Differential Development of Facial and Hind Paw Allodynia in a Nitroglycerin-Induced Mouse Model of Chronic Migraine: Role of Capsaicin Sensitive Primary Afferents

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Despite the relatively high prevalence of migraine or headache, the pathophysiological mechanisms triggering headache-associated peripheral hypersensitivities, are unknown. Since nitric oxide (NO) is well known as a causative factor in the pathogenesis of migraine or migraine-associated hypersensitivities, a mouse model has been established using systemic administration of the NO donor, nitroglycerin (NTG). Here we tried to investigate the time course development of facial or hindpaw hypersensitivity after repetitive NTG injection. NTG (10 mg/kg) was administrated to mice every other day for nine days. Two hours post-injection, NTG produced acute mechanical and heat hypersensitivity in the hind paws. By contrast, cold allodynia, but not mechanical hypersensitivity, occurred in the facial region. Moreover, this hindpaws mechanical hypersensitivity and the facial cold allodynia was progressive and long-lasting. We subsequently examined whether the depletion of capsaicin-sensitive primary afferents (CSPAs) with resiniferatoxin (RTX, 0.02 mg/kg) altered these peripheral hypersensitivities in NTG-treated mice. RTX pretreatment did not affect the NTG-induced mechanical allodynia in the hind paws nor the cold allodynia in the facial region, but it did inhibit the development of hindpaw heat hyperalgesia. Similarly, NTG injection produced significant hindpaw mechanical allodynia or facial cold allodynia, but not heat hyperalgesia in transient receptor potential type V1 (TRPV1) knockout mice. These findings demonstrate that different peripheral hypersensitivities develop in the face versus hindpaw regions in a mouse model of repetitive NTG-induced migraine, and that these hindpaw mechanical hypersensitivity and facial cold allodynia are not mediated by the activation of CSPAs.

Key words: allodynia; nitroglycerin; migraine; resiniferatoxin; transient receptor potential type V1 (TRPV1)

Migraine, a multifactorial primary headache disorder, is characterized by one-sided, severe, and pulsatile headaches. 1) During the headache phase, stimuli that are generally innocuous, such as ambient light or sound, can be unpleasant during headache. Most patients with migraines experience increased cutaneous sensitivity to non-noxious mechanical, cold, and thermal stimulation of the skin of the cephalic or non-cephalic regions of the body. 2) While central sensitization could contribute to the mechanisms underlying this cutaneous hypersensitivity, 2,3) the exact mechanisms are poorly understood.

Nitroglycerin (NTG), a nitric oxide (NO) donor and effective vasodilator, is generally used to treat patients with angina. One of the main side effects of NTG is migraine, which may develop within minutes, or up to four hours post-administration. 4) Systemic NTG activates nociceptive neurons in areas of the mouse brain. 5) In mice, a single injection of NTG induces acute mechanical, cold, and thermal hypersensitivity, which persists up to four hours post-injection. 6,7) Recently Pradhan et al. reported that repetitive injections of NTG induced chronic mechanical hypersensitivity, mimicking the chronic migraine observed in human patients. 7) Since mechanical and thermal hyperalgesia are indicators of migraine-related sensory abnormalities, 8,9) a rodent model of NTG-evoked hypersensitivity is useful for investigating the mechanisms involved in the development of these abnormalities.

Recently, transient receptor potential type V1 (TRPV1) receptor antagonists have been identified as potential therapeutic agents for the management of migraines. 9,10) TRPV1 antagonists have been shown to significantly prevent the upregulation of c-fos in the trigeminal nucleus caudalis in a migraine model. 11) Capsaicin injection has also been used to stimulate the trigeminovascular system, and significantly increases c-fos expression in the trigeminal ganglion of a rodent migraine model. 12) Conversely one study reported that there is no significant effect on either alpha- or C-fiber firing in the trigeminal nerve elicited by electrical stimulation of the middle meningeal artery, following systemic injection of a TRPV1 antagonist. 13) Accordingly, the involvement of the TRPV1 receptor, or capsaicin-sensitive primary afferents (CSPAs), in the development of migraine or migraine related hypersensitivities remains controversial.

In the present study, we utilized a mouse model of chronic migraine-associated peripheral hypersensitivities induced by repetitive injections of NTG. We tried to investigate whether the types of NTG-induced peripheral hypersensitivities present in the hind paw differ from those present in the face. We further investigated the potential involvement of CSPAs in the development of these hypersensitivities. In this regard, the ad-
ministration of capsaicin depletes most unmyelinated afferent fibers in neonatal rats.\textsuperscript{14,15} Similarly, resiniferatoxin (RTX), a potent capsaicin analog that binds to TRPV1,\textsuperscript{16–18} can deplete CSPAs in adult mice, when administered systemically. Taking advantage of this, we evaluated the role of CSPAs and TRPV1 receptors in the development of these facial and hind paw peripheral hypersensitivities, using both RTX-treated mice and TRPV1 knockout (KO) mice.

**MATERIALS AND METHODS**

**Animals** Male C57BL/6 mice (25–30 g) were purchased from DBL Animal Inc. (Seoul, Korea), and TRPV1 KO and wild-type (WT) mice were obtained from Seoul National University. Mice were habituated to colony cages with free access to water and pelleted feed. They were housed in a standard animal facility maintained on a 12-h light/dark cycle (lights on at 07:00 a.m.) with a constant room temperature (23±2°C). Mice were acclimated for at least one week prior to the experiment. The protocols for this study were approved by the Kyung Hee University Institutional Animal Care and Use Committee (KHUASP[SE]-16-058). All experimental procedures using animals were performed in accordance with National Institutes of Health guidelines (NIH publication No. 86-23, revised 1985).

**Drug Administration** Repetitive injection of NTG was used to produce peripheral hypersensitivity associated with chronic headache or migraine. Before intraperitoneal injection, the stock solution of NTG (5.0 mg/mL NTG in 30% alcohol, 30% propylene glycol, and water) was dissolved in 0.9% saline to a concentration of 10 mg/kg. For chronic experiments, NTG was repetitively administered every other day for nine days. Pre-injection basal pain responses were measured prior to injection of NTG or vehicle on days 1–9, and then the responses were also observed from days 11 to 21 following the 9-d NTG injection period (Pre-injection Basal Response). On the other hand, the measurements made two hours after NTG injection were considered the post treatment response (Post-injection Response). All experiments were performed blinded to the pharmacological treatment of the mice.\textsuperscript{19}

In order to examine whether TRPV1 expressing sensory nerve fibers are involved in the development of NTG-induced hypersensitivity, resiniferatoxin (RTX, LC Laboratory, Woodland Hills, CA, U.S.A.) was applied on hind paws, or the whisker pad, with a 1 cc syringe connected to PE-10 tubing. The duration (s) of withdrawal response (e.g., lifting, shaking, or licking the hind paws, and facial grooming behavior) was counted.

To test heat hyperalgesia, mice were placed into a plastic chamber with a glass floor and radiant heat was applied to the plantar surface of the hind paw (HITC Life Science Inc., Woodland Hills, CA, U.S.A.). Paw withdrawal latency was measured, with a cut off time of 20 s.\textsuperscript{24}

**Immunohistochemistry** Immunohistochemistry was performed to determine whether TRPV1-immunoreactive neurons are present in the trigeminal ganglia in RTX-treated mice. Under general anesthesia (5% isoflurane, in a mixture of nitrous oxide/oxygen gas), mice were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, 50 mL, pH 7.4). The trigeminal ganglion was removed and submerged in cryoprotectant (30% sucrose in PBS, pH 7.4) overnight at 4°C. Frozen serial frontal sections (16 µm) were cut through the trigeminal ganglion. After pre-blocking with 5% normal donkey serum and 0.3% Triton X-100 in PBS for one hour at room temperature, the sections were incubated in 1:250 goat polyclonal TRPV1 antibody (Santa Cruz Biotechnology Inc., Dallas, TX, U.S.A.) for 24 h at 4°C, followed by incubation in 1:500 Cy3-conjugated anti-goat secondary antibody (Invitrogen, Carlsbad, CA, U.S.A.) for two hours at room temperature. Positive TRPV1 neurons were visualized using a fluorescent microscope (ECLIPSE 80i, Nikon Corp., Kanagawa, Japan) at X200 and digitized using a cooled CCD camera (CoolSnap ES model, Nikon Roper, Tokyo, Japan).

**Statistical Analysis** All values are expressed as the mean± standard error of the mean (S.E.M.) Prism 5.01 (Graph Pad Software, San Diego, CA, U.S.A.) was used for data analysis and statistical comparisons. All pain behavioral tests were analyzed by repeated measured two-way ANOVA, followed by post-hoc Bonferroni’s test. In this study, p<0.05 was considered statistically significant.

**RESULTS**

**Induction of NTG-Induced Hypersensitivity in the Hind Paw** Pain behaviors were measured before (pre-injection basal responses, Figs. 1A, C, E) and two hours after (post-injection responses, Figs. 1B, D, F) NTG administration, on each NTG injection day. To determine the time course for possible recovery, mechanical and thermal responses were assessed every other day for 12 d, after the final treatment on day nine. Mechanical threshold, cold allodynia, and heat hypersensitivity were measured in the hind paw. When compared to control mice, repetitive intermittent NTG administration evoked significant mechanical allodynia on each test day.
Mechanical withdrawal threshold was measured prior to NTG injection on days 1–9 or measured from days 11 to 21 after termination of NTG treatment (A), and measured two hours after nitroglycerin (NTG) treatment (B). **p<0.01, ***p<0.001 vs. vehicle. Hind paw cold allodynia was not induced by repetitive NTG injection (D) or when measured over the 21d period (C). When compared to control, NTG induced significant thermal hyperalgesia both during the pre-injection basal response periods (E) and the post-injection period (F). *p<0.05, ***p<0.001 vs. vehicle.

(Fig. 1B, ***p<0.001 as compared to vehicle mice). In addition, mice that received NTG for nine days developed a basal mechanical allodynia that persisted over the entire 21-d testing period (Fig. 1A; **p<0.01 or ***p<0.001). Repetitive NTG administration did not increase either basal or nine day paw responses to the cold stimulus (Figs. 1C, D). Paw withdrawal latency to heat was significantly reduced after administration of NTG (Figs. 1E, F). When compared to control, NTG significantly reduced the withdrawal latency in both the initial nine-day treatment (Fig. 1F; ***p<0.001) and pre-injection basal response periods (Fig. 1E; *p<0.05 or ***p<0.001). Thermal hypersensitivity developed quickly and was sustained throughout the testing period.

**Induction of NTG-Induced Hypersensitivity in the Facial Region** Pain behaviors were measured before (pre-injection basal response, Figs. 2A, C) and two hours after (post-injection response, Figs. 2B, D) NTG administration, on each injection day. Following NTG injection, facial withdrawal threshold to mechanical stimulation was not significantly different, when compared to control (Figs. 2A, B). In contrast,
facial withdrawal responses to cold stimuli were significantly increased in mice treated with NTG, when compared to control (Figs. 2C, D; *p < 0.05, **p < 0.01 and ***p < 0.001 as compared to vehicle-treated mice). Cold allodynia peaked at day nine, gradually decreased, and returned to baseline levels by day 21.

**Depletion of CSPAs and Reduction in Eye-Wiping Following RTX Treatment** In order to verify the depletion of CSPA, we performed the eye-wiping test in RTX-treated mice. When compared to control, RTX treated mice showed no eye-wipe response to capsaicin (Fig. 3A). Histologically, treatment with RTX abolished CSPAs, including TRPV1 positive neuronal cell bodies, in the trigeminal ganglia (Fig. 3C). Conversely, TRPV1 immuno-positive cells were present in the trigeminal ganglia of control mice (Fig. 3B).

**Effect of RTX on NTG-Induced Hypersensitivity in the Hind Paw** We measured mechanical threshold, cold allodynia, and heat hyperalgesia in the hind paw. In both the RTX and control group, NTG injection significantly decreased the mechanical withdrawal threshold. This decrease was evident from day one, and was sustained for 19d after the first injection. Thus, RTX treatment did not modify the development of mechanical allodynia caused by NTG (Figs. 4A, B, ***p < 0.001 as compared to vehicle+vehicle treated mice). Pretreatment with RTX, or the vehicle control, did not produce cold allodynia in the hind paw of NTG-treated mice (Figs. 4C, D). Treatment with RTX significantly increased the baseline paw withdrawal latency to heat as well as the post-injection response in both vehicle- and NTG-treated mice (Figs. 4E, F; *p < 0.05, **p < 0.01, ***p < 0.001 as compared to vehicle+vehicle group, +++p < 0.001 compared to vehicle+NTG group).

**Effect of RTX on NTG-Induced Hypersensitivity in the Facial Region** We measured mechanical hypersensitivity and cold allodynia in the face region of NTG-treated mice. Facial mechanical hypersensitivity was not produced by NTG in naïve mice (Figs. 2A, B). However, mechanical hypersensitivity was evident by NTG in the RTX treated mice (Fig. 5A). Before NTG treatment, facial thresholds were similar in both RTX and vehicle control mice. However, one day after NTG treatment, the mechanical threshold decreased in the RTX treated NTG group (Figs. 5A, B; **p < 0.01, ***p < 0.001 when compared to RTX+vehicle, +p < 0.05, ++p < 0.01, +++p < 0.001 when compared to vehicle+NTG mice). There was also a prominent increase in cold hypersensitivity (allodynia) in both the RTX treated NTG group and the vehicle treated NTG group. (Figs. 5C, D; *p < 0.05, **p < 0.01 or ***p < 0.001 as compared to vehicle+vehicle group).

**Changes in NTG-Induced Hypersensitivity in the Hind Paw of TRPV1 KO Mice** Basal paw withdrawal threshold levels were not significantly different between TRPV1 KO and wild-type control mice. NTG injection significantly decreased the mechanical withdrawal threshold (Fig. 5A), and increased cold allodynia (Fig. 5B). Pretreatment with RTX significantly increased the baseline paw withdrawal latency to heat as well as the post-injection response in both vehicle- and NTG-treated mice (Figs. 5E, F; *p < 0.05, **p < 0.01, ***p < 0.001 as compared to vehicle+vehicle group, +++p < 0.001 compared to vehicle+NTG group).
WT and KO mice. One day after NTG treatment, the paw withdrawal threshold began to decrease in both TRPV1 WT and KO mice (Fig. 6B, ***p<0.001 as compared to TRPV1 WT+vehicle group). This mechanical allodynia was evident for 19 d following the first NTG injection (Fig. 6A; **p<0.01 or ***p<0.001). Mechanical thresholds returned to the basal level by day 21. Conversely, NTG did not produce cold allodynia in either TRPV1 WT or KO mice (Figs. 6C, D). In WT mice, but not KO mice, NTG injection produced significant heat hyperalgesia (Figs. 6E, F; *p<0.05, **p<0.01 or ***p<0.001 as compared to TRPV1 WT+vehicle group, +++p<0.001 compared to TRPV1 WT+NTG group).

**Changes in NTG-Induced Hypersensitivity in the Facial Region of TRPV1 KO Mice** Prior to NTG treatment, mechanical and cold stimulus-induced withdrawal responses were similar in all groups. Following NTG treatment, mechanical allodynia did not develop in either TRPV1 WT or KO mice (Figs. 7A, B). Following the initial injection of NTG, the response to a cold stimulus was significantly increased (Fig. 7D; *p<0.05 or ***p<0.001 as compared to TRPV1 WT+vehicle group), and maintained for 19 d, in both TRPV1 WT and KO mice (Fig. 7C; *p<0.05, **p<0.01 or ***p<0.001 compared to TRPV1 WT+vehicle group).

**DISCUSSION**

Migraine patients experience altered processing of sensory stimuli and display lower than normal discomfort thresholds in response to mechanical and thermal noxious stimuli.25) In clinical observations of migraine patients, skin hypersensitivity occurs in trigeminal, as well as extracephalic sites.26–28) We utilized a mouse migraine model to measure pain hypersensitivity in both the hind paw and facial region. Our results indicate that NTG induces hypersensitivities to different pain modalities, and that the hypersensitivities vary depending on the site of measurement (hind paw versus face). Mechanical allodynia developed in the hind paw, but not in the face, while cold allodynia developed in the face, but not in the hind paw (Figs. 1, 2). Bates et al. showed the mechanical allodynia and heat hyperalgesia developed in the hind paw after injection of NTG, which was similar to results in the present study.3)

On the other hand, Galeotti and Ghelardini reported that NO donors NTG and sodium nitroprusside induced cold allodynia and heat hyperalgesia in the hind paw.6) The induction of hind paw cold allodynia was different from our study, but it seems to be associated with the difference of mice strain or experimental protocol (C57BL/6 male mice vs. Swiss albino male mice, and acetone solution test vs. cold plate test).

In addition, our results are in agreement with clinical observations demonstrating that specific pain modalities in
migraine patients may differ, depending on the body site tested.28 In the clinical study, Guy et al. reported that among 67 episodic migraine patients, 73% patients cited one or more allodynic symptoms during or immediately after the migraine attack.26 These patients reported cephalic cutaneous allodynia, whereas 24 patients also reported extracephalic cutaneous allodynia. Modalities of cephalic and extracephalic cutaneous allodynia were also different, extracephalic cutaneous allodynia being mostly thermal whereas cephalic cutaneous allodynia was mostly mechanical.26 These findings including our current study suggest that peripheral hypersensitivities in patients with migraine may differ based on the physical location and the stimulus modality, and these cephalic and extracephalic cutaneous hypersensitivities involved different mechanisms.

It has been hypothesized that allodynia in the cephalic region is a type of referred pain induced by the activation of trigeminal neurons.27,28 On the other hand, activation of thalamic neurons might be related to the development of alldynia in extracephalic regions.27,28 Since the mechanisms of cephalic and extracephalic cutaneous allodynia appear to be different, the specific pain modalities reported by migraine patients could differ by location. In this regard, more investigation is necessary to draw conclusions regarding the neural

![Figure 4](image-url)

**Fig. 4.** Involvement of Capsaicin-Sensitive Primary Afferents (CSPAs) in Nitroglycerin (NTG) Induced Mechanical, Cold, and Heat Hypersensitivity in the Hind Paw

Resiniferatoxin (RTX) pre-treatment did not affect mechanical allodynia in NTG treated mice (A and B). *** p < 0.001 vs. vehicle + vehicle. Cold allodynia was not produced by NTG and was not affected by RTX (C and D). In contrast, RTX blocked the development of NTG-induced heat hyperalgesia in the hind paw (E and F). * p < 0.05, ** p < 0.01, *** p < 0.001 vs. vehicle + vehicle, +++ p < 0.001 vs. vehicle + NTG.
mechanisms responsible for region dependent mechanical and cold hypersensitivity. The NTG migraine model used here provides a convenient animal model that can be utilized to investigate the region specific mechanisms underlying NTG migraine-associated peripheral hypersensitivities.

As part of our effort to discern mechanisms that would contribute to regional differences in hypersensitivity, we examined the involvement of CSPAs and TRPV1 in NTG-induced migraine, using both RTX injected mice and TRPV1 KO mice. Depletion of CSPAs using RTX treatment blocked the development of thermal hyperalgesia, but did not affect the development of mechanical allodynia in the hind paw, or the development of cold allodynia in the face (Figs. 4, 5). Several papers have reported that a single injection of RTX could not only induce thermal hypoalgesia in sham control animals but also abolish the development of thermal hyperalgesia in rodent neuropathic pain models.29,30) In addition, our previous study also reported that RTX treatment could prevent lamina-dependent increases in spinal N-methyl-D-aspartate receptor subunit 1 expression and phosphorylation, which was closely associated with thermal hyperalgesia in neuropathic rats.20) Interestingly, mechanical hypersensitivity of the face did not develop in NTG-treated naive mice, while the facial mechanical hypersensitivity was significantly developed in RTX-treated NTG mice (Figs. 5A, B). Although these data suggest that the loss of CSPAs induces facial mechanical hypersensitivity in NTG-treated mice, the underlying mechanism is not clear. Pan et al. reported that an intraperitoneal injection of RTX rapidly produced an increase in the paw withdrawal latency to a heat stimulus, while profound tactile allodynia developed in all the RTX-treated rats in 3 weeks.31) In addition, they suggested that the delayed tactile allodynia induced by RTX is likely attributable to damage to myelinated afferent fibers and their abnormal sprouting in lamina II of the spinal dorsal horn. This damage of myelinated primary afferent fiber by systemic RTX injection may contribute to the increased sensitivity to mechanical stimulus, which ultimately leads to mechanical allodynia by repetitive nitroglycerine injection. Another possible hypothesis relates to local irritation due to the RTX treatment. For example, subcutaneous injection of capsaicin into newborn rat pups leads to long-lasting cutaneous lesions.32) The mechanical hypersensitivity we observed in the face (Figs. 5A, B) could be due, in part, to the pruritic action induced by the loss of TRPV1-expressing nociceptive afferents. To exclude the potential pruritic action of RTX, and to further evaluate the specific action of the TRPV1 receptor in NTG-induced cutaneous hypersensitivities, we performed additional experiments with TRPV1 KO mice. Our results show that mechanical allodynia did not develop in the face in either TRPV1 WT or KO mice (Figs. 7A, B). These results indicate that the mechanical allodynia observed in the face of RTX-treated mice was induced by a TRPV1-independent mechanism. In addition, knockout of TRPV1 did not affect mechanical allodynia in the hind paw (Figs. 6A, B), or the
development of cold allodynia in the face (Figs. 7C, D). Only heat hypersensitivity was significantly reduced in the TRPV1 KO mice (Figs. 6E, F).

The role of TRPV1 and capsaicin-sensitive fibers in migraine remains controversial. Summ et al. reported that there was no significant effect on either A- or C-fiber firing in the trigeminal nerve elicited by electrical stimulation of the middle meningeal artery, following systemic injection of the TRPV1 antagonist, A-993610.13) In clinical studies, administration of the TRPV1 receptor antagonist, SB-705498, or the TRPV1 receptor agonist, civamide, had minimal beneficial effect in the acute migraine phase.10,33) However, TRPV1 antagonists have been shown to significantly downregulate the increased expression of c-fos in the trigeminal nucleus caudalis in a migraine model.11) As a chemical stimulant, capsaicin has also been used to stimulate the trigeminovascular system, and significantly increases c-fos expression in the trigeminal ganglion of a rodent migraine model.12) Capsaicin-induced meningeal nerve activity and vasodilation are inhibited by the application of a TRPV1 antagonist.34,35) Our current study provides direct evidence suggesting that CSPAs and TRPV1 receptors play a role in migraine induced cutaneous thermal hypersensitivity. On the other hand, the development of hind paw mechanical allodynia and facial cold allodynia is not mediated by CSPAs in long-term NTG treated mice.

Importantly, there are no clinical or experimental evidence that peripheral receptors or nociceptors that supply the skin (face and hind limbs) respond differently to quantitative sen-
sory stimuli during migraine. It is well known that the only group of peripheral nociceptors affected during migraine are those supplying the meninges. However, the present study shows that repeated administration of NTG may affect cutaneous primary afferent receptors or nociceptors directly and differently. Although we did not verify which type of primary afferents or receptors are involved in the distinct development of hind paw mechanical and facial cold hypersensitivity, we speculate that a variety of neuronal mechanisms may be related to the development of these hypersensitivities in migraine patients. In the further studies, we plan to examine the potential direct involvement of acid-sensing ion channels (ASICs), transient receptor potential cation channels, subfamily A, member 1 (TRPA1), and subfamily M, member 8 (TRPM8) in paw mechanical hypersensitivity and/or facial cold allodynia in NTG-treated mice.

In conclusion, the present study demonstrates that repetitive NTG administration produces acute and long-lasting mechanical, thermal, and cold hypersensitivities. These hypersensitivities are differentially expressed in the hind paw as compared to the face. Moreover, NTG-induced heat hypersensitivity is dependent on CSPAs, while mechanical hypersensitivity in the hind paw and cold allodynia in the facial region are not. These results suggest that the development of peripheral hypersensitivities in migraine patients can be differentially expressed depending on the body site and the modality tested, as well as the primary afferent fiber types that innervate that particular body region.

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Conflict of Interest The authors declare no conflict of interest.

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