TITLE:

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CITATION:

Tobori, Shota ...[et al]. MrgprB4 in trigeminal neurons expressing TRPA1 modulates unpleasant sensations. Journal of Pharmacological Sciences 2021, 146(4): 200-205

ISSUE DATE:

2021-08

URL:

http://hdl.handle.net/2433/274020

RIGHT:

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Journal of Pharmacological Sciences 146 (2021) 200–205

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

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

Full Paper

MrgprB4 in trigeminal neurons expressing TRPA1 modulates unpleasant sensations

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ARTICLE INFO

Article history:
Received 12 February 2021
Received in revised form
18 April 2021
Accepted 22 April 2021
Available online 28 April 2021

Keywords:
Sensory behavior
Hairy skin
MrgprB4
TRPA1
Trigeminal ganglion

ABSTRACT

Gentle touch such as stroking of the skin produces a pleasant feeling, which is detected by a rare subset of sensory neurons that express Mas-related G protein-coupled receptor B4 (MrgprB4) in mice. We examined small populations of MrgprB4-positive neurons in the trigeminal ganglion and the dorsal root ganglion, and most of these were sensitive to transient receptor potential ankyrin 1 (TRPA1) agonist but not TRPV1, TRPM8, or TRPV4 agonists. Deficiency of MrgprB4 did not affect noxious pain or itch behaviors in the hairless plantar and hairy cheek. Although behavior related to acetone-induced cold sensing in the hind paw was not changed, unpleasant sensory behaviors in response to acetone application or sucrose splash to the cheek were significantly enhanced in Mrgprb4-knockout mice as well as in TRPA1-knockout mice. These results suggest that MrgprB4 in the trigeminal neurons produces pleasant sensations in cooperation with TRPA1, rather than noxious or cold sensations. Pleasant sensations may modulate unpleasant sensations on the cheek via MrgprB4.

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1. Introduction

Animals possess a sensory nervous system to monitor change in their surrounding environment. The sensory nervous system can detect specific stimuli via a variety of sensor molecules, including ion channels in primary sensory neurons.[1–3] For example, transient receptor potential (TRP) channels are among the major cation-permeable channel families, and some TRP channels act as thermal or mechanical sensors.[4] TRP ankyrin 1 (TRPA1) is a Ca^{2+}-permeable cation channel that is activated by a wide spectrum of noxious external stimuli, including pungent compounds such as cinnamaldehyde and allyl isothiocyanate, reactive chemical species such as reactive oxygen/nitrogen species, and cold in the noxious range.[5] TRPA1 is expressed by primary sensory neurons and was first identified as a candidate cold nociceptor and mechanosensor,[6,7] but there are several lines of conflicting evidence for these functions *in vivo*.[8–11]

The Mas-related G protein-coupled receptor (Mrgpr) family is a large family of G protein-coupled receptors (GPCRs) that has more than 50 members.[12,13] This family evolved in tetrapods, which acquired arms and legs, enabling removal of harmful substances from the skin,[13] and has evolved along with the surrounding environments. Mrgprs are expressed by subsets of nociceptive sensory neurons and contribute to sensations of itch or pain,[14–16] but some Mrgpr family members may also contribute to sensations on the skin perceived as pleasant. For example, MrgprB4 is an orphan GPCR expressed by a small subset of unmyelinated sensory neurons in mice.[17] These neurons detect massage-like stroking of hairy skin, which can be interpreted as a pleasant feeling.[18] MrgprB4-positive neurons, so far only observed in rodents,[13] have physiological and

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Peer review under responsibility of Japanese Pharmacological Society.
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https://doi.org/10.1016/j.jphs.2021.04.006
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functional similarities with human C-tactile fibers.[17,19] Accumulating evidence suggests that C-tactile fibers respond to dynamic touches, such as stroking of the skin that produces pleasant sensations,[20–26] and that stimulation of C-tactile afferents activates the insular cortex[27] that projects to emotional cortical areas.[28] MrgprB4-positive neurons also express the mechanosensitive ion channel Piezo2,[29] but the role of MrgprB4 in sensing mechanical inputs has not been clarified.

In this study, we used a transgenic mouse line in which MrgprB4-expressing cells are labeled by red fluorescent protein[18] to examine how MrgprB4-positive primary sensory neurons respond to various TRP channel agonists *in vitro*. We also explored how this receptor influences behaviors induced by pain or other unpleasant sensations *in vivo* using MrgprB4-knockout (KO) mice.

2. Materials and methods

2.1. Animals

All experiments were conducted in accordance with the ethical guidelines of the Kyoto University animal experimentation committee and the guidelines of the Japanese Pharmacological Society. Mrgprb4$^{tm3(cre)And}$/J mice and TRPA1-knockout (TRPA1-KO) mice, obtained from Jackson Laboratory (Bar Harbor, ME), were backcrossed with C57BL/6J mice (Japan SLC) for ten generations to eliminate any background effects on the phenotype. In Mrgprb4$^{tm3(cre)And}$/J mice, the entire open reading frame of the *Mrgprb4* gene was replaced by an mtdTomato-2A-NLSCre-Frt-PGK-neomycin-FRT cassette. Mrgprb4$^{tm3(cre)And}$ heterozygous and homozygous mice were used as MrgprB4-tdTomato and MrgprB4-knockout (MrgprB4-KO), respectively. We used the C57BL/6J strain of mice as the wild-type (WT) control. Both sexes of mice (6–9 weeks of age) were used in the present study. Animals were maintained at constant ambient temperature (22 ± 2 °C) under a 12 h light/dark cycle, with food and water available *ad libitum*.

2.2. Reagents

Menthol, cremophore EL, poly-L-lysine, and D-mannitol were purchased from Sigma–Aldrich (St. Louis, MO). Laminin was acquired from Life Technologies (Carlsbad, CA). Other drugs and chemicals were obtained from Nacalai Tesque (Kyoto, Japan).

2.3. Primary cultures of mouse trigeminal ganglion (TG) neurons

Bilateral TGs were harvested from a freshly killed adult MrgprB4-tdTomato male or female mouse. TGs were incubated for 1 h at 37 °C in Hank's balanced salt solution containing 137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM D-glucose, and 2.4 mM HEPES (adjusted to pH 7.4 with NaOH), which contained 0.3% collagenase and 0.4% dispase. A Percoll (Sigma–Aldrich) gradient was used to separate TG neurons from myelin and nerve debris as follows. Solutions of 30% and 60% Percoll were prepared with L15 medium. The 30% Percoll solution was gently layered over the 60% Percoll solution, and the cell suspension was gently layered over the Percoll gradient. After 10 min of centrifugation at 1800×*g*, the cells were harvested from the Percoll interface, suspended in 8 mL of L15 medium, and centrifuged again for 5 min at 1800×*g*. The supernatant was removed, and the cell pellet was resuspended in 70 μL of DMEM (D6046; Sigma) containing 10% heat-inactivated fetal bovine serum (Sigma), penicillin G (100 U/mL), and streptomycin (100 μg/mL). The cells were plated onto laminin-coated coverslips and incubated at 37 °C in a humidified 5% CO_2 atmosphere. After 4 h of incubation, 1.5 mL of DMEM was added and the cells were incubated again, but this time overnight at 37 °C in a humidified 5% CO_2 atmosphere.

2.4. Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

Cells on coverslips were loaded for 30–40 min with 5 μM Fura-2 acetoxymethyl ester (Fura-2 AM; Dojindo Laboratories) in Krebs–Ringer bath solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, which contained 0.005% cremophore EL. Fluorescence images were captured every 5 s using alternating excitation at 340 and 380 nm and emission at 510 nm with an AQUACOSMOS/ORCA-AG imaging system (Hamamatsu Photonics, Shizuoka, Japan). Experiments were conducted at room temperature. The ratio of the fluorescence intensity obtained by the excitation/emission of 340 nm/510 nm (F_{340}) to the fluorescence intensity obtained by the excitation/emission of 380 nm/510 nm (F_{380}), namely, F_{340}/F_{380}, was calculated to evaluate the $[Ca^{2+}]_i$. Cells with an F_{340}/F_{380} ratio greater than 1.5 at baseline before treatment were excluded.

2.5. Behavioral tests

Animals were acclimatized to the testing room for at least 1 h before all behavioral tests. The same experimenter handled and tested all animals in each experiment and was blinded to the genotype of each animal.

In the capsaicin injection test, mice were acclimated to a clear acrylic cylinder before 10 μL of 10 μg or 0.5 μg capsaicin (diluted in saline with 20% ethanol and 7% Tween 80) was injected into the cheek subcutaneously or the hind paw, respectively. Capsaicin-induced pain behaviors were measured as the number of the wiping behaviors on the cheek or the durations of licking and biting behaviors to the hind paw for 10 min.

In the hot plate test, mice were acclimatized to the testing apparatus for 1 h. They were placed individually in a transparent Plexiglas cylinder on the center of the hot plate analgesimeter (Ugo Basile) maintained at 52 °C. The latency to the first response either by jumping or by licking or flicking the hind paw was measured to the nearest 0.1 s. After the measurement, the mice were immediately removed from the hot plate and returned to the home cage.

In the tail immersion test, each mouse was gently restrained in a 50 mL conical tube, and the protruding tail was dipped into a 44 °C water bath. The latency to respond to the heat stimulus (clambering up the wall of the tube) was measured.

The acetone test was carried out as previously described with slight modification.[30] Briefly, mice were placed onto a mesh grid and a drop (30 μL) of acetone was applied to the hairy cheek or hairless plantar surface of the hind paw using a 1 mL syringe. The frequencies of wiping the cheek or flinching or licking the paw were measured for 5 min, and the average from three responses was calculated.

In the chloroquine injection test, mice were acclimatized to a clear acrylic cylinder, and then 10 μL of chloroquine (100 μg diluted in PBS) was injected subcutaneously into the cheek. Chloroquine-induced itch behaviors were measured as bouts of scratching behaviors for 40 min.

In the sucrose splash test, mice were acclimated to a clear acrylic cylinder, and then 10% (w/v) sucrose was applied to the hairy cheek using a small mist spray bottle. The number of times the mice wiped their cheeks was measured for 10 min.

2.6. Immunohistochemistry

Mice were intraperitoneally injected with 64.8 mg/kg pentobarbital and perfused transcardially with PBS (K^+ free) followed

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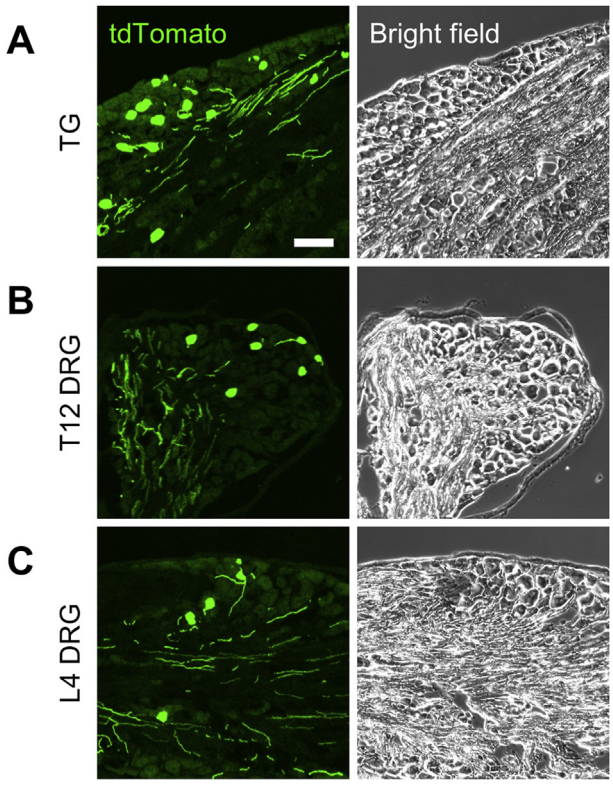

Fig. 1. Expression of MrgprB4 in the TG and DRG. (A–C) Representative immunostaining of tdTomato in the TG (A) and thymus level 12 (T12) (B) and lumbar level 4 (L4) (C) DRGs from MrgprB4-tdTomato mice. The corresponding bright field images are shown on the right. Scale bar: 100 μm.

by PBS (K^+ free) containing 4% (w/v) paraformaldehyde. TGs or thymus level 12 (T12, middle) or lumbar level 4 (L4, bottom) dorsal root ganglions (DRGs) were stored in the fixative for 4 h and then transferred to 30% sucrose overnight. Sections of 16 μm thickness were cut using a cryomicrotome (Leica). The coronal sections were then incubated with primary antibodies for tdTomato (rabbit anti-DsRed antibody, 1:500; Clontech) or MAP2 (mouse anti-MAP2 antibody, 1:500; Chemicon) at 4 °C overnight. Sections were then labeled with fluorescence-labeled secondary antibodies (AlexaFluor 594-labeled donkey anti-rabbit or AlexaFluor 488-labeled donkey anti-mouse IgG, 1:500; Invitrogen) at room temperature for 1 h in the dark. Images were captured with a confocal fluorescence microscope.

2.7. Statistical analysis

Statistical analysis was performed using Prism 9 software (GraphPad Software). For comparisons between a single experimental group and a control group, an unpaired *t*-test was used. In all cases, a *p*-value of <0.05 was considered statistically significant. Data are given as means ± SEMs.

3. Results

3.1. Location of MrgprB4-positive neurons

To determine the areas where MrgprB4-positive neurons are localized, we assessed ganglia from a transgenic mouse line whose MrgprB4-expressing cells are labeled by tdTomato.[18] We observed an abundance of tdTomato-expressing cells in the TG as well as

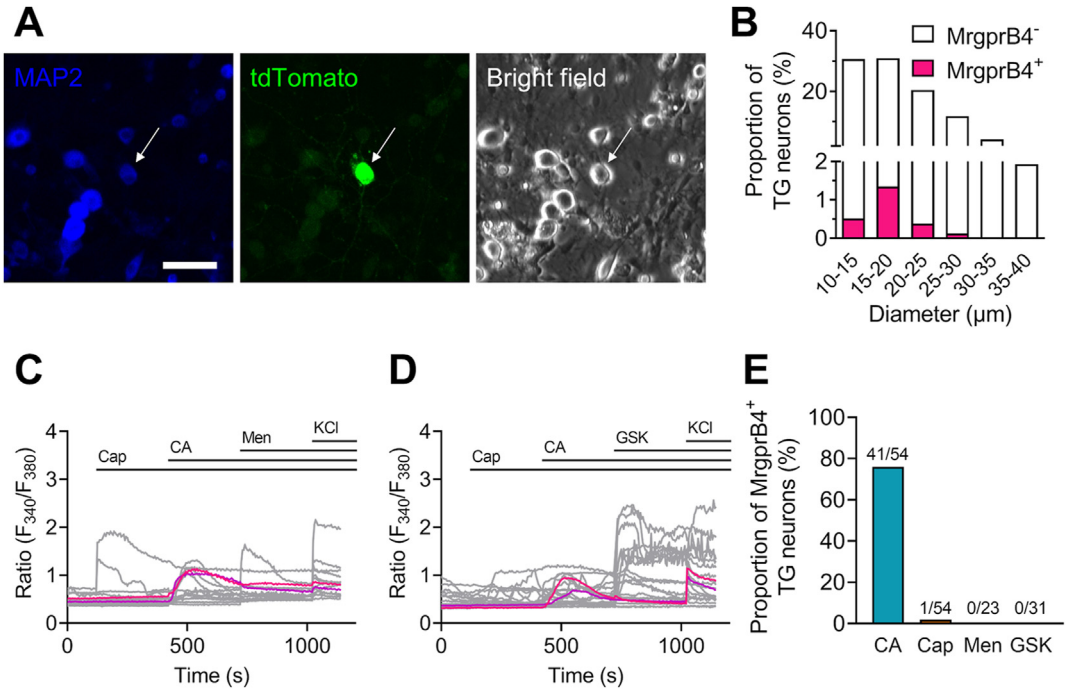

Fig. 2. MrgprB4-expressing TG neurons are small-diameter neurons and exclusively respond to TRPA1 stimulation. (A) Representative Immunostaining of MAP2 (left) or tdTomato (middle) and bright field (right) image of cultured TG cells derived from MrgprB4-tdTomato mice. White arrows indicate the same cell. Scale bar: 50 μm. (B) Diameter frequency distribution of MrgprB4-positive or -negative cultured TG neurons derived from MrgprB4-tdTomato mice (summarized data from two mice). Mean diameters of $MrgprB4^+$ and $MrgprB4^-$ neurons were 17.8 μm ($n = 37$ cells) and 19.3 μm ($n = 1768$ cells), respectively. (C, D) Representative traces obtained from fura-2 Ca^{2+} imaging experiments. Each trace was acquired from an individual $MrgprB4^+$ (colored, $n = 2$ cells) or $MrgprB4^-$ (gray, $n = 13–18$ cells) neuron. Capsaicin (Cap, a TRPV1 agonist), 0.3 μM; cinnamaldehyde (CA, a TRPA1 agonist), 100 μM; menthol (Men, a TRPM8 agonist), 0.5 mM; GSK1016790A (GSK, a TRPV4 agonist), 1 μM; KCl, 50 mM. (E) The proportion of $MrgprB4^+$ neurons displaying the response to each selective TRP channel agonist ($n = 54$ cells).

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some cells in the T12 and L4 DRG (Fig. 1). Therefore, we focused on TG in subsequent experiments.

3.2. Characteristics of MrgprB4-positive neurons

The double-immunostaining experiments in TG cells derived from MrgprB4-tdTomato mice revealed that tdTomato fluorescence overlapped with that for a neuronal marker, MAP2, indicating that MrgprB4-positive cells are neurons (Fig. 2A). We then measured the distributions of cell body diameters of the MrgprB4-positive and -negative cells. The population of TG cells comprised a variety of cell sizes between 10 and 40 μm. The average size of MrgprB4-positive cells was 17.8 ± 0.62 μm, indicating that the small population of MrgprB4-positive neurons is small-diameter TG neurons (Fig. 2B). To determine whether MrgprB4-positive TG neurons are responsive to TRP channel agonists; we performed ratiometric calcium imaging of TG neurons dissociated from MrgprB4-tdTomato mice (Fig. 2C, D). Forty-one of 54 MrgprB4-positive cells (75.9%) responded to a selective TRPA1 agonist, cinnamaldehyde (100 μM), whereas only 1 of 54 MrgprB4-positive cells (1.9%) responded to a selective TRPV1 agonist, capsaicin (0.3 μM). Furthermore, MrgprB4-positive cells did not respond to either the TRPM8 agonist menthol (0.5 mM) or the TRPV4 agonist GSK1016790A (1 μM) (Fig. 2E).

3.3. MrgprB4-KO mice show normal responsiveness to sensory stimuli on glabrous skin

No phenotypic abnormalities have been reported in association with MrgprB4 deficiency.[17,18] However, the responses of MrgprB4-KO mice to a variety of sensory stimuli have not been investigated. We examined whether MrgprB4 deficiency alters responses to stimuli applied to the plantar surface, which is often used for sensory and pain research.[18] The glabrous skin in this region is not innervated by MrgprB4-positive neurons.[17] Accordingly, responses evoked by a variety of stimuli, including capsaicin injection, thermal responses to placement on a hot plate or immersion of the tail in hot water, and cold stimulus by acetone application, did not differ between WT and MrgprB4-KO mice (Fig. 3A–D). Vehicle treatment for capsaicin test did not affect the durations of licking and biting behaviors in both WT and MrgprB4-KO mice. Similarly, TRPA1-KO mice showed no difference in response to acetone application compared with that of WT mice (Fig. 3E) despite the fact that TRPA1 is reported as a cold nociceptor.[10]

3.4. MrgprB4 deficiency increases an unpleasant sensation in hairy skin

We investigated behavioral phenotypes of MrgprB4 transgenic mice in response to sensory stimulation of areas for which MrgprB4-expressing neurons innervate, such as the cheek, which is hairy skin innervated by TG neurons.[31] Subcutaneous injections of capsaicin similarly elicited wiping behaviors in both WT and MrgprB4-KO mice, whereas no difference was observed in the number of wiping behaviors with vehicle treatment for both WT and MrgprB4-KO mice (Fig. 4A). Likewise, itch behaviors (scratching) evoked by subcutaneous injections of chloroquine, which is an agonist of MrgprA3 and induces acute itch by activating TRPA1,[15] were not different between WT and MrgprB4-KO mice, while vehicle-injected mice rarely scratched their cheeks for chloroquine test (Fig. 4B). However, MrgprB4-KO mice showed significantly more wiping of the cheek in response to acetone application than WT mice (Fig. 4C). Interestingly, these mice also showed more wiping than WT controls in response to the discomfort elicited by a sticky sucrose solution splash (Fig. 4E). The behavioral responses of MrgprB4-KO mice resembled those exhibited by TRPA1-KO mice, which also showed increased wiping in response to acetone application and sucrose splash (Fig. 4D, F).

4. Discussion

In the present study, we found that MrgprB4 is expressed by a small subset of TG and DRG neurons and that most MrgprB4-positive TG neurons respond to TRPA1 agonists but not TRPV1, TRPM8, or TRPV4 agonists. Furthermore, the results from the behavioral experiments suggest that MrgprB4 in TG neurons may modulate

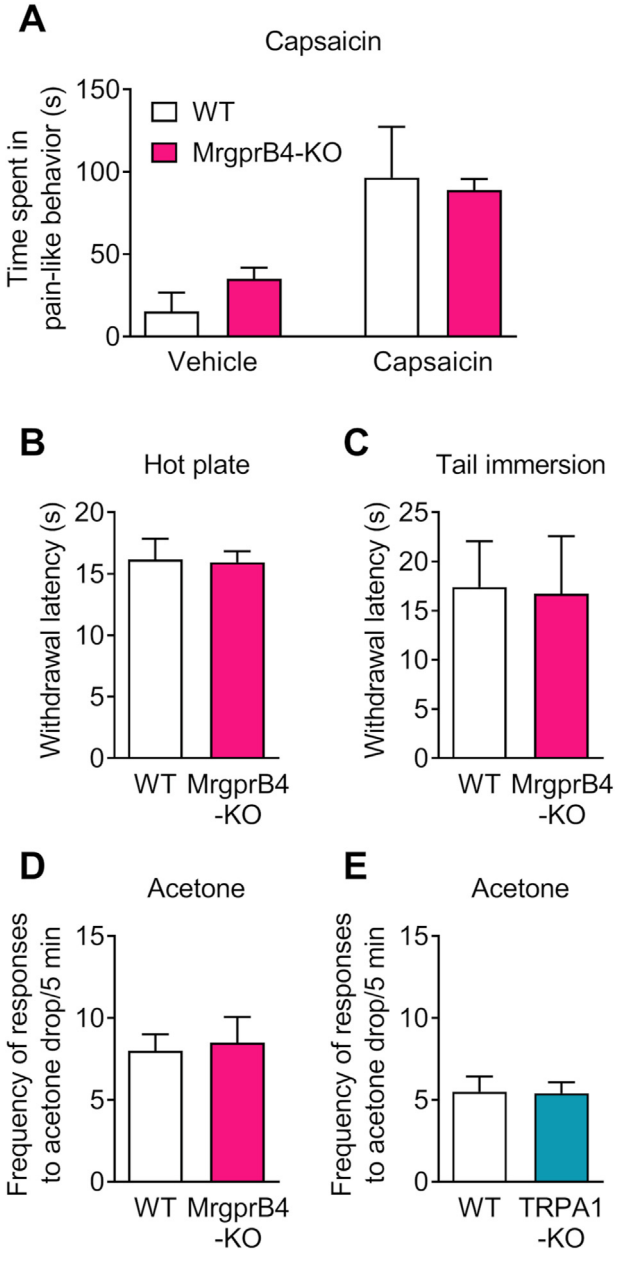

Fig. 3. Responsiveness to painful stimuli on the hairless plantar region in mice. (A) The duration of nocifensive behavior (licking or biting) evoked by intraplantar injection of 0.5 μg capsaicin was measured for 10 min (n = 4–5). (B, C) Responses to noxious thermal stimulation were measured by paw withdrawal latency in the hot plate test (B; n = 6) and tail-flick latency in the water immersion test (C; n = 3–5). (D, E) The frequencies of responses to a 30 μL acetone drop applied to the hairless plantar region of WT mice compared with those of MrgprB4-KO mice (D; n = 3–4) and TRPA1-KO mice (E; n = 5–6) were measured for 5 min. Data are expressed as means ± SEMs.

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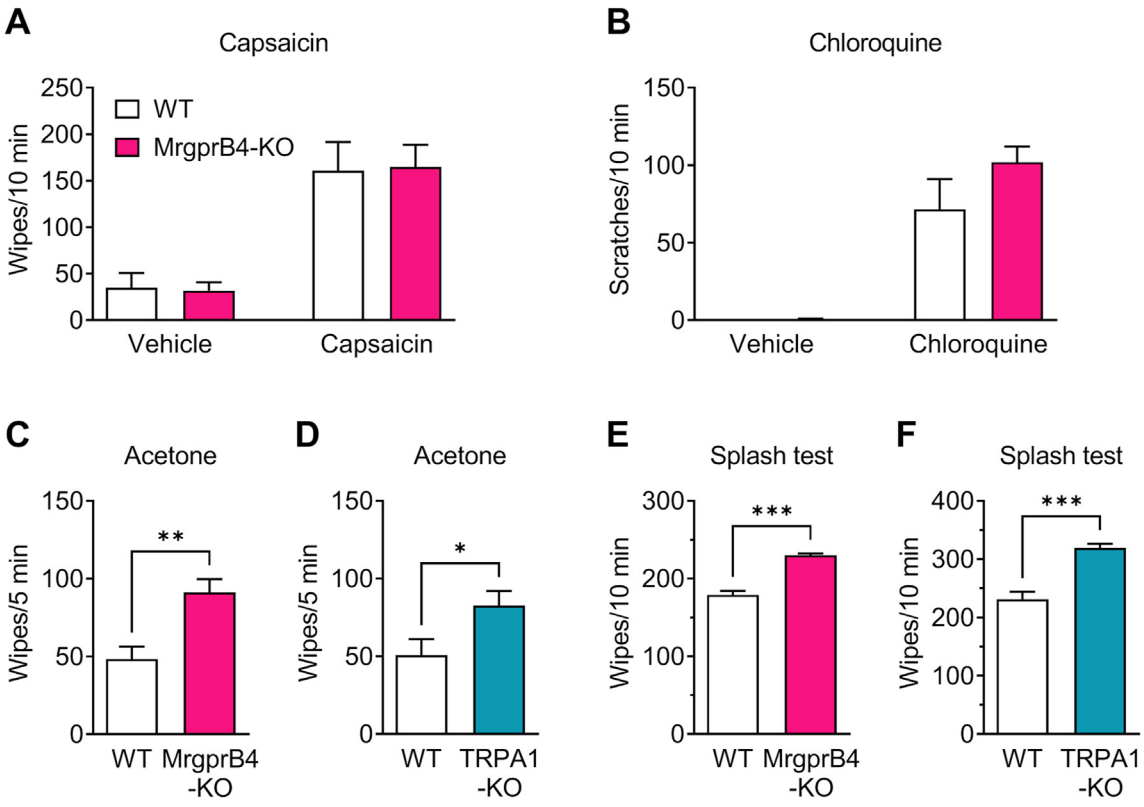

Fig. 4. Responsiveness to painful, itch, and unpleasant sensory stimuli on the hairy cheek. (A, B) Mice received subcutaneous injections of 10 μg capsaicin (A; $n = 5–6$) or 100 μg chloroquine (B; $n = 4–7$) into the cheek, and the number of wipes or scratches was counted for 10 or 40 min, respectively. (C, D) Acetone (30 μL drop) was applied to the hairy cheek of WT and MrgprB4-KO (C; $n = 10$) or TRPA1-KO (D; $n = 7–8$) mice, and the number of wipes was measured for 5 min. (E, F) A 10% sucrose solution was applied by spray onto the hairy cheeks of WT and MrgprB4-KO (E; $n = 4–5$) or TRPA1-KO (F; $n = 6$) mice, and the number of wipes was measured for 10 min. Data are expressed as means ± SEMs. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

unpleasant sensations, such as those induced by acetone application or sucrose splash on the cheek. Together, these findings suggest that MrgprB4 cooperates with TRPA1 to modulate unpleasant sensations.

We found that only a small proportion of neurons in the TG express MrgprB4 and that these neurons had small cell body diameters, consistent with previous findings in the DRG.[17,18,29] However, the immunohistochemical results in those studies also indicated that MrgprB4-positive neurons do not express TRPV1.[17,32] By contrast, Huang et al. reported that MrgprB4 transcript levels were higher in TG neurons than in DRG neurons, and approximately half of the DRG neurons were MrgprB4-positive and also expressed TRPV1.[33] The reasons for the discrepancies between the previous reports are not clear. In the present study, we clearly demonstrate that most MrgprB4-positive neurons from the TG are sensitive to TRPA1 agonists but insensitive to a TRPV1 agonist. A recent large-scale single-cell RNA sequencing study in mouse DRG neurons revealed that MrgprB4-positive neurons are rare among DRG neurons (10 of 622 total cells).[34] Among these ten cells, six expressed TRPA1, two expressed TRPV1, and none expressed either TRPV4 or TRPM8. Our findings for cellular responses to agonists support this study, demonstrating that a small subset of sensory neurons in the TG is MrgprB4 and TRPA1-positive but TRPV1-negative. Although TRPA1/TRPV1 double-positive neurons have been investigated, the properties of TRPA1-positive and TRPV1-negative neurons have not been clarified. Further studies are also needed to ascertain whether MrgprB4-positive neurons express functional TRPA1.

We found that both MrgprB4-KO and TRPA1-KO mice exhibited more wiping behaviors than WT controls when acetone was applied to the hairy cheek. This apparent increase in responsiveness to acetone could be considered an enhanced sensitivity to an unpleasant cold stimulus (cold hypersensitivity). Although a deficiency of TRPA1 does not alter sensitivity to a cold stimulus,[8–11] TRPA1-KO mice show a greater preference for warm zones, which may reflect a higher sensitivity to a cold environment than WT mice.[35,36] In addition, TRPA1-KO mice in our study did not show increased responsiveness to acetone applied to the hairless plantar region, suggesting that the enhanced responsiveness to acetone on the hairy cheek is not due to cold hypersensitivity. As stimulation of MrgprB4- and TRPA1-coexpressing TG neurons produces pleasant sensations in hairy skin,[18] the unpleasant sensations induced by acetone application on the hairy cheek might not be inhibited in MrgprB4-KO or TRPA1-KO mice, leading to the increase in unpleasant sensory behaviors. Similar results were observed in the sucrose splash test, in which the unpleasant sensation was induced by a sticky sucrose solution rather than cold. These findings support the hypothesis that pleasant sensations through MrgprB4 activated by dynamic touches such as wiping may modulate unpleasant sensations in the hairy cheek. However, since MrgprB4 is an orphan receptor whose ligand is unknown, further investigations are needed to reveal what stimulates or activates MrgprB4.

By contrast, our findings show that MrgprB4 does not contribute to noxious pain, such as that from capsaicin and heat, or cold sensation in the hind paw, a region in which MrgprB4-positive neurons do not innervate. This is consistent with previous reports.[18,37] As the behavioral response to capsaicin applied to the hairy cheek was not altered in MrgprB4-KO mice and MrgprB4-positive neurons were not sensitive to capsaicin, we consider that pain evoked by stimulation of TRPV1-positive nociceptive neurons is not affected by the enhancement of unpleasant sensation in MrgprB4-KO mice. Similarly, the present results suggest no relation

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between MrgprB4 and the chloroquine-induced itch behavior in the cheek. Chloroquine induces acute intense itch by activating TRPA1 through MrgprA3[15]; therefore, the intense itch elicited by chloroquine may mask the behavioral changes evoked by the enhancement of unpleasant sensation in MrgprB4-KO mice.

In conclusion, most of the MrgprB4-positive sensory neurons in TG express TRPA1, and MrgprB4 may cooperate with TRPA1 to modulate unpleasant sensations in hairy skin. Our findings provide further insight into the relationship between pleasant and unpleasant sensations and may lead us to reconsider the physiological roles of TRPA1, which is thought to be just an ion channel that mediates unpleasant sensations.

Declaration of competing interest

The authors indicated no potential conflicts of interest.

Acknowledgments

This study was supported by MEXT/JSPS KAKENHI Grant Numbers JP19H03377, JP19K22494 (to H.S.), and JP20H00491 (to S.K.). This work was also supported by the Nakatomi Foundation and the Takeda Science Foundation (to H.S.).

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