Role of P2X3 receptors in scratching behavior in mouse models

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To the Editor:

Itch is an unpleasant cutaneous sensation that elicits a desire to scratch or scratch reflex. Itch generally serves as a warning and a self-protection mechanism against harmful external agents. However, chronic itch caused by multiple dermatological or systemic diseases (eg, atopic dermatitis) has strongly deleterious effects on the quality of life of millions of people worldwide.[1]

Sensory information from the peripheral endings of primary afferent sensory neurons in the skin is conveyed through these neurons to the spinal dorsal horn (SDH), where it activates brain-projecting neurons via neural circuits. Neuronal pathways from the periphery to the brain selective for itch are still poorly understood, but recent studies have identified neuronal populations for itch at the levels of primary afferents and the SDH.[2] Han et al[3] showed that deleting a subpopulation of primary afferent sensory neurons that express Mas-related G protein–coupled receptor A3 (MrgprA3) led to a selective deficit in behaviors related to itch but not to pain, indicating that MrgprA3^{+} primary sensory neurons are essential for itch sensation. However, little is known about endogenously expressed molecules that control the activity of this population. Because MrgprA3^{+} neurons are also necessary for chronic itch,[3] the identification of such molecules controlling MrgprA3^{+} neurons will help our understanding of the mechanism for acute and chronic itch and also provide a new therapeutic strategy for chronic itch.

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In this study, we focused on P2X3 purinoceptors (P2X3Rs), a subtype of ATP-gated cation channels, because immunoreactivity for P2X3Rs has previously been detected in small-diameter dorsal root ganglion (DRG) and trigeminal (TG) neurons, including the MrgprA3$^+$ population.[3] However, it remains unknown whether P2X3Rs are functional in the MrgprA3$^+$ population and, more importantly, whether they play a role in itch sensation. Consistent with previous observations in lumbar DRG neurons,[3] we observed P2X3R immunofluorescence in cervical DRG neurons positive for green fluorescent protein (GFP) in *Mrgpra3$^{GFP-Cre}$* mice (Fig 1, A) in which GFP is expressed in the MrgprA3$^+$ neuronal population.[3] P2X3Rs were also observed in MrgprA3$^+$ fibers (tdTomato$^+$) in the skin of *Mrgpra3$^{GFP-Cre}$;ROSA26^{tdTomato}* mice (Fig 1, B), but P2X3R mRNA was extremely low in the skin (see Fig E1 in this article's Online Repository at www.jacionline.org). Thus, P2X3Rs are expressed in MrgprA3$^+$ nerve endings in the skin. We also found that 61.5% and 55.2% of chloroquine (an MrgprA3 agonist)-responded DRG and TG neurons, respectively, showed intracellular Ca^{2+} transients after application of α,β-methylene ATP (α,β-meATP), which activates P2X3Rs (Fig 1, C). These percentages were similar to those of DRG and TG neurons with double-immunoreactivity for P2X3R and GFP in *Mrgpra3$^{GFP-Cre}$* mice (P2X3R$^+$ DRG and TG neurons: 57.4% and 44.9% of total GFP$^+$ neurons, respectively; see Fig E2 in this article's Online Repository at www.jacionline.org). These results indicate that approximately half of the MrgprA3$^+$ DRG and TG neurons expressed functional P2X3Rs.

To assess the ability of P2X3R agonists to produce itch, we monitored scratching behavior after intradermal injection of ATP or α,β-meATP to the rostral back in mice. Scratching directed to the injection site was elicited by either ATP or α,β-meATP (Fig 2, A). Similarly, in the cheek model,[4] an alternative behavioral assay for itch, these agonists when administered to the cheeks of mice increased scratching with the hindlimb (Fig 2, B; see Fig E3 in this article's Online Repository at www.jacionline.org). The α,β-meATP–induced scratching was prevented by A317491, a P2X3R-selective antagonist (Fig 2, B; see Fig E4 in this article's Online Repository at www.jacionline.org). By contrast, ATP and α,β-meATP did not induce wiping, a behavior that reflects pain-related responses in the cheek model (Fig 2, C). These results indicate that activation of P2X3Rs in the skin elicits scratching. To determine whether MrgprA3$^+$ neurons mediate P2X3R-dependent scratching, we intradermally coadministered the lidocaine derivative QX-314 with chloroquine, a treatment that selectively silences MrgprA3$^+$ sensory neurons.[5] This treatment markedly suppressed scratching evoked by ATP and α,β-meATP (Fig 2, D). Chloroquine or QX-314 alone had no effect on the α,β-meATP–induced scratching (data not shown). These results indicate that activation of P2X3Rs expressed in MrgprA3$^+$ sensory neurons induces scratching.

Itch information received at the terminals of primary afferents in the skin is transmitted to the SDH. A recent study revealed that gastrin-releasing peptide receptors (GRPRs) are crucial for processing itch information in the SDH.[6] We thus next examined whether GRPRs mediate P2X3R-derived scratching using *Grpr*-knockout mice. The ATP- and α,β-meATP–induced scratching seen in wild-type mice was markedly suppressed in *Grpr*-knockout mice (Fig 2, E), indicating that GRPRs are required for P2X3R-dependent scratching.

GRPR signaling in the SDH has been implicated not only in acute but also in chronic itch.[7] We therefore predicted that P2X3Rs would also participate in chronic pathological itch using NC/Nga mice, a model of atopic dermatitis. These mice are known to develop spontaneous severe dermatitis and show repetitive scratching when maintained under conventional (CV) conditions, mimicking human atopic dermatitis.[8] We found that P2X3R mRNA was increased in the DRG (but not skin) of CV-NC/Nga mice (Fig 2, F) and that compared with vehicle, intradermal treatment with A317491 decreased scratching in CV-NC/Nga mice (Fig 2, G) without affecting skin inflammation (see Fig E5 in this article's Online Repository at www.jacionline.org), suggesting that P2X3Rs play a role in chronic scratching.

The present findings demonstrate that activation of P2X3Rs at the nerve endings of MrgprA3^{+} primary afferent sensory neurons in the skin produces scratching behavior through GRPR signaling in the SDH. Therefore, it is possible that P2X3Rs are important regulators of MrgprA3^{+} sensory neurons linked to itch. Reduction of repetitive scratching in atopic dermatitis by pharmacological blockade of P2X3Rs implies that chronic itch related to atopic dermatitis involves ongoing signaling via P2X3Rs, likely activated by extracellular ATP that may be released from mechanically stimulated keratinocytes or from damaged skin tissues.[9] In inflammatory skin diseases including atopic dermatitis, skin lesions induced by scratching can lead to more severe itch and further scratching (the itch-scratch vicious cycle). It is thus conceivable that ATP-P2X3R signaling may be involved in this cycle in a pathway via MrgprA3^{+} sensory neurons and spinal GRPRs, which are also implicated in chronic itch.[3,7] Thus, our findings provide the possibility that targeting P2X3Rs may be a novel strategy for treating chronic itch (for additional discussion including the role of P2X3Rs in MrgprA3^{−} neurons, see this article's Online Repository at www.jacionline.org).

METHODS

Animals

C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan) and Charles River Japan (Kanagawa, Japan). Specific pathogen free and CV-NC/Nga mice (male, 15 weeks old) were purchased from SLC Japan (Hamamatsu, Japan). B6.129X1-*Grpr^tm1Jfb*/J (*Grpr*-knockout) and B6;129S6-*Gt(ROSA) 26Sor^tm14(CAG-tdTomato)Hze*/J mice were purchased from the Jackson Laboratory (Bar Harbor, Me). *Mrgpra3^GFP-Cre* mice were kindly provided by Prof. Xinzhong Dong (Johns Hopkins University). All animals were housed at a temperature of 22°C ± 1°C with a 12-hour light-dark cycle (light on 8:00–20:00) and fed food and water ad libitum. All animal experiments were conducted according to relevant national and international guidelines contained in the "Act on Welfare and Management of Animals" (Ministry of Environment of Japan) and "Regulation of Laboratory Animals" (Kyushu University) and under the protocols approved by the Institutional Animal Care and Use Committee review panels at Kyushu University.

Immunohistochemistry

Mice were deeply anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg) and perfused transcardially with 20 mL of PBS, followed by 50 mL ice-cold 4% paraformaldehyde. The third to fifth cervical segments of the DRG, TG, or skin were

removed, postfixed in the same fixative at 4°C, and placed in 30% sucrose solution for 48 hours at 4°C. DRG, TG (15 μm), or skin (30 μm) sections were incubated in blocking solution (3% normal goat or donkey serum) for 2 hours at room temperature and then incubated for 48 hours (DRG and TG) or 120 hours (skin) at 4°C with primary antibodies: anti-P2X3R (rabbit polyclonal, 1:4000; Chemicon (Temecula, Calif); guinea pig polyclonal, 1:12000; Neuromics, Edina, Minn), anti-GFP (chicken polyclonal, 1:20000; Aves Labs, Tigard, Ore), anti-RFP (rabbit polyclonal, 1:5000; MBL, Nagoya, Japan), and anti-TRPV1 (goat polyclonal, 1:2000; Neuromics). Following incubation, tissue sections were washed and incubated for 3 hours at room temperature in secondary antibody solution (Alexa Fluor 405, 488, or 546, 1:1000). The tissue sections were washed, slide mounted, and subsequently coverslipped with Vectashield Hardmount with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, Calif). Immunofluorescence images were obtained with confocal laser microscope (LSM510 or LSM700, Carl Zeiss, Thornwood, NY).

Real-time RT-PCR

Mice were anesthetized with pentobarbital and perfused transcardially with PBS. The third to fifth cervical segments of the DRG, TG, and skin were removed immediately. Total RNA was extracted using TRIsure (Bioline, London, United Kingdom) according to the manufacturer's protocol. The amount of total RNA was quantified by OD260 using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, Mass). For reverse transcription, 250 ng of total RNA was transferred to the reaction with Prime Script reverse transcriptase (Takara, Kusatsu, Japan) and random 6-mer primers. Quantitative PCR was carried out with Fast Start Essential DNA Probes Master (Roche, Mannheim, Germany) using a LightCycler 96 system (Roche) according to the manufacturer's specifications, and the data were analyzed by LightCycler 96 Software (Roche) using standard curves. All values were normalized to glyceraldehyde 3-phosphate dehydrogenase expression. The TaqMan probe, forward primer, and reverse primer used in this study were as follows: P2X3R, probe, 5′-FAM-TCCTGAAGGCTTTTGGCATCCGCTT-TAMRA-3′; forward primer, 5′-ATCGGCTGGGTGTGTGATCT-3′; reverse primer, 5′-GTTGAACTTGCCAGCGTTCC-3′; glyceraldehyde 3-phosphate dehydrogenase, probe, 5′-FAM-ACCACCAACTGCTTAGCCCCCCTG-TAMRA-3′; forward primer, 5′-TGCCCCCATGTTTGTGATG-3′; reverse primer, 5′-GGCATGGACTGTGGTCATGA-3′.

Acute dissociation of DRG and TG neurons

The DRGs from the third cervical to the second thoracic level or the TGs of 4-week-old mice were collected in cold Dulbecco modified Eagle medium/F-12 medium and treated with collagenase (Worthington, Freehold, NJ) at 37°C. After trituration and centrifugation, cells were resuspended in Dulbecco modified Eagle medium/F-12 and plated at 0.5 to 2.0 × 10^6 cells/well on poly-L-lysine and laminin-coated glass coverslips surrounded by silicon rubber walls (Flexiperm, Greiner Bio-One GmbH, Frickenhausen, Germany). Cells were maintained in an incubator at 37°C and used within 3 hours.

Intracellular Ca^{2+} imaging

Ca^{2+} imaging was performed as previously described.[E1] Briefly, neurons were loaded with 2.5 μM Fura 2-AM (Molecular Probes, Eugene, Ore) containing 0.01% pluronic F-127

(Molecular Probes) in a balanced salt solution (150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose, and 25 mM HEPES, pH 7.4) for 30 minutes in the dark at 37°C. After washing, neurons were imaged at 340- and 380-nm excitation to detect intracellular free Ca^{2+}. Data were calculated using the relative increase ratio (F340/F380) from the basal level before the first application.

Behavioral tests

Injections of chemicals into the rostral back or cheek were performed as previously described.[E2] Briefly, mice were shaved at the rostral back or the right cheek at least the day before injection. After intradermal injection into the skin of the back (α,β-meATP [200 μg/50 μL, Sigma, Poole, United Kingdom] with or without A317491 [200 μg/50 μL, Sigma] and ATP [200 μg/50 μL, Sigma]) or the cheek (α,β-meATP [50, 100, and 200 μg/10 μL, Sigma] and ATP [50, 200, and 500 μg/10 μL, Sigma] with or without A317491 [200 μg/10 μL, Sigma]), each mouse was placed in a plastic chamber (11 cm in diameter, 10 cm high). Behavioral responses were video-recorded for 30 (back) or 40 (cheek) minutes.[E3,E4] Hindlimb scratching behaviour and, for cheek, wiping with the forepaw, directed toward the injection site, were counted. One scratch was defined as a lifting of the hindlimb toward the injection site and then placing the limb back on the floor, regardless of how many scratching strokes took place between those 2 movements.

For activity-dependent neuronal silencing, *N*-ethyl-lidocaine (QX-314, Sigma) was used.[E5] Mice received cheek injections (conditional injection) of chloroquine with or without QX-314 (2%, 30 μL in PBS) for 30 minutes before injection of the agonists αβ-meATP (100 μg/10 μL) or ATP (200 μg/10 μL) into the same site as the conditional injection (test injection). After the test injection, behavioral responses were video-recorded for 40 minutes and the number of hindlimb scratching behaviors directed toward the injection site was counted.

In chronic itch model using CV-NC/Nga mice, scratching behavior was automatically detected and objectively evaluated using MicroAct (Neuroscience, Tokyo, Japan) in accordance with a method described previously.[E2] Briefly, a small Teflon-coated magnet (1 mm in diameter, 3 mm in length; Neuroscience) was implanted subcutaneously into the hindpaws of the mice at least 1 day before the first recording and placed in an observation chamber (11 cm in diameter, 18 cm high) with food and tap water, surrounded by a round coil. Movements of magnets implanted into the hindpaws were recorded for 24 hours after intradermal injection of vehicle or A317491 (200 μg/50 μL in PBS) into the rostral back, and the characteristic waves by the movements of magnets (reflecting scratching behavior) were analyzed by MicroAct software.[E6,E7]

Skin histology

Hairs on the back skin were shaved off. Skin biopsies were fixed with 10% phosphate-buffered formalin and embedded in paraffin. Deparaffinized sections of 3 μm thickness were stained with hematoxylin and eosin, and analyzed by light microscopy. Inflammatory cells were counted and the epidermal thickness was measured.

Statistical analysis

Statistical analyses of the results were performed using the unpaired *t* test, the Mann-Whitney *U* test, and 1-way ANOVA followed by post hoc Tukey multiple comparisons test. Values were considered significantly different at *P* values of less than .05.

DISCUSSION

Percentage of P2X3R expression in MrgprA3$^+$ DRG neurons

This study demonstrated that approximately half of MrgprA3$^+$ neurons expressed P2X3Rs in the cervical DRG. However, Han et al[E8] have previously shown that approximately 80% of MrgprA3$^+$ neurons coexpress P2X3Rs in the lumbar DRG of *Mrgpra3*$^{GFP-Cre}$;*ROSA26*^{tdTomato} mice. The reason for this discrepancy remains unclear, but one possibility may be related to the segment of the DRG used for immunohistochemical analyses; that is, we used cervical segments, whereas Han et al used lumbar segments. In fact, in *Mrgpra3*$^{GFP-Cre}$;*ROSA26*^{tdTomato} mice we found that 54.9% (113 of 206 cells) and 70.2% (106 of 151 cells) of tdTomato$^+$ neurons coexpressed P2X3Rs in the cervical and lumbar DRGs, respectively, suggesting that the percentage of P2X3R expression in MrgprA3$^+$ DRG neurons is slightly higher in the lumbar segment.

Role of P2X3R$^+$ MrgprA3$^-$ primary afferent neurons in somatosensory information

We demonstrated that a small population of the DRG neurons expressed both P2X3R and MrgprA3 (3.4% of total neurons), whereas 34.9% of total DRG neurons were P2X3R$^+$ MrgprA3$^-$ (Fig E2). In addition, we performed triple-labeling experiments using antibodies to P2X3R, GFP, and TRPV1 in the DRG of *Mrgpra3*$^{GFP-Cre}$ mice and found that 50% of P2X3R$^+$ MrgprA3$^+$ DRG neurons express TRPV1.

Because P2X3Rs have previously been implicated in pain,[E9,E10] P2X3R$^+$ MrgprA3$^-$ neurons may have a role in transducing nociceptive information from the skin to the spinal cord. However, our study failed to detect a significant pain-related wiping behavior by α,β-meATP injected into the cheek (Fig 2, C). The cheek model has been reported to assess not only itch but also pain by measuring 2 distinct behaviors: scratching and wiping, respectively.[E11] Indeed, pain-evoking substances (such as capsaicin and bradykinin) injected into the cheek produce wiping behavior.[E11,E12] However, a previous report showed that formalin, which is well known to produce pain, does not produce wiping behavior when injected into the cheek.[E3] From these findings, it is conceivable that not all pain-producing substances robustly elicit wiping behavior. Considering the previous findings demonstrating that the formalin-induced pain behavior involves P2X3R signaling,[E9,E10] it is possible that pain behavior caused by P2X3R agonists may be difficult to be detected in the cheek model.

Role of MrgprA3 in itch

Mrgprs are a family of G protein–coupled receptors (GPCRs) that are composed of more than 50 members in the mouse genome.[E13] The expression of some members including MrgprA3 is restricted to primary afferent sensory neurons.[E14] Furthermore, some of the Mrgpr members are activated not only by mammalian peptide but also by nonmammalian peptide such as molluscan FMRF amide (Phe-Met-Arg-Phe).[E14] However, endogenous

ligands for MrgprA3 have not been identified to date. Moreover, the role of MrgprA3 itself in chronic itch is yet to be determined, although MrgprA3-expressing sensory neurons are crucial for acute and chronic itch.[E8] In addition, our study revealed a subset of sensory neurons coexpressing P2X3R and MrgprA3. However, whether P2X3R and MrgprA3 interact at nerve endings in the skin and whether such interaction contributes to chronic itch remains unknown; these will be important subjects for future studies.

Other factors involved in chronic itch

Intradermal treatment with the selective P2X3R antagonist A317491 significantly reduced spontaneous scratching in atopic dermatitis model mice. However, considering the fact that A317491 reduced scratching by approximately 30%, it seems that ATP-P2X3R signaling contributes partially to chronic scratching in atopic dermatitis. Indeed, previous reports have shown that other factors (eg, T-cell–derived IL-31,[E15,E16] substance P,[E17] and thymic stromal lymphopoietin[E18]) contribute to chronic itch associated with atopic dermatitis by acting on nerve endings of primary afferent sensory neurons in the skin.

P2X3R and cough

P2X3Rs have been implicated in cough via activation of pulmonary vagal afferent fibers.[E19,E20] A recent study has shown that MrgprC11-expressing vagal sensory neurons innervating the airway mediate cholinergic bronchoconstriction and airway hyperresponsiveness.[E21] The MrgprC11^{+} subset is known to be an almost identical population that expresses MrgprA3,[E22] which suggests the possibility that MrgprC11^{+} neurons could express P2X3Rs. Thus, cough and itch may have a common mechanism in activation of primary afferents (especially the MrgprA3/C11-expressing subset) by P2X3Rs.

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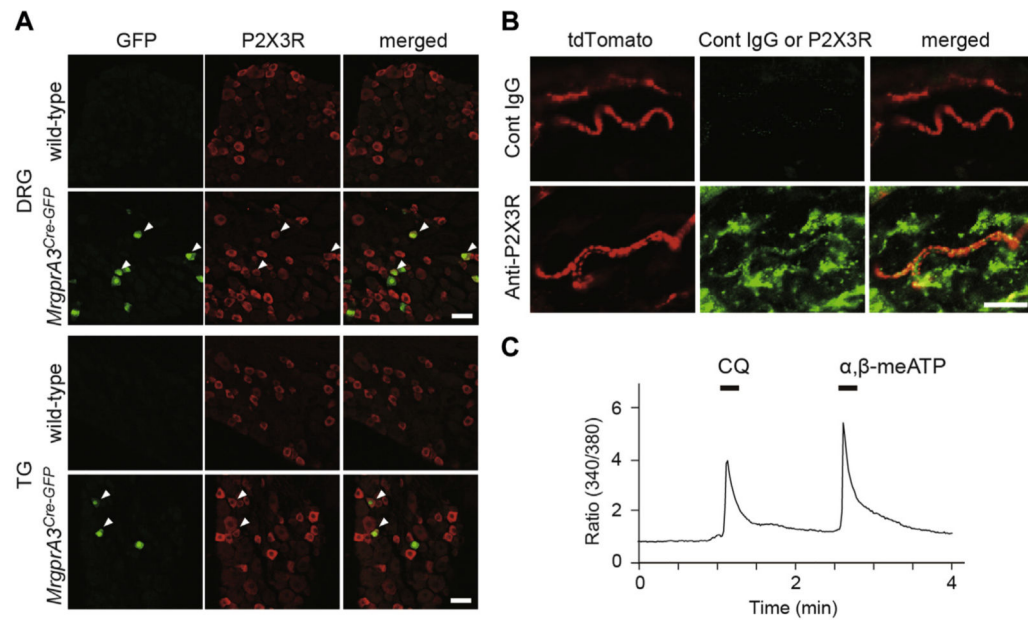
FIG 1.
MrgprA3-positive sensory neurons express functional P2X3Rs. **A**, Immunofluorescence images of DRG/TG neurons of wild-type and *MrgprA3*[GFP-Cre] mice. The MrgprA3^{+} neurons (GFP^{+} cells, green) were counterstained for P2X3R immunoreactivity (red). Arrowheads indicate double-positive neurons. **B**, Immunofluorescence images of the skin of *Mrgpra3*[GFP-Cre];*ROSA26*[tdTomato] mice. MrgprA3^{+} fibers (tdTomato[+], red) were counterstained for P2X3R (bottom, green) or isotype control (Cont IgG, top, green). Scale bar, 20 μm. **C**, Representative intracellular Ca^{2+} responses to chloroquine (CQ) and α,β-meATP in DRG neurons.
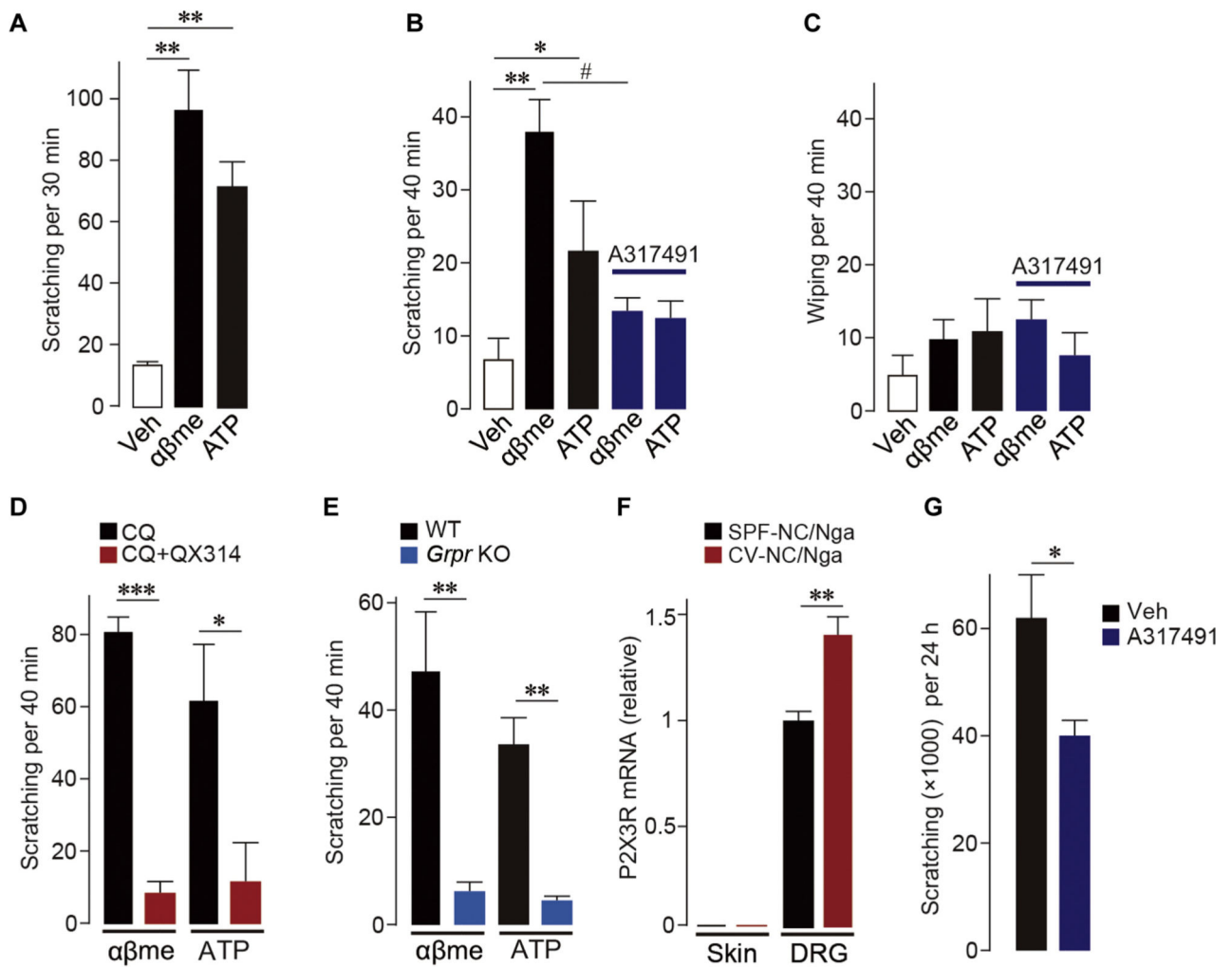
FIG 2.

Activation of P2X3Rs in MrgprA3-positive sensory neurons induces scratching behavior mediated via GRPR signaling. **A,** Scratching induced after intradermal injection of ATP and α,β-meATP (αβme) into the rostral back (n = 6). **B** and **C,** Scratching (Fig 2, *B*) or wiping (Fig 2, *C*) induced after cheek injection of ATP and α,β-meATP (αβme) with or without A317491, a selective antagonist for P2X3Rs (n = 6–10). **D,** Effect of coinjection of chloroquine (CQ) and QX-314 on ATP and α,β-meATP (αβme)-induced scratching (n = 5–6). **E,** Scratching evoked by ATP and α,β-meATP (αβme) in *Grpr*-knockout (KO) mice (n = 6–7). **F,** P2X3R mRNA in the skin or DRG of SPF (control, maintained in specific pathogen-free condition) or CV-NC/Nga mice (n = 4). **G,** Effect of intradermal injection of A317491 on scratching in CV-NC/Nga mice (n = 7–9). *Veh*, Vehicle; *WT*, Wild-type. Data are shown as means ± SEM. Mann-Whitney *U* test (Fig 2, *A, E,* and *G*), unpaired *t* test (Fig 2, *D* and *F*), and 1-way ANOVA followed by post hoc Tukey multiple comparisons test (Fig 2, *B* and *C*) were used for statistical analyses. *,#*P* < .05, **P* < .01, ***P* < .001.
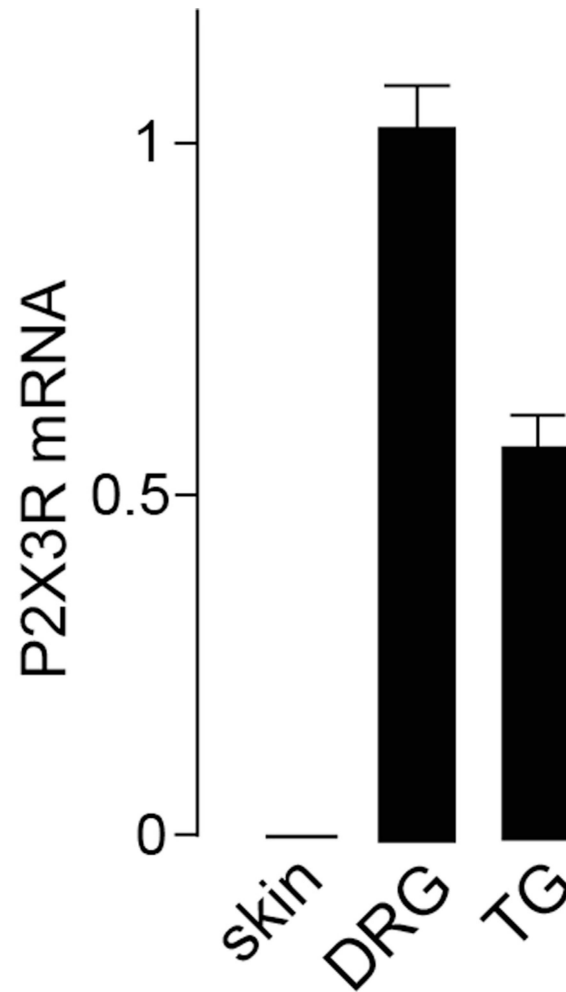
FIG E1.

P2X3R mRNA expression in the skin, DRG, and TG. P2X3R mRNA in the skin, cervical DRG, or TG of C57BL/6J mice (n = 3). Values represent the relative ratio of P2X3R mRNA (normalized to GAPDH mRNA value) to the value of the DRG. P2X3R mRNA expression levels in the skin were very low compared with those in the DRG ($<0.02\%$). *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase. Data are shown as means ± SEM.
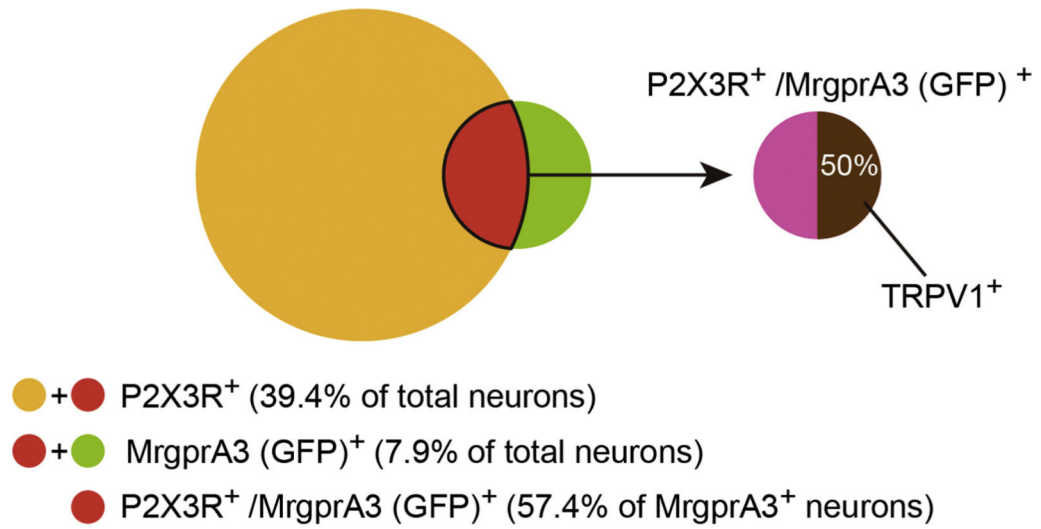
FIG E2.

Expression profiles of P2X3R and MrgprA3+ neurons. The Venn diagram illustrating the expression profiles of DRG neurons positive for P2X3R, MrgprA3, and/or TRPV1 in *MrgprA3Cre-GFP* mice. The sizes of the circles represent the proportion of each neuronal population.
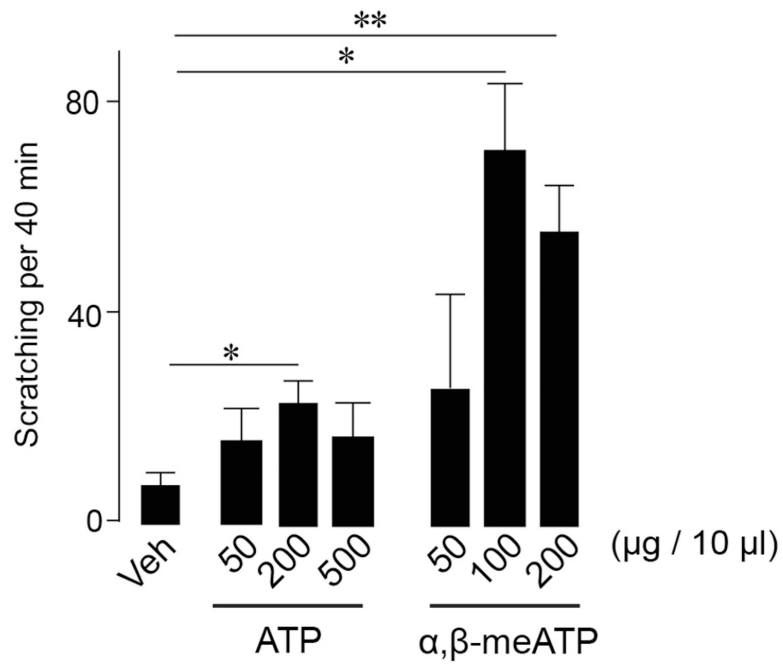
FIG E3.
Dose-dependent increase in scratching induced by P2X3R agonists. Dose-responses for increase in scratching after cheek injection of ATP or α,β-meATP (n = 4–6). *Veh*, Vehicle. Data are shown as means ± SEM. *$P <$ .05, unpaired *t* test; **$P <$ .01, Mann-Whitney *U* test.
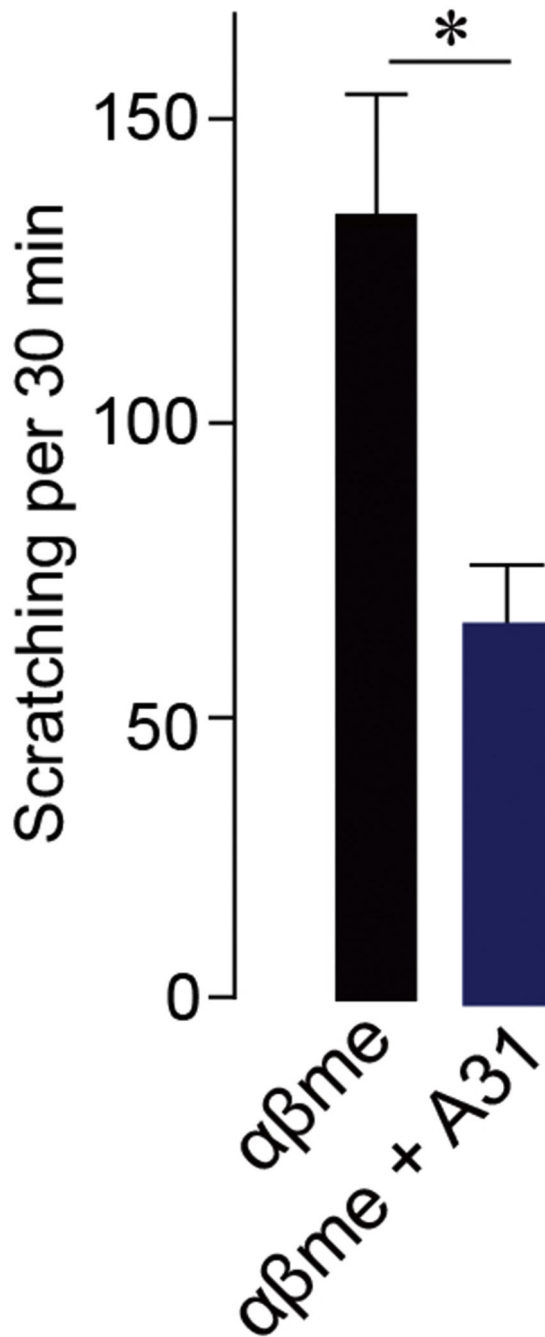
FIG E4.

Effect of A317491 on scratching caused by intradermal injection of α,β-meATP into the rostral back. α,β-meATP (αβme: 200 μg/50 μL) was intradermally injected into the rostral back with or without A317491 (A31: 200 μg/50 μL), a selective antagonist for P2X3Rs (n = 6). Scratching behavior was counted for 30 minutes after α,β-meATP injection. Data are shown as means ± SEM. *$P < .05$, unpaired t test.</_Caption>
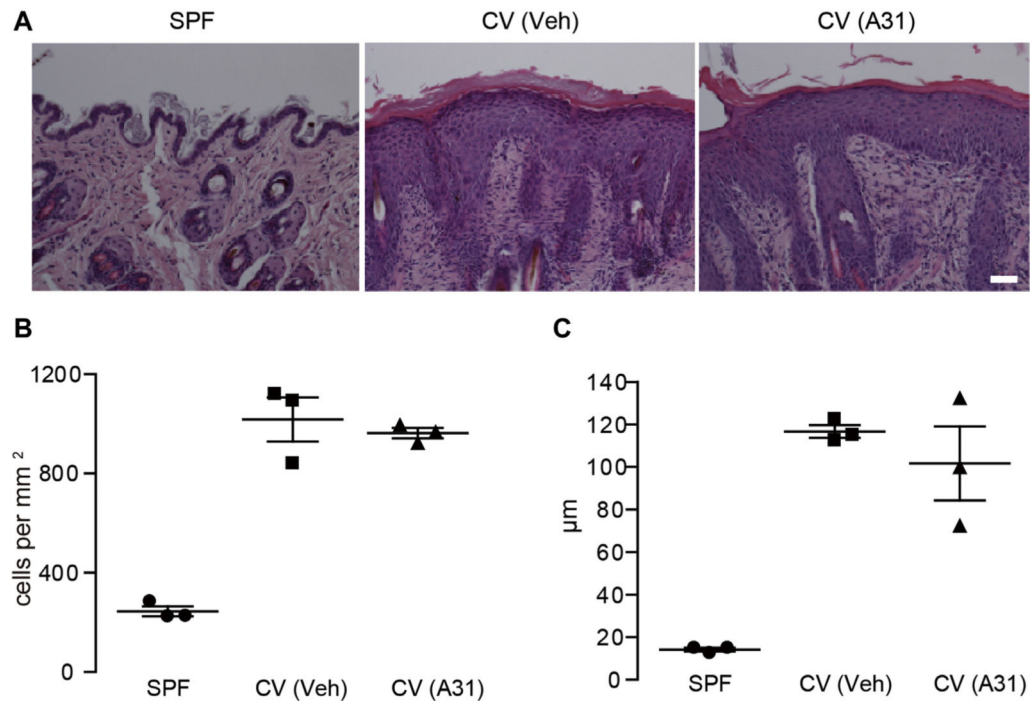
FIG E5.

Effect of A317491 on skin inflammation of NC/Nga mice. **A,** Photographs of hematoxylin-eosin–stained deparaffinized sections of the rostral back skin taken from SPF (control) and CV (chronic scratching)-NC/Nga mice 24 hours after intradermal administration of vehicle (Veh) or A317491 (A31). Scale bar, 50 μm. **B,** Number of inflammatory cells in the skin of SPF, CV (Veh), and CV (A31)-NC/Nga mice (n = 3). **C,** Epidermal thickness of SPF, CV (Veh), and CV (A31)-NC/Nga mice (n = 3). *SPF*, Specific pathogen free. Data are shown as means ± SEM.