected mice, respectively. Repeated NTG injections decreased the hindpaw mechanical threshold and increased the facial response to acetone (Fig. 1A, B). These results were similar to the results described in our previous studies. DBV injections at the ST36 and LI4 acupoints did not attenuate mechanical allodynia in the hindpaw (Figs. 1A, C)

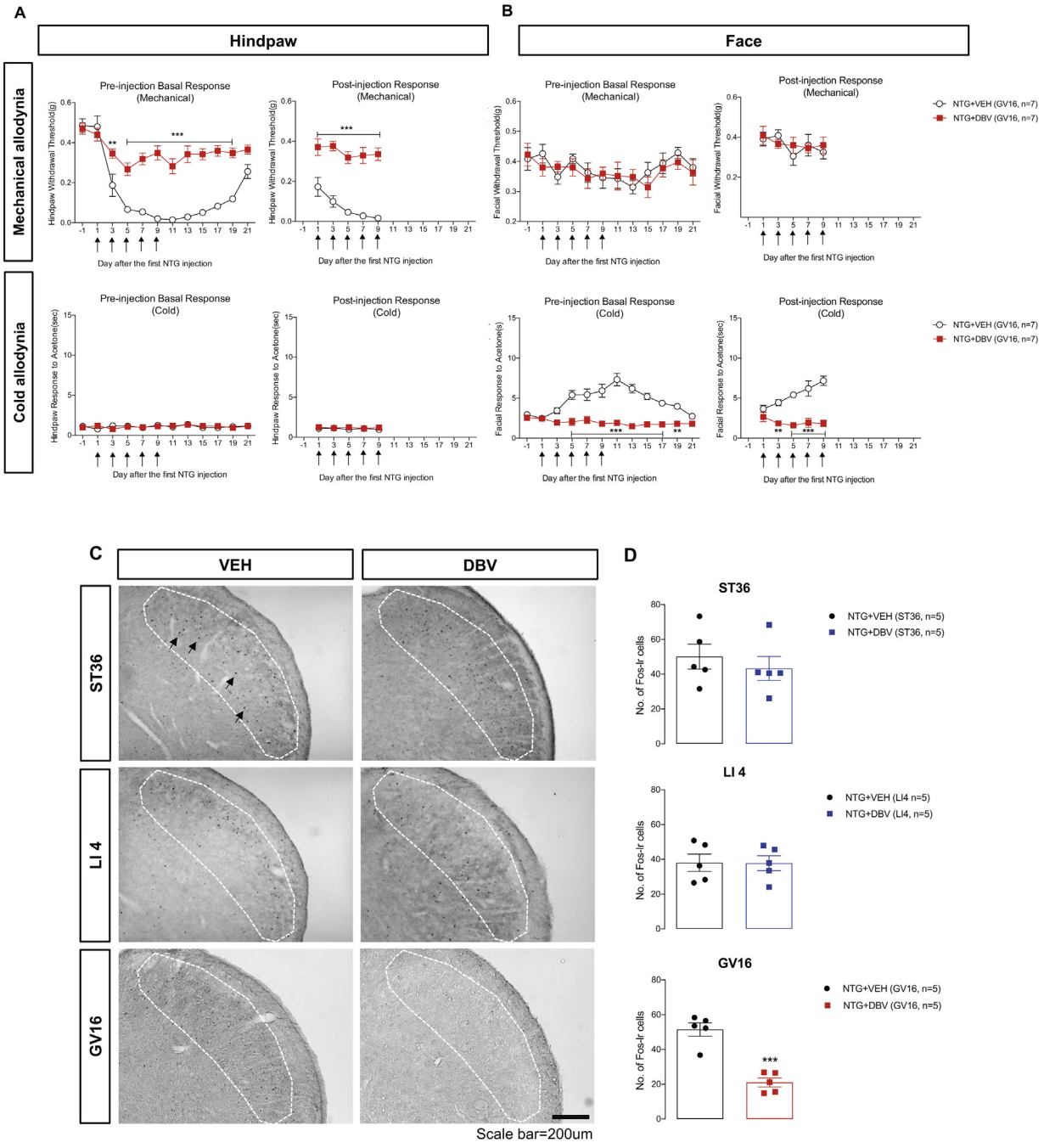

Fig. 2. The effects of diluted bee venom (DBV) stimulation at GV16 on pain responses to mechanical and cold stimulation in hindpaw (A) and facial region (B), respectively, after repetitive NTG injections. NTG injections decreased the mechanical withdrawal threshold in the hindpaw (A) and induced facial cold allodynia (B). DBV injection at GV16 significantly increased the mechanical threshold for both the basal and post-treatment response of hindpaw (A, ** $p < 0.01$, *** $p < 0.001$ compared with the NTG+VEH group). Moreover, DBV injection at GV16 reduced the response time to acetone in the facial region (B, ** $p < 0.01$, *** $p < 0.001$ compared with the NTG+VEH group). All values are expressed as means ± standard errors of the mean (SEM). A two-way repeated-measures analysis of variance followed by posthoc Bonferroni tests were used. The effects of DBV stimulation at ST36, LI4, and GV16 on NTG-induced increases in c-Fos expression in the trigeminal nucleus caudalis (TNC). Repetitive DBV treatment at ST36 or LI4 did not reduce the number of c-Fos-immunoreactive (ir) cells in the TNC (C and D). In contrast, the number of c-Fos-ir cells significantly decreased in mice treated with DBV at the GV16 acupoint (C and D, *** $p < 0.001$ compared with the NTG+VEH group). All values are expressed as means ± SEM and unpaired *t*-tests were used to compare the number of c-Fos-ir cells. The arrows indicate representative c-Fos-ir cells. Scale bar = 200 μm.

or cold allodynia in the facial region (Figs. 1B, D) induced by repeated NTG injections. In contrast, repeated DBV injections at the GV16 acupoint significantly reduced acute and chronic mechanical allodynia (pre-injection basal response and Post-injection Response, respectively) in the hindpaw induced by repeated NTG injections (Fig. 2A, ** $p < 0.01$, *** $p < 0.001$ compared to vehicle treatment at the GV16 acupoint). Furthermore, NTG-induced acute and chronic cold allodynia responses were completely blocked in mice treated with DBV at the GV16 acupoint (Fig. 2B, ** $p < 0.01$, *** $p < 0.001$ compared to the vehicle-treated group).

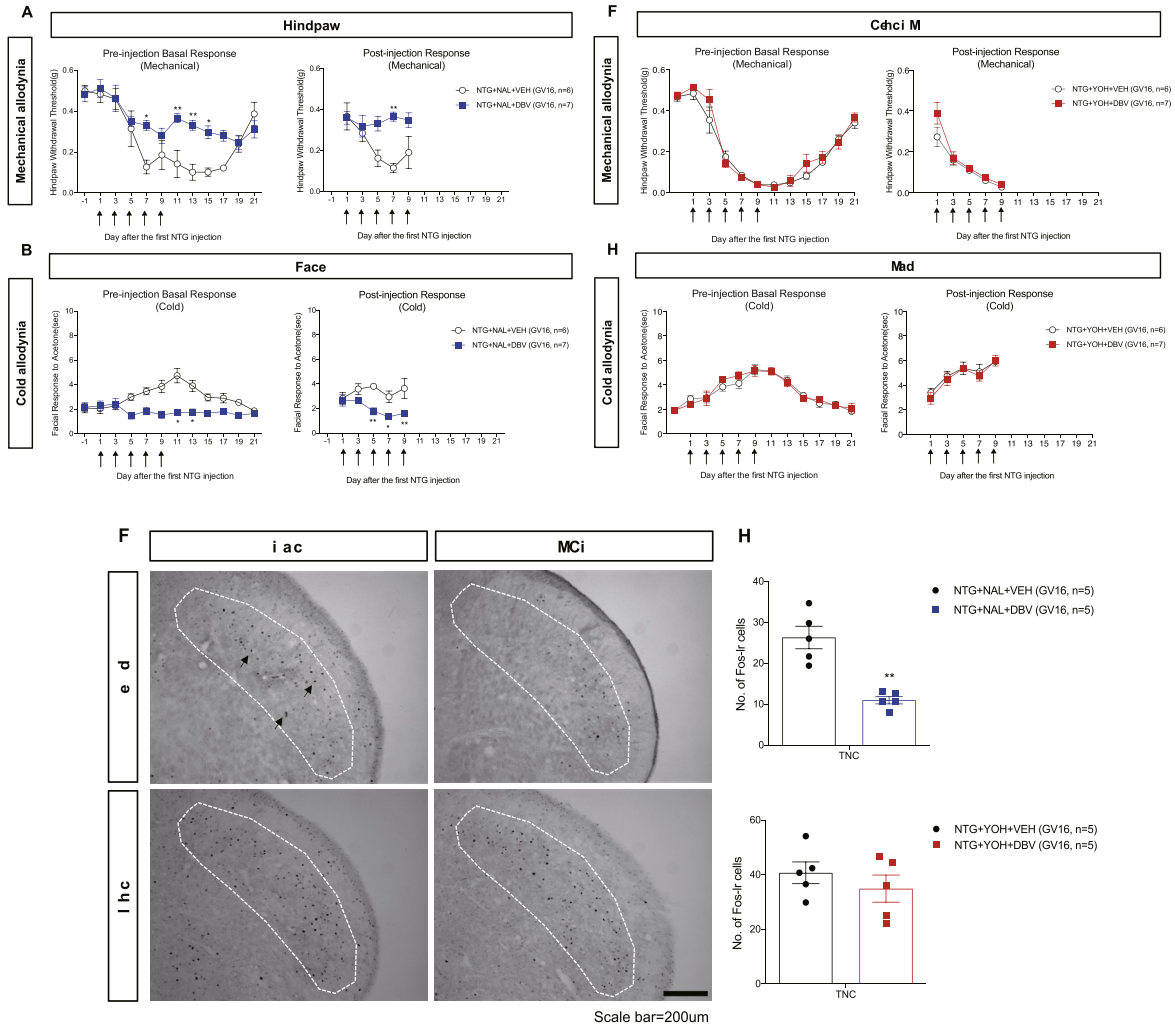

Figure 3. Kim et al.

Fig. 3. The effects of naloxone (NAL, A and B) or yohimbine (YOH, C and D) pretreatment on the anti-allodynic effect of diluted bee venom (DBV) stimulation at GV16 in NTG-treated mice. NAL pretreatment (5 mg/kg, i.p.) did not affect the development of hindpaw mechanical allodynia (A) or facial cold allodynia (B) induced by repeated NTG injections (NTG-NAL-VEH), and anti-allodynic effects induced by DBV stimulation into GV16 were also observed in both the hindpaw and facial region of NAL-pretreated DBV mice (NTG-NAL-DBV) (* $p < 0.05$, ** $p < 0.01$ compared to NTG-NAL-VEH). YOH pretreatment (5 mg/kg, i.p.) did not affect the development of hindpaw mechanical allodynia (C) or facial cold allodynia (D) induced by repeated NTG injections (NTG-YOH-VEH), whereas YOH pretreatment blocked the anti-allodynic effects induced by DBV stimulation at GV16 in both the hindpaw (C) and facial region (D) (NTG-YOH-DBV). All values are expressed as means ± standard errors of the mean (SEM). A two-way repeated-measures analysis of variance followed by posthoc Bonferroni tests were used. The effects of NAL and YOH pretreatments on the reduction in c-Fos expression by DBV at GV16 was shown in E and F. The inhibitory effects of DBV stimulation at GV16 on NTG-induced increases in c-Fos-ir cells were observed in NAL-pretreated (5 mg/kg, i.p.) DBV mice (NTG-NAL-DBV) (E and F, ** $p < 0.01$ compared to NTG-NAL-VEH). In contrast, YOH pretreatment (5 mg/kg, i.p.) attenuated the DBV (GV16)-induced suppressive effects on NTG-induced c-Fos expression (E and F). All values are expressed as means ± SEM and unpaired *t*-tests were used to compare the number of c-Fos-ir cells. The arrows indicate representative c-Fos-ir cells. Scale bar = 200 μm.

3.2. Acupoint-dependent effects of DBV on NTG-induced increases in c-Fos expression in the TNC

Repetitive DBV injections at ST36 or LI4 did not reduce NTG-induced c-Fos expression in the TNC (Fig. 2C, D). However, the number of c-Fos-ir cells significantly decreased in mice treated with DBV at the GV16 acupoint (Fig. 2C and D, *** $p < 0.001$ compared to the NTG+VEH group).

3.3. Involvement of alpha-2 adrenoceptors, but not opioid receptors, in DBV-induced anti-allodynic effects

NAL pretreatment (5 mg/kg, i.p.) did not affect the development of acute and chronic mechanical allodynia in hindpaws induced by repeated NTG injections or the DBV-induced anti-allodynic effects (at GV16) (Fig. 3A, * $p < 0.05$, **$p < 0.01$, NTG-NAL-DBV vs. NTG-NAL-VEH). In addition, the anti-allodynic effects of DBV (GV16 acupoint) on acute and chronic cold allodynia in the facial region were not attenuated in NAL-pretreated DBV mice (Fig. 3B, * $p < 0.05$, **$p < 0.01$, NTG-NAL-DBV vs. NTG-NAL-VEH). In contrast, YOH pretreatment (5 mg/kg, i.p.) completely blocked the DBV-induced anti-allodynic effects (GV16) in both the hindpaw (Fig. 3C) and facial region (Fig. 3D). Moreover, YOH did not affect the development of hindpaw mechanical allodynia or facial cold allodynia induced by repeated NTG injections.

3.4. Involvement of alpha-2 adrenoceptors in DBV (GV16) inhibition of c-Fos expression

The DBV-induced (GV16) attenuation of the NTG-induced increases in c-Fos-ir cells was not restored in NAL-pretreated DBV mice (Fig. 3E and F, ** $p < 0.01$ NTG-NAL-DBV vs. NTG-NAL-VEH). In contrast, YOH

pretreatment (5 mg/kg, i.p.) completely blocked the DBV (GV16) attenuation of NTG-induced c-Fos expression (Fig. 3E, F). NAL or YOH alone did not alter NTG-induced c-Fos expression.

4. Discussion

Our results demonstrate that repetitive DBV treatment at GV16, but not ST36, or LI4 acupoints, suppress NTG-induced hindpaw mechanical allodynia and facial cold allodynia. DBV treatment at GV16, but not ST36, or LI4, also suppressed the increased c-Fos protein expression induced by NTG injections.

Migraines are the most common neurological disorder and one of the most common chronic pain conditions. Despite the prevalence of migraines, the pathophysiology leading to migraines is poorly understood and the identification of new therapeutic targets has been slow.[2,32] Although several articles reported that alternative therapeutic approaches, including manual acupuncture and electroacupuncture, produce significant analgesic effects in headache and migraine animal models and human studies,[33-36] a systematic review and meta-analysis also reported that the effectiveness of acupuncture is still uncertain and further investigation is needed.[37] In this regard, the current study demonstrated for the first time that chemical stimulation with DBV at a specific acupoint (GV16) relieved migraine symptom-like peripheral hypersensitivity in an NTG-injected mouse model.

Of note, DBV stimulation at GV16, but not at ST36, and LI6, reduced both facial cold allodynia and hindpaw mechanical allodynia in the NTG-induced migraine mouse model (Figs. 1 and 2). Our previous studies demonstrated that DBV injection at ST36 elicited significant anti-nociceptive and anti-allodynic effects in the hindpaws in acute and chronic inflammatory pain rat and mouse models.[14,21] In addition, we reported that single or repetitive stimulation with DBV at the ST36 acupoint elicits potent anti-allodynic effects against neuropathic pain induced by chronic constriction injury of the sciatic nerve in rats[17,38] or oxaliplatin-induced neuropathy in mice.[19,20] Several other research groups also reported that acupuncture at ST36 induced analgesic effects in animal and human studies.[39,40] Thus, we speculated that DBV stimulation at the ST36 acupoint may produce anti-allodynic effects against NTG-induced pain in the hindpaw and facial regions. In addition, electroacupuncture stimulation at LI4 reduced complete Freund's adjuvant-induced inflammatory pain in mice,[23] and low-level laser stimulation at the LI4 acupoint controlled pain during local anesthesia in children.[41] We tried to verify the anti-allodynic effects of DBV at ST36 or LI4, but neither acupoint showed analgesic effects against NTG-induced peripheral hypersensitivity. On the other hand, the GV16 acupoint is located at the occipital posterior occipital ridge atlas joint and is most frequently used for the treatment of disorders caused by exogenous factors, such as the common cold, neck pain, headache, epilepsy, mania, dizziness, and sore throat.[42] The GV16 acupoint is also associated with reduced stress-induced depressive behavior.[26] These studies combined with our results demonstrate that different acupuncture points may induce different effects depending on the type or classification of pain (inflammatory and neuropathic pain vs. migraine-related pain). In addition, DBV acts as a stimulant rather than a drug and can cause injection site-specific analgesic effects in several animal models of pain.

Notably, it would be useful to determine whether the injection of other chemicals into the GV16 point could produce an anti-allodynic effect in an NTG-injected mouse model with migraine symptoms. However, we consider these experiments to be separate studies because their results may differ depending on which type of chemical stimulant was chosen or which dose of chemical are selected. For example, we recently reported that pharmaco-puncture using Scolopendra subspinipes on the ST36 acupoint produced anti-nociceptive effects similar with those of DBV stimulation on the same acupoint in a chemotherapy-induced neuropathic pain model.[19,20,43] In this regard, we believe that DBV is effective not as a drug but as an acupoint stimulant.

Moreover, the present study did not show the effect of non-acupoint stimulation with DBV on the same segment of the GV16 acupoint. Since DBV is one of the pharmaco-punctures and is a stimulation in a liquid state, the range of stimulation sites may be somewhat wider than that of manual acupuncture or electroacupuncture stimulation. Thus, it was considered difficult to validate and interpret the effect of DBV stimulation in the same segment on the GV16 acupoint. Although there were other acupuncture points (ST36 or LI4) where the effect of DBV was not observed, the lack of a control group for non-acupuncture point in the same segment of GV16 acupoint in verifying the efficacy of acupuncture points stimulation with DBV is a limitation of this study.

In a previous study, we demonstrated that systemic injection of NTG increased c-Fos protein expression in the TNC, but not the spinal dorsal horn.[6] NTG injections also caused mechanical allodynia in the hindpaw and cold allodynia in the facial region. These findings suggest that peripheral hyperalgesia in non-cephalic regions might be linked to neuronal activity in the TNC region, and NTG-induced hindpaw mechanical allodynia may not be related to neuronal modulation within the spinal cord. In the present study, Fig. 2C and D also shows that DBV stimulation at the GV16, but not ST36, or LI4 acupoints, significantly reduced c-Fos-ir cells in the TNC. These results imply that DBV stimulation at GV16 elicits anti-allodynic effects against both hindlimb mechanical allodynia and facial cold allodynia via modulation of central sensitization of second-order neurons in the TNC or upper brain center, such as the thalamus.[44]

It is important to note that the development of mechanical allodynia in the hindpaws in several studies are closely related to the activation of TNC pathway after single or repeated injections of NTG.[10-12] Thus, NTG-treated mouse has been used as one of the animal models for migraine or headache. Our previous studies also examined that hindpaw mechanical allodynia induced by NTG injection was associated with the increase of activated neurons in TNC, but not in lumbar spinal cord.[6] In that study, we verified that repetitive NTG injection did not increase the number of c-Fos-immunoreactive cells in the spinal cord, whereas c-Fos-positive cells in TNC were increased by NTG injections. In this regard, we believe that mechanical allodynia in the hindpaw is caused by activated TNC pathways rather than secondary hyperalgesia mediated in the spinal cord. c-Fos is a nuclear phosphoprotein product of the c-fos proto-oncogene and its expression in neuronal cells can be induced by various factors, including nerve growth factor, several second messengers and cell membrane events such as NMDA receptor activation. In this regard, their increases at the level of the spinal cord have been used as important markers of neuronal activation induced by noxious stimuli.[45] Therefore, we considered that the increase of c-Fos expression in the TNC was associated with the activated neurons ascending in the TNC, where they receive information about deep/crude touch, pain, and temperature from the face. In addition, they can receive information of meningeal blood vessels from the trigeminal nerve, which was considered one of the mechanisms of migraine.[46] Based on these literature, increased c-Fos expression in TNC may be a consequence of activation of TNC neurons synapsing with the meningeal trigeminal nerves that detect vasodilation in the dura meter after repeated injections of NTG.

The increase in c-Fos expression in TNC has been also used as an indicator of headache or migraine itself 2 h after injection of NTG. Thus, in this study, the increase in TNC nerve activity was not thought to be caused by cold stimulation to the face, whereas the development of cold allodynia was believed to be a central sensitization phenomenon caused by repetitive trigeminal nerve stimulation in a migraine condition. In this regard, mechanical allodynia in the hind paws as well as cold allodynia in the face can be seen as symptoms of peripheral hypersensitivity occurring during headache. However, the mechanism of how peripheral pain can occur differently depending on stimuli of different modalities (mechanical and cold stimuli) on the face and hind limbs needs to be elucidated in detail through further studies in the future.

Subsequently, we examined whether the anti-allodynic effects of DBV stimulation at GV16 on NTG-induced hindpaw mechanical allo-

dynia and facial cold allodynia were mediated by components of the endogenous analgesia system involving activation of opioid receptors and/or alpha-2 adrenoceptors. Our previous studies showed that the prominent anti-nociceptive and anti-allodynic effects of DBV injection at ST36 were mainly mediated by the activation of alpha-2 adrenoceptor signaling,[17,38,47] which is a well-known descending pain modulatory system.[48] However, injection of DBV at the ST36 acupoint showed no effects on migraine-related peripheral hypersensitivity. Thus, the involvement of opioid receptors and/or alpha-2 adrenoceptors in migraine-related anti-allodynic effects of DBV injections at GV16 was investigated. Interestingly, pretreatment with yohimbine not only reversed the anti-allodynic effects but also restored the reduced c-Fos expression in mice treated with DBV at GV16, while naloxone pretreatment did not affect the anti-allodynic effects or the decreases in c-Fos-ir cells in mice with DBV stimulation at the GV16 acupoint. These findings demonstrate that the effects of DBV stimulation at the GV16 acupoint are related to the systemic activation of alpha-2 adrenoceptors, but not opioid receptors.

The previous studies have shown that DBV stimulation at ST36 acupoint potently activates catecholaminergic neurons in locus coeruleus,[38] ultimately leading to the activation of descending noradrenergic neuron.[17,47] One of possible mechanisms underlying anti-migraine effect of DBV stimulation into GV16 is that it may induce the increased activity of noradrenergic neurons, which activate trigeminospinal alpha-2 adrenoceptors. The activation of alpha-2 adrenoceptors may ultimately lead to suppression of meningeal vascular signaling from the trigeminal nerve and reduction of TNC central sensitization, resulting in anti-allodynic effects in the face (cephalic) and hind paws (non-cephalic).[44] However, the detailed mechanisms need to be investigated by further experiments. Taken together with data from previous studies, our results suggest that although acupoints responding to several different types of pain models are different, the underlying mechanism of the anti-allodynic effects of acupoint stimulation with DBV on chronic inflammatory or neuropathic pain and migraine-associated hypersensitivity may be similar. Of note, the present study is the first report showing the modification of alpha-2 adrenoceptors using DBV acupuncture as a possible therapeutic target for migraine-associated peripheral hypersensitivity.

However, it is important to note that bee venom can cause severe allergic reactions in some individuals and can be life threatening in extreme cases. Bee stings and other insect stings can cause an anaphylactic reaction in people with allergies. Therefore, the use of bee venom for medical purposes should be approached cautiously and in a controlled medical environment where emergency measures can be taken if necessary. Moreover, although our study suggests that DBV therapy might be beneficial for certain headache conditions, it is important to understand that the safety and effectiveness of DBV stimulation at GV16 for migraine patients have not been fully demonstrated in large-scale clinical trials.

In the previous studies, we also found that systemic NTG injection produced both mechanical allodynia in hind paw and cold allodynia in facial region, whereas facial mechanical allodynia and hind paw cold allodynia were not shown in the same mouse.[5,6] As there was no experimental evidence that the peripheral receptors or nociceptors that supply the skin (face and hind limbs) respond differently to quantitative sensory stimuli during migraines, we recently investigated which type of primary afferents or receptors are involved in the distinct development of hind paw mechanical and facial cold hypersensitivity, and finally found that facial cold allodynia and hindpaw mechanical allodynia are differentially mediated by activation of TRPA1 and ASIC, respectively, in mice with repetitive NTG-induced hypersensitivity.[6] However, it is still unclear how NTG injection causes the difference in the activity of TRPA1 and ASIC in the head and hind limb regions, and it would be clarified through further studies. In addition, how bee venom therapy on the GV16 acupoint may affect these mechanisms will be further investigated.

In conclusion, the present study demonstrates that repetitive DBV stimulation at the GV16 acupoint alleviates both hindlimb mechanical allodynia and facial cold allodynia in mice with NTG-induced peripheral hypersensitivity and the increased c-Fos expression in the TNC. Furthermore, the effects of DBV injections at GV16 are mediated by the activation of alpha-2 adrenoceptors, but not endogenous opioid receptors. Taken together, the current study indicates that chemo-acupuncture with DBV at GV16 is a potential strategy for the management of migraine-related peripheral pain.

CRediT authorship contribution statement

Sol-Ji Kim: Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing. **Ji-Hee Yeo:** Methodology, Investigation. **Seo-Yeon Yoon:** Formal analysis, Writing – original draft, Writing – review & editing. **Dae-Hyun Roh:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing.

Conflict of interests

The authors declare that there are no conflicts of interest.

Funding

This research was supported by The National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (No. NRF-2022R1F1A1073652 and 2021R1F1A1055082).

Ethical statement

The study was approved by the Kyung Hee University Institutional Animal Care and Use Committee (KHUASP[SE]-16-058) and conformed to the National Institutes of Health guidelines (NIH publication No. 86-23, revised 1985).

Data availability statement

The original data presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

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