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Sensory neuron expressed TRPC3 mediates acute and chronic itch

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Abstract

Chronic pruritus is a prominent symptom of allergic contact dermatitis (ACD) and represent a huge unmet health problem. However, its underlying cellular and molecular mechanisms remain largely unexplored. TRPC3 is highly expressed in primary sensory neurons and has been implicated in peripheral sensitization induced by proinflammatory mediators. Yet, the role of TRPC3 in acute and chronic itch is still not well defined. Here, we show that, among mouse trigeminal ganglion (TG) neurons, Trpc3 mRNA is predominantly expressed in nonpeptidergic small diameter TG neurons of mice. Moreover, Trpc3 mRNA signal was present in the majority of presumptively itch sensing neurons. TRPC3 agonism induced TG neuronal activation and acute nonhistaminergic itch- and pain-like behaviors in naïve mice. In addition, genetic deletion of Trpc3 attenuated acute itch evoked by certain common nonhistaminergic pruritogens, including endothelin-1 and SLIGRL-NH2. In a murine model of contact hypersensitivity (CHS), Trpc3 mRNA expression level and function were upregulated in the TG following CHS. Pharmacological inhibition and global knockout of Trpc3 significantly alleviated spontaneous scratching behaviors without affecting concurrent cutaneous inflammation in the CHS model. Furthermore, conditional deletion of Trpc3 in primary sensory neurons but not in keratinocytes produced similar antipruritic effects in this model. These findings suggest that TRPC3 expressed in primary sensory neurons may contribute to acute and chronic itch via a histamine independent mechanism and that targeting neuronal TRPC3 might benefit the treatment of chronic itch associated with ACD and other inflammatory skin disorders.

Keywords: TRPC3, itch, pain, primary sensory neurons, dermatitis

1. Introduction

Persistent pruritis is a major symptom of allergic contact dermatitis (ACD) with a profound impact on patients' quality of life[45]. Despite its prevalence, chronic itch is underappreciated and difficult to manage. Although antihistamines are the first choice for antipruritic treatments, many of them can produce severe side effects, such as drowsiness and dizziness. In addition, most chronic itch conditions are resistant to antihistamines, indicating the existence of nonhistaminergic itch mechanisms [31]. Yet, such mechanisms remain incompletely undefined.

TRPC3 is a member of the canonical subfamily of TRP channels (subtypes 1–7) [9]. Within sensory ganglia, TRPC3 is predominantly expressed in a subset of nonpeptidergic (NP) small diameter primary sensory neurons [6; 9]. Single cell RNAseq analysis has identified that the *Trpc3* gene is expressed across all three distinct populations of itch sensory neurons: NP1 [which expess Mas-related G protein–coupled receptor D (MrgprD)]; NP2 (which express MrgprA3); and NP3 [which express the peptides natriuretic peptide B (Nppb) and somatostatin (Sst)] [19; 50], indicating a potential role of TRPC3 in itch processing. However, this notion is challenged by recent findings that genetic deletion and local pharmacological blockade of TRPC3 did not alter MrgprD- or MrgprA3-mediated acute itch, respectively[6; 41]. Double knockout of *Trpc3/6* had no significant effects on CQ- evoked cutaneous sensory fiber firing [39]. Although these studies failed to establish a clear role of TRPC3 in acute itch elicited by CQ or the MrgprD agonist β-alanine[6; 41], it is unclear whether activation of TPRC3 alone induces acute itch. In addition, the role of TRPC3 in chronic itch in inflammatory skin disorders remains controversial. Pharmacological inhibition of TRPC3 was shown to alleviate chronic itch in a mouse model of

contact dermatitis [43]. A more recent study found that deletion of *Trpc3* increased scratching responses in mice with contact dermatitis induced by squaric acid dibutylester (SADBE) [4]. Unlike the published standard SADBE-induced dermatitis model [10; 13], this study remarkably prolonged SDABE challenge which may cause different immune responses and pathology in the inflamed skin and peripheral neural circuits.

Pruritogens are thought to induce itch by exciting sensory neurons largely via activation of Gq protein-coupled receptors (GPCRs) [48]. TRPC3 channels are commonly activated by Gq protein coupled GPCRs and serve as a versatile downstream effector in multiple signaling pathways, including those activated by endotherlin-1(ET-1), ATP and proteinase [2; 34; 47]. Given that many of these mediators and their receptors are key players in nonhistaminergic itch [1; 12; 42], it is conceivable that TRPC3 might function as an integrator of these itch signaling pathways. In addition, TRPC3 was shown to be functionally coupled to receptors for multiple inflammatory mediators that trigger peripheral sensitization in sensory neurons [2]. Our previous study identified TRPC3 as a key downstream transduction channel of IgG immune complex (IgG-IC)/FcγRI signaling in sensory neurons, suggesting that TRPC3 might be involved in sensory symptoms associated with inflammatory diseases [38]. Therefore, we hypothesized that TRPC3, particularly in primary sensory neurons, contributes to both acute and chronic itch.

2. Material and methods

2.1. Animals

Adult mice of both genders that were 2 to 4 months old and 20 to 30 g weight were used for all the experiments. Animals were housed under a 12-hour light/12-hour dark cycle with ad libitum access to food and water. The breeders of global *Trpc3* knockout and *Trpc3*$^{fl/fl}$ mice were

provided by Drs. Barbara Miller (Pennsylvania State University) and Lutz Birnbaumer (National Institute of Environmental Health Sciences), respectively. Conditional deletion of *Trpc3* in primary sensory neurons or in keratinocytes was achieved by crossing *Trpc3*$^{fl/fl}$ mice with a *PirtCre* mouse line [18] or a K14Cre mouse line (Stock No: 018964; Jackson lab). All mice were maintained on a C57BL/6 background. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Johns Hopkins School of Medicine and were consistent with the guidelines provided by the National Institute of Health and the International Association for the Study of Pain.

2.2. RNAScope in situ hybridization and immunohistochemistry

Mice were deeply anaesthetized with 20% urethane and transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Mouse trigeminal ganglia (TG) were harvested and postfixed for 2 hrs in 4% PFA at 4 °C. TG were subsequently washed three times with PBS, followed by cryopreservation in 30% sucrose overnight at 4 °C. Tissues were then embedded in Tissue-Tek OCT compound (Sakura Finatek, Torrance, CA) and cryosectioned at 12 µm. In situ hybridization (RNAScope) was performed using the Multiplex Fluorescent Kit v.2. [Advanced Cell Diagnostics (ACD), Newark, CA] according to the manufacturer's instructions. Pre-hybridization, hybridization and washing were performed according to standard protocols as described previously[21].The probes were designed by the manufacturer and available from ACD (Newark, CA). The following probes were used in this study: Mm-Trpc3-C1 (#572151), Mm-Trpa1-C2 (#400211-C2), Mm-Nppb-C3 (#42502-C3), and Mm-Sst-C2 (#404631-C2). A negative control probe against the bacterial DapB gene (cat#700141, ACD, Newark, CA) was used to check for non-specific/background signals. The RNAScope signal was visualized by using Opal 570 or 690 (Akoya Biosciences, Marlborough,

MA). Following the completion of the RNAScope protocol, immunohistochemistry was performed. After blocking with 10% donkey serum in PBST (0.1% Triton X-100 in PBS) for 1 hr at room temperature, sections were incubated overnight at 4^{o}C with the following primary antibodies: chicken anti-NeuN (1:200; Aves); rabbit anti-CGRP (1:500; Immunostar); sheep anti-TH (1:1000; Millipore); rabbit anti-GS (1:1000; Abcam); and guinea pig anti-P2X3 receptor (1:1000; Millipore). Sections were then washed three times and incubated with the following complementary secondary antibodies: Donkey anti-rabbit IgG Cy3 or Alexa 647 (1:500; Jackson ImmunoResearch); Donkey anti-sheep IgG Alexa 647 (1:500; Jackson ImmunoResearch); Donkey anti-Guinea pig IgG Alexa 488 (1:500; Jackson ImmunoResearch); and Donkey anti-chicken IgG Alexa 488 (1:500; Jackson ImmunoResearch) at RT for 1 hr. For IB4 staining, sections were incubated with Alexa Fluor 647-conjugated isolectin B4 (IB4; 1:200; Invitrogen) along with other secondary antibodies. To visualize MrgprA3^{+} TG neurons, TG tissues from MrgprA3$^{eGFP\text{-}Cre}$ mice [14; 22] were stained with chicken anti-GFP (1:1000; Aves) followed by Donkey anti-chicken IgG Alexa Fluor 488 (1:500; Jackson ImmunoResearch). Images were captured using a confocal microscope (Nikon A1+, Melville, NY). Three sections per TG at different depth were analyzed in a blinded manner using NIS-Elements software (Nikon, Melville, NY). A semi-quantitative scoring system provided by the manufacturer (ACD, Newark, CA) was used to analyze RNAScope signals (Score 0: no staining or <1 punctum/10 cells; Score 1: 1–3 puncta/cell; Score 2: 4–9 puncta/cell and none or very few dot clusters; Score 3: 10–15 puncta/cell and/or <10% dots in clusters; Score 4: >15 puncta/cell and/or >10% puncta in clusters). For the expression of a given gene (e.g., *Trpc3* mRNA), positive signals were defined as puncta or clusters present around the nucleus and/or cytoplasm of a single neuron with a score ≥2. For the coexpression of *Trpc3* and other genes (i.e., *Trpa1*, *Sst*, and *Nppb*), puncta clusters of

C1 and C2/C3 channels associated within a TG cell body were considered as double positive, whereas neurons with only C1 or C2/C3 puncta clusters were counted as single positive [3].

2.3. Culture of dissociated mouse TG neurons

Mouse TG neurons were isolated and cultured as previously described [37]. Briefly, mouse TGs were removed quickly and transferred to ice-cold complete saline solution (CSS) for cleaning and mincing. The CSS contained (in mM): NaCl 137, KCl 5.3, $MgCl_2$ 1, $CaCl_2$ 3, Sorbitol 25, and HEPES 10, adjusted to pH 7.2 with NaOH. Mouse TGs were then digested with Liberase TM (0.35U/ml; Roche, Mannheim, Germany) for 20 minutes, and for another 15 minutes with Liberase TL (0.25 U/ml; Roche, Mannheim, Germany) and papain (30 U/ml; Worthington, Lakewood, NJ) in CSS containing 0.5 mM EDTA (Invitrogen, Vilnius, Lithuania) at 37°C. After enzymatic digestion, cells were dissociated by gentle trituration in a cultured medium containing 1 mg/ml bovine serum albumin (Sigma, St Louis, MO) and 1 mg/ml trypsin inhibitor (Sigma, St Louis, MO), and placed on poly-D-lysine (100 µg/ml; Sigma, St Louis, MO)/laminin (80 µg/ml; Sigma, St Louis, MO) coated glass coverslips. The culture medium contained equal amounts of DMEM and F12 (Gibco, Grand Island, NY) with 10% heat-inactivated FBS (Hyclone, Marlborough, MA), 1% penicillin and streptomycin (Gibco, Grand Island, NY) and nerve growth factor (50 ng/ml; Sigma, St Louis, MO). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2 and used within 16-24 hrs.

2.4. Calcium imaging of cultured TG neurons

Mouse TG neurons were loaded with Fura-2-acetoxymethyl ester (Fura-2 AM; 1 µM; Invitrogen, Vilnius, Lithuania) in the dark for 30 minutes at 37°C. After loading, TG neurons were washed in HEPES buffer to remove extracellular dye and placed in a recording chamber continuously perfused with HEPES buffer at a flow rate of 1 ml/min at room temperature. The HEPES buffer

contained (in mM): NaCl 145, KCl 3, $MgCl_2$ 2, $CaCl_2$ 2, glucose 10 and HEPES 10 (adjusted to pH 7.4 with NaOH). Ratiometric calcium imaging was performed at room temperature (20–22°C) using an invert microscope (Nikon ECLIPSE TE200; Melville, NY). TG neurons were alternatively excited at 340 and 380 nm and images were recorded at 2-s intervals using a cooled CCD camera (DC-152Q-C00-FI; ANDOR, Belfast, Northern Ireland) controlled by a computer with NIS-Elements AR software (Nikon, Melville, NY). The ratio of 340 nm to 380 nm fluorescence intensity [$R_{(340/380)}$] within a certain region of interest was used as a relative measure of the intracellular concentration of calcium ($[Ca^{2+}]_i$). At the end of the experiment, the viability of the neurons was confirmed by an increase in $[Ca^{2+}]_i$ evoked by a 20-s application of 50 mM KCl. Neurons were considered to be responsive to a chemical if an increase in R340/380 was equal or greater than 15% above baseline [53]. GSK1702934A (GSK, FOCUS biomolecules, PA or TOCRIS, Bristol, UK) was dissolved in 100% ethanol and then diluted to the concentration of 0.1, 1, 10 and 100 µM. The dose of other chemicals was chosen based on previous studies [25; 37]: 1 mM Chloroquine (CQ; Sigma, St Louis, MO), 100 µM allyl isothiocyanate (AITC; Sigma, St Louis, MO), and 1 µM capsaicin (Cap; Sigma, St Louis, MO).

2.5. Mouse model of contact hypersensitivity (CHS)

CHS was induced using the contact sensitizer SADBE (Tokyo Chemicals Inc., Japan) or 1-Chloro-2,4-dinitrobeneze (DNCB, Acros Organics, Belgium, NJ) as reported previously [29; 33]. DNCB induced CHS was used only for pharmacological experiments. Briefly, mice were sensitized by topical application of 1% SADBE (25 µl in acetone) or 2% DNCB (50 µl in acetone) onto the abdominal skin once daily for three consecutive days. Five days later, mice were challenged with 1% SADBE or 1% DNCB (25 µl in acetone) topically applied, either to the right ear (for behavioral tests) or cheek (for in vitro studies) once daily for three consecutive days.

Separate groups of mice were sensitized and challenged with acetone alone and served as controls. For pharmacological experiments, TRPC3 antagonist Pyr3 (0.1 mg/kg, 100 µl; TOCRIS, Bristol, UK) or vehicle (0.1% DMSO in saline) was administered intraperitoneally (i.p.) once daily starting on the day of the 1st challenge for six consecutive days.

2.6. Behavioral testing

For behavioral measurements, the experimenters were blinded to genotypes and treatments. Animals were habituated to the behavioral testing apparatus for 1 h once daily for three consecutive days before the testing day.

For the cheek model of acute itch, the fur on the cheek was shaved 3 days before testing. On the day of the experiment, 10 µl of GSK at different doses (5, 10, and 20 µg) was subcutaneously (s.c.) injected into the left cheek of awake naïve mice. Control animals received the equivalent volume of the vehicle (20% HPCD). Immediately after injections, the mouse was placed in the recording chamber and video recorded for 30 mins. The total number of spontaneous scratching with the hind paw and wiping with the forepaw were quantified in a blinded manner. A bout of scratching was defined as a hind paw scratching directed at the area of injection, and a bout of wiping was defined as a single forepaw stroking the injection site. In some experiments, either histamine receptor 1 (H1R) antagonist, cetirizine (30 mg/kg, 100 µl, TOCRIS, Bristol, UK) or vehicle (saline; 100 µl) was administered i.p. 30 min before GSK was injected into the cheek.

For the neck model of acute itch, the fur on the rostral back was shaved 3 days before behavioral testing. Mice were administered with an intradermal (i.d.) injections of 50 µl of the following pruritogens into the nape: histamine (200 µg), PAR2 agonist SLIGRL-NH2 (100 µg), ET-1 (25 ng), and α-Me-5-HT (20 µg). All agents were dissolved in saline. Scratching responses

were video recorded for 30 min and subsequently quantified by observers blinded to genotype and treatment.

For the CHS model, site-directed spontaneous scratching was videoed for 30 min on day 3 after the 3rd challenge and the total number of scratches was counted by observers blinded to treatments and genotypes. Mouse ear thickness was measured three times with a digital caliper before and after each challenge.

2.7. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

TG, DRG, thalamus, cerebellum, and ear tissues from either control or CHS mice were collected at day 3 after the 3rd challenge. Total RNA was extracted using the RNeasy lipid tissue mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's suggestions and then reverse-transcribed to complimentary DNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time PCR was performed on a real-time PCR system (QuantStudio 3; Applied Biosystems Corp., Beverly Hills, CA) using the PowerUp SYBR Green Master Mix (Applied Biosystems, Beverly Hills, CA). The primers used are summarized in Supplemental Table 1 (available at http://links.lww.com/PAIN/B634). Each sample was performed in duplicate. The expression levels of the target genes were quantified relative to the level of β-actin (*Actb*) gene expression using the $2^{-\Delta\Delta CT}$ method.

2.8. Skin histology

Mouse ears were collected on day 3 after the 3rd challenge, post-fixed in 4% paraformaldehyde for 24 hours, and dehydrated in ethanol and then embedded in paraffin. Sections were cut at a thickness of 5 µm and stained with hematoxylin & eosin (H&E). Three sections at different depth from each mouse were chosen for analysis. Ear epidermal thickness was evaluated by observers blinded to treatments and genotypes using ImageJ software (NIH).

2.9. Cell preparation and flow cytometry

On day 3 after the 3rd challenge with either acetone or SADBE, the ear tissue was excised using a 4 mm biopsy punch (Miltex, Inc., York, PA), cut into small pieces and placed in RPM1640 media containing 1.25 mg/ml Dispase II (Sigma, St Louis, MO), 2 mg/ml Collagenase II (Gibco, Grand Island, NY) and 2 mg/ml Collagenase IV (Gibco, Grand Island, NY). The ear tissue was incubated at 37°C for ~75 min. The digested ear tissues were passed through a 70 µm cell strainer, pelleted and washed in phosphate buffered saline. Cells were then stained with Aqua Live/Dead viability dye (Biolegend, San Diego, CA) to exclude non-viable cells. Cells were treated with CD16/CD32 Fc block (1:100; eBioscience, Santa Clara, CA) for 10 min prior to staining with CD45-FITC (1:100, Biolegend, San Diego, CA). Data were acquired with a CytoFLEX LX (Beckman Coulter, Indianapolis, IN) flow cytometer, and analyzed using FlowJo v10 software (TreeStar).

2.10. Measurement of extravasation of Evans Blue in the skin

Evans Blue dye (1.5 mg/ml; 200 µl in saline; Sigma) was injected retro-orbitally to adult mice anesthetized with ketamine/xylazine (i.p; ketamine 100 mg/kg; xylazine 10 mg/kg). Five minutes later, GSK (20 µg, 10 µl in 20% HPCD) and vehicle (20% HPCD; 10 µl) were injected s.c. into each mouse hind paws, respectively. Substance P (SP; 50 µM, 10 µl in saline) served as a positive control. Photographs were taken 15 minutes after injection. Mice were then euthanized by CO_2 inhalation, paw tissue was collected, dried for 24 h at 50°C, and weighed. The Evans Blue dye was extracted from the tissue by a 24 h incubation in 1 ml of formamide at 50°C, and the OD was read at 600 nm with the correction wavelength at 740 nm using a FlexStation3 plate reader (Molecular Devices) [28]. ***2.11. Statistical analysis***

Data are presented as means ± SEM. Sample sizes were chosen based on our pilot experiments and field conventions to accurately detect statistical significance and considering ethical animal use, experiment design, resource availability, and technical feasibility. Comparisons of proportions were made using Chi-square test. A two-tailed Student's t test was used to test the significance of differences between two groups. Comparisons for multiple groups or multiple time points were carried out using a one-way or two-way ANOVA for random measures, or repeated measures followed by Bonferroni's post hoc test comparisons. P value less than 0.05 was considered significant. The types of statistical tests used for each comparison are noted in each figure legend.

3. Results

3.1. TRPC3 is expressed in a subset of mouse TG neurons

To define the expression profile of TRPC3 in primary sensory neurons, we performed RNAScope in situ hybridization on mouse TGs using commercial *Trpc3* RNA probes. *Trpc3* mRNA signal was expressed in 37.2 ± 2.5% of TG neurons, the majority of which were small-sized neurons (Fig. 1A, B). The specificity of *Trpc3* mRNA detection was validated by a loss of RNAScope signal in wildtype TG sections stained with a negative probe (Fig. 1C). To further map the expression pattern of TRPC3 in TG neurons, we performed double staining for *Trpc3* mRNA and markers of different cell populations (Fig. 1D-E). *Trpc3* mRNA signal was highly co-expressed with IB4 (a maker for non-peptidergic neurons) but had a little overlap with CGRP (a marker for peptidergic neurons). However, we did not observe obvious colocalization of *Trpc3* mRNA signal with tyrosine hydroxylase (TH; a marker for C-type low-threshold mechanoreceptors) or glutamine synthetase (GS; a satellite glial cell marker).

Since TRPA1 and P2X3 receptor (P2X3R) are considered as itch related receptors [42; 53; 54], we asked whether TRPC3 is colocalized with these receptors. Approximately 50% of $Trpa1^{+}$ or $P2X3R^{+}$ neurons coexpressed *Trpc3* mRNA signal (Fig. 2A, B). Given that MrgprA3, Nppb and SST are exclusively expressed in small-diameter neurons and sensory neurons that express these markers are critical to transmit itch signals [14; 16; 30], we next sought to assess whether TRPC3 is expressed in these itch-specific neurons. As expected, the majority of Sst^{+}, or all $Nppb^{+}$ and $MrgprA3^{GFP+}$ neurons coexpressed *Trpc3* mRNA signal (Fig. 2C-E). These results provide an anatomical basis for a potential role of TRPC3 in itch sensation.

3.2. TRPC3 agonism directly activates mouse TG neurons

We next performed ratiometric Ca^{2+} imaging to determine whether TRPC3 is functional in mouse TG neurons. TRPC3 agonist GSK1702934A (GSK) but not vehicle caused a significant increase in $R_{340/380}$ in a subset of TG neurons from $Trpc3^{+/+}$ mice in a dose dependent manner (Fig. 3A-G). Such effect was significantly diminished in global $Trpc3^{-/-}$ mice (Fig. 3G). Since the majority of pruritogen receptors are usually coupled to TRPV1 or TRPA1 [17; 53], we further determined whether GSK -responsive neurons overlapped with these two cell populations . We found that 55.9% and 86.1% of GSK-responsive neurons also responds to the TRPV1 agonist capsaicin and the TRPA1 agonist AITC, respectively (Fig. 3H). In addition, about half of GSK-responsive neurons were sensitive to CQ, an agonist for MrgprA3. These data suggest that functional TRPC3 is expressed in a heterogenous subset of primary sensory neurons that may play distinct roles in itch processing.

3.3. TRPC3 activation elicits acute non-histaminergic itch

Given that TRPC3 was expressed in a subpopulation of nociceptors and pruriceptors [6; 7], we next asked whether activation of peripheral TRPC3 by GSK is sufficient to trigger itch and/or

pain behaviors in the naïve state. To test this possibility, we used a mouse cheek model of itch that allows us to distinguish pain versus itch, as indicated by distinct pain-like wiping by forelimbs and itch-like scratching by the hind limbs (Fig. 4) [36]. In naïve mice, subcutaneous (s.c.) injection of GSK into the cheek of wildtype mice significantly increased the number of site-directed scratching bouts in a dose-dependent manner, compared to vehicle (Fig. 4A-C). Notably, mice injected with GSK (20 µg; 10 µl) scratched immediately and intensively for 15 min (Fig. 4C). In addition, s.c. injection of GSK also evoked a significant greater number of bouts of wiping than vehicle (Fig. 4B). However, there were no significant differences in the number of wiping bouts evoked by GSK among doses. The peak time of GSK induced wiping responses occurred within the first 5 min after the injection (Fig. 4D). To further examine whether GSK induced scratching behavior is histamine-dependent, H1R antagonist cetirizine (Ctz) or vehicle was administered i.p. 30 min before i.d. injection of GSK to the mouse cheek. However, no significant differences were observed in GSK-induced scratching behaviors between treatments, suggesting that GSK-evoked itch is non-histaminergic (Fig. 4E).

Since GSK was shown to activate both TRPC3 and TRPC6[49; 55], we further assessed whether TRPC3 is necessary for pruriceptive and pronociceptive effects of GSK using global *Trpc3*$^{-/-}$ mice. GSK-induced scratching and wiping responses observed in *Trpc3*$^{+/+}$ mice was markedly diminished in global *Trpc3*$^{-/-}$ mice, indicating the involvement of TRPC3 in this process (Fig. 4F, G). Overall, these data indicated that activation of TRPC3 by GSK is sufficient to evoke both acute itch and pain behaviors.

To determine whether GSK induces edema and inflammation, we investigated the effects of GSK on vascular permeability in the hind paw of wildtype mice using Evans Blue dye. Intraplantar injection of either GSK or a positive control, Substance P, induced obvious

extravasation of Evans Blue in the hind paw of wildtype mice compared to vehicle (Supplemental Figure 1A, B, available at http://links.lww.com/PAIN/B634). To further examine whether sensory neuron expressed TRPC3 is involved in this process, we compared GSK-induced extravasation of Evans Blue between wildtype and sensory neuron specific *Trpc3* knockout mice. This mouse line was achieved by crossing $Trpc3^{fl/fl}$ mice with a *PirtCre* mouse line ($PirtCre::Trpc3^{fl/fl}$; Supplemental Figure 1C, available at http://links.lww.com/PAIN/B634). qRT-PCR analysis in $PirtCre::Trpc3^{fl/fl}$ mice confirmed that the decrease of *Trpc3* mRNA expression specifically occurred in the TG and DRG but not in the thalamus or cerebellum (Supplemental Figure 1D, available at http://links.lww.com/PAIN/B634). There were no significant differences in Evans Blue extravasation in the hind paw evoked by GSK between $PirtCre::Trpc3^{fl/fl}$ and *PirtCre* negative control littermates (Supplemental Figure 1E, F, available at http://links.lww.com/PAIN/B634). This result suggests that GSK is able to induce edema and inflammation, but that it does so independently of neuronal TRPC3.

3.4. TRPC3 mediates acute itch evoked by non-histaminergic pruritogens

To further explore a potential role of TRPC3 in itch sensation, we next set out to evaluate scratching behaviors in wildtype and global $Trpc3^{-/-}$ mice after i.d injection of histaminergic or non-histaminergic pruritogens into the nape of the neck. Compared with wildtype animals, global $Trpc3^{-/-}$ mice exhibited a significant reduction in scratching behaviors evoked by histaminergic-independent pruritogens, including endothelin-1(ET-1), the PAR2 agonist SLIGRL-NH_2 (Fig. 5A, B). However, we did not observe obvious differences in scratching responses elicited by histamine and the serotonin receptor 2B agonist α-Me-5-HT between genotypes (Fig. 5C, D). We next asked whether neuronally expressed TRPC3 is responsible for acute itch evoked by these two nonhistaminergic pruritogens using $PirtCre::Trpc3^{fl/fl}$ mice. Specific deletion of *Trpc3* in

primary sensory neurons attenuated scratching behaviors evoked by both pruritogens (Fig. 5E, F), suggesting that TRPC3 may serve as a pruritogenic effector for those itch signaling pathways.

3.5. Genetic deletion and pharmacological inhibition of TRPC3 attenuates allergic itch in the CHS model

Given that TRPC3 has been implicated in allergic diseases[51; 52], we next asked whether TRPC3 plays a role in allergic itch associated with CHS, a mouse model of ACD, induced by SADBE (Fig. 6A). On day 3 after the 3rd challenge, wildtype mice subjected to SADBE challenge displayed more site-directed spontaneous scratching behaviors on the ear than acetone-treated control animals (Fig. 6B). However, global *Trpc3*$^{-/-}$ mice scratched much less than wildtype littermates during CHS (Fig. 6B). To further dissect the contributions of neuronal and non-neuronal TRPC3 to chronic itch, we genetically deleted *Trpc3* expression in one or more selected cell populations before the induction of dermatitis, including: *K14Cre::Trpc3*$^{fl/fl}$ (to target keratinocytes) and *PirtCre::Trpc3*$^{fl/fl}$ (to target peripheral sensory neurons). In the context of CHS, *PirtCre::Trpc3*$^{fl/fl}$ mice showed significantly reduced spontaneous scratching responses compared to *PirtCre* negative controls. (Fig. 6C). However, no significant differences in spontaneous scratching responses were observed between wildtype and *K14Cre::Trpc3*$^{fl/fl}$ mice (Fig. 6D). These results support the notion that TRPC3 expressed in sensory neurons but not keratinocytes mediates inflammatory itch associated with CHS. To circumvent potential confounds of genetic deletion of *Trpc3*, we explored whether acute pharmacological blockade of TRPC3 attenuated allergic itch following CHS. In mice with CHS, systemic administration of Pyr3 but not vehicle significantly attenuated spontaneous scratch responses (Fig. 6E).

3.6. TRPC3 is not involved in skin inflammation in the CHS model

Given that TRPC3 is widely expressed in immune cells and acts as a key player in the regulation of immunity [8; 15; 46], we assessed whether diminished scratching responses in *Trpc3*$^{-/-}$ mice are secondary to reduced skin inflammation. Interestingly, we did not observe obvious differences between genotypes in ear swelling over time (Fig. 7A) or in epidermal thickening as analyzed by H&E staining on day 3 after the 3rd challenge (Fig. 7B, C). In addition, qPCR revealed an upregulation of mRNAs encoding a number of proinflammatory markers (e.g. *Il-6*, *Il-1b*, *cxcl1*, and *cxcl10*) in mouse ear tissues on day 3 after the 3rd SADBE challenge compare to vehicle controls (Fig. 7D). However, these changes were not significantly different between genotypes (Fig. 7D). We next performed flow cytometry to compare immune cell infiltration in the ear skin between wildtype and global *Trpc3*$^{-/-}$ mice. Compared to vehicle, SADBE treatment caused an enrichment of immune cells in the ear as measured by CD45^{+} cell percentage on day 3 after the 3rd challenge. However, we did not observe obvious differences in CD45^{+} cell percentage between genotypes (Fig. 7E, F).

3.7. CHS upregulates TRPC3 signaling in TG neurons

We performed qPCR on TG tissues from wildtype mice to assay for alterations in *Trpc3* mRNA expression level following CHS. On day 3 after the 3rd challenge, the mean expression level of *Trpc3* mRNA in the TG was significantly greater in mice with CHS than in vehicle-treated mice (Fig. 8A). To determine whether the function of neuronal TRPC3 is upregulated in the setting of CHS, we compared Ca^{2+} responses evoked by GSK in TG neurons from control versus CHS mice on day 3 after the 3rd challenge. The percentage of GSK responsive neurons was significantly greater in CHS mice compared to that in control animals (Fig. 8B-D), Among GSK responsive TG neurons, we observed larger proportions of CQ- and Cap-responding neurons in CHS mice compared to those in control animals (Fig. 8E).

4. Discussion

In this study, we provide novel evidence that activation of TRPC3 in sensory neurons is sufficient to trigger acute histamine-independent itch in naïve states. TRPC3 is also involved in acute itch evoked by certain types of nonhistaminergic pruritogens. In addition, neuronal TRPC3 contributes to spontaneous itch independent of skin inflammation in the context of CHS.

TRPC3 is predominantly expressed in nonpeptidergic small diameter sensory neurons, including MrgprA3^{+} and MrgprD^{+} itch-mediating neurons[6]. Although previous studies failed to establish that TRPC3 was involved in MrgprA3 and MrgprD mediated acute itch[6; 40], little is known about whether activation of TRPC3 itself induces acute itch and whether neuronal TRPC3 contributes to chronic itch in the context of dermatitis. In this study, we provide several complementary lines of evidence that support a potential role of peripheral neuronal TRPC3 in itch processing. First, RNAScope revealed that *Trpc3* mRNA is coexpressed with certain itch related receptors, such as TRPA1 and P2X3R. Also, *Trpc3* mRNA signal is present in the majority of NP3 itch sensory neurons (Nppb+ and Sst+ neurons), consistent with previous single cell RNAseq data[50]. In addition, RNAseq analysis on human tissues has revealed TRPC3 expression in human TG and DRG [11; 32]. Moreover, *Trpc3* mRNA transcripts were coexpressed with itch-related transcripts in human DRG neurons[32]. These findings provide an anatomical basis for a potential role of TRPC3 in itch sensation. Second, the TRPC3 agonist GSK directly induced sensory neuron activation. In agreement with above anatomical observations, GSK responsive neurons also overlapped with the neurons that responded to the agonists for MrgprA3 and TRPA1. Third, in naïve mice, exogenous administration of GSK evoked scratching responses in the cheek of the mouse. Such effects were diminished in mice

lacking *Trpc3*, suggesting the involvement of TRPC3. In addition, GSK-evoked scratching responses were likely independent of histamine since an H1 receptor antagonist failed to inhibit this effect. Given the time window of rapid modulation of neuronal activity by ligand (minutes to hours), these transient changes in itch behaviors evoked by GSK are likely mediated by neuronal activation. Given that TRPC3 and TRPC6 have similar structures, there are no commercial specific TRPC3 agonists available. Thus, we cannot completely rule out the additional involvement of TRPC6 in neuronal and/or behavioral effects of GSK. Further experiments are needed to test this possibility using available *Trpc6*$^{-/-}$ mice. Fourth, TRPC3 is involved in certain forms of histamine-independent itch elicited by common pruritogens, including ET-1 and SLIGRL-NH_2. Given that TRPC3 channels are commonly activated by Gq protein-coupled GPCRs[2; 34; 47], it is possible that TRPC3 acts as a downstream target of signaling pathways activated by ET-1 and SLIGRL-NH_2. Indeed, TRPC3 was reported to be functionally coupled to receptors for these two molecules[2; 34].

A previous study reported that intrathecally delivered pharmacological inhibition of TRPC3 alleviated chronic itch in mice with contact dermatitis induced by diphenylcyclopropenone, possibly through effects on spinal microglia [43]. We have complemented these findings by showing that global deletion and conditional deletion of *Trpc3* in primary sensory neurons each significantly alleviated spontaneous itch in the CHS model. Other than sensory neurons, many other cell types express TRPC3, including keratinocytes[8; 15; 20]. Yet, genetic deletion of *Trpc3* in keratinocytes failed to attenuate chronic itch in the setting of CHS, suggesting that TRPC3 expressed in keratinocytes is not required for allergic itch.

Unlike another recent study [4], we observed that deletion of *Trpc3* attenuated rather than enhanced scratching responses in mice with SADBE-induced contact dermatitis. This

discrepancy might be explained by different procedures for SADBE challenge. Our present study strictly followed a published procedure in which sensitized mice were challenged by topical application of SADBE for three consecutive days [10; 13]. By contrast, the other study involved longer (i.e., 5 days) SADBE challenge, which might have evoked different inflammatory processes and pathology in the inflamed skin and peripheral neural circuits [4]. In addition, observation time points and affected skin areas differed between our studies. In the other study, the investigators also found that the somata and central terminals of MrgprD neurons were lost in *Trpc3* $^{-/-}$ mice after SADBE challenge[4]. They also reported that both peptidergic and MrgprD epidermal nerve fibers were lost in the SADBE model regardless of genotype, though there was no difference between wildtype and Trpc3$^{-/-}$ mice in dermal MrgprD nerve fiber density[4]. Given that MrgprD neurons are reportedly critical for itch transmission [24], the loss of MrgprD neurons might have been expected to reduce pruriceptive input and thus diminish, rather than increase itch responses. Indeed, in our previous in vivo and in vitro studies, MrgprD neurons became hyperexcitable following SADBE challenge [36]. However, loss of epidermal innervation or ablation of MrgprD neurons increases mast cell degranulation and cutaneous inflammation [56]. The other study proposed that this effect might explain the enhanced scratching responses observed in *Trpc3*$^{-/-}$ mice during dermatitis [4], although this interpretation appears inconsistent with the apparently similar loss of epidermal MrgprD fibers between genotypes [4]. Our present study found that knockout of *Trpc3* did not alter skin inflammation in the CHS model. Another potentially important difference between studies was the use of different Cre mouse lines for the generation of conditional TRPC3 knockout mice. We employed *PirtCre* mice to genetically omit TRPC3 expression in almost all primary sensory neurons whereas the *Trpv1Cre* mouse line was used in the other study [4]. It should be noted that TRPC3

is expression is low in TRPV1 expressing adult sensory neurons [6]. Although the entire TRPV1 lineage is targeted in *TRPV1Cre* mice, potentially including nonpeptidergic MrgprD expressing neurons, TRPC3 deletion in this population of neurons might be less efficient in *Trpv1Cre::Trpc3*$^{fl/fl}$ mice than in *PirtCre::Trpc3*$^{fl/fl}$ mice. In addition to sensory neurons, TRPV1 is expressed in various types of non-neuronal cells (i.e., immune cells, astrocytes and microglia) [44]. These genetic differences may contribute to the apparent discrepancies between studies. All mice used in present study were on a C57 background. It is unclear whether the same strain was used in the other study.

Given that TRPC3 is expressed in a heterogenous subpopulation of nociceptors and pruriceptors [6; 7], TRPC3 likely signals both itch and pain sensations. In support of this notion, GSK induces both pain and itch in the cheek model. Since GSK injection may activate multiple cell types, including neurons, keratinocytes and immune cells, there are multiple pathways potentially activated by GSK. We also cannot conclude whether our data support a labeled line or intensity theory of pain and itch coding. Regardless, a lack of specificity for sensory modality does not weaken the potential importance of TRPC3 in itch sensation. Indeed, many receptors or ion channels (e.g., histamine receptors, endothelin receptors, TRPV1 and TRPA1) that are involved in itch transduction also play pivotal roles in pain processing [26]. In addition, even the same cell population (e.g., MrgprA3^{+} neurons) can encode pain or itch in response to different stimuli [41].

Our study also provides transcriptional and functional evidence that supports the enhancement of Trpc3 signaling within TG after the development of CHS. First, qRT-PCR revealed an upregulation of *Trpc3* mRNA expression in the mouse TG following CHS. Second, calcium imaging experiments clearly demonstrated a considerable degree of functional TRPC3

upregulation in TG neurons following CHS. A larger proportion of dissociated TG neurons became responsive to GSK after CHS. The mechanisms by which CHS induces the upregulation of TRPC3 signaling in the cell bodies of sensory neurons require further study. They might include the activation of a signaling cascade of inflammatory cytokines (i.e., IL-1β) in the context of skin inflammation, which upregulates TRPC3 expression [5]. Also, certain inflammatory cytokines are able to sensitize neuronal TRPC3 and enhance its functional activity [2; 38]. Neuroinflammation within the TG may also upregulate TRPC3 expression and function in non-cutaneous TG neurons.

In this study, we did not define the mechanisms by which TRPC3 channels mediate chronic itch in the setting of CHS. Dermal inflammation, epidermal hyperplasia, and immune cells infiltration appear to be a key contributor to the induction and maintenance of chronic itch in allergic skin disorders [27; 57]. Yet, we found that global deletion of *Trpc3* had no obvious effects on skin inflammatory processes in the CHS model. Thus, these anti-pruritic effects seem to be dissociable from skin inflammation. We suggest that TRPC3 signaling, specifically in primary sensory neurons, contributes to chronic itch through a mechanism that parallels skin inflammation. Given that nociceptive neurons are capable of regulating innate and adaptive immune responses[35; 56], we cannot completely exclude the contributions of neuronal TRPC3 to inflammatory process beyond the sensitivity of our histological and biochemical assays.

In summary, we have demonstrated that TRPC3 in primary sensory neurons mediates non-histaminergic itch and may contribute to the pathogenesis of chronic itch accompanying CHS via a mechanism that parallels skin inflammatory process. We suggest that TRPC3 may serve as a new potential therapeutic target for the treatment of chronic itch associated with contact dermatitis in which antihistamines are poorly tolerated or ineffective.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Author contributions

Y. Liu performed RNAScope, behavioral experiments, flow cytometry and qRT-PCR and wrote the draft; YT. Liu conducted calcium imaging, behavioral experiments, ear H&E staining, qRT-PCR, analyzed data, and wrote the draft. N. Limjunyawong carried out flow cytometry, Evans Blue assays, and analyzed data; C. Narang, H. Jamaldeen, S. Yu, S. Patiram analyzed behavioral data; H. Nie edited the manuscript. M. Caterina and X. Dong facilitated experimental design and revised the manuscript. L. Qu conceived and supervised the project, designed the experiments, carried out behavioral experiments, and wrote the manuscript.

References

[1] Akiyama T, Lerner EA, Carstens E. Protease-activated receptors and itch. Handb Exp Pharmacol 2015;226:219-235.

[2] Alkhani H, Ase AR, Grant R, O'Donnell D, Groschner K, Séguéla P. Contribution of TRPC3 to Store-Operated Calcium Entry and Inflammatory Transductions in Primary Nociceptors. Molecular Pain 2014;10:1744-8069-1710-1743.

[3] Barry DM, Liu XT, Liu B, Liu XY, Gao F, Zeng X, Liu J, Yang Q, Wilhelm S, Yin J, Tao A, Chen ZF. Exploration of sensory and spinal neurons expressing gastrin-releasing peptide in itch and pain related behaviors. Nat Commun 2020;11(1):1397.

[4] Beattie K, Jiang H, Gautam M, MacVittie MK, Miller B, Ma M, Liu Q, Luo W. TRPC3 Antagonizes Pruritus in A Mouse Contact Dermatitis Model. J Invest Dermatol 2021.

[5] Dalrymple A, Slater DM, Poston L, Tribe RM. Physiological Induction of Transient Receptor Potential Canonical Proteins, Calcium Entry Channels, in Human Myometrium: Influence of Pregnancy, Labor, and Interleukin-1β. The Journal of Clinical Endocrinology & Metabolism 2004;89(3):1291-1300.

[6] Dong P, Guo C, Huang S, Ma M, Liu Q, Luo W. TRPC3 Is Dispensable for beta-Alanine Triggered Acute Itch. Sci Rep 2017;7(1):13869.

[7] Dong X, Dong X. Peripheral and Central Mechanisms of Itch. Neuron 2018;98(3):482-494.

[8] Dube PR, Chikkamenahalli LL, Birnbaumer L, Vazquez G. Reduced calcification and osteogenic features in advanced atherosclerotic plaques of mice with macrophage-specific loss of TRPC3. Atherosclerosis 2018;270:199-204.

[9] Elg S, Marmigere F, Mattsson JP, Ernfors P. Cellular subtype distribution and developmental regulation of TRPC channel members in the mouse dorsal root ganglion. Journal of Comparative Neurology 2007;503(1):35-46.

[10] Feng J, Yang P, Mack MR, Dryn D, Luo J, Gong X, Liu S, Oetjen LK, Zholos AV, Mei Z, Yin S, Kim BS, Hu H. Sensory TRP channels contribute differentially to skin inflammation and persistent itch. Nature communications 2017;8(1):980-980.

[11] Flegel C, Schobel N, Altmuller J, Becker C, Tannapfel A, Hatt H, Gisselmann G. RNA-Seq Analysis of Human Trigeminal and Dorsal Root Ganglia with a Focus on Chemoreceptors. PLoS One 2015;10(6):e0128951.

[12] Gomes LO, Hara DB, Rae GA. Endothelin-1 induces itch and pain in the mouse cheek model. Life Sci 2012;91(13-14):628-633.

[13] Gong X, Xiong H, Liu S, Liu Y, Yin L, Tu C, Wang H, Zhao Z, Chen W, Mei Z. Qingpeng Ointment Ameliorates Inflammatory Responses and Dysregulation of Itch-Related Molecules for Its Antipruritic Effects in Experimental Allergic Contact Dermatitis. Front Pharmacol 2019;10:354.

[14] Han L, Ma C, Liu Q, Weng HJ, Cui Y, Tang Z, Kim Y, Nie H, Qu L, Patel KN, Li Z, McNeil B, He S, Guan Y, Xiao B, Lamotte RH, Dong X. A subpopulation of nociceptors specifically linked to itch. Nat Neurosci 2013;16(2):174-182.

[15] Heiner I, Eisfeld J, Halaszovich CR, Wehage E, JÜNgling E, Zitt C, LÜCkhoff A. Expression profile of the transient receptor potential (TRP) family in neutrophil granulocytes: evidence for currents through long TRP channel 2 induced by ADP-ribose and NAD. Biochemical Journal 2003;371(3):1045-1053.

[16] Huang J, Polgar E, Solinski HJ, Mishra SK, Tseng PY, Iwagaki N, Boyle KA, Dickie AC, Kriegbaum MC, Wildner H, Zeilhofer HU, Watanabe M, Riddell JS, Todd AJ, Hoon MA. Circuit dissection of the role of somatostatin in itch and pain. Nat Neurosci 2018;21(5):707-716.

[17] Imamachi N, Park GH, Lee H, Anderson DJ, Simon MI, Basbaum AI, Han SK. TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms. Proc Natl Acad Sci U S A 2009;106(27):11330-11335.

[18] Kim AY, Tang Z, Liu Q, Patel KN, Maag D, Geng Y, Dong X. Pirt, a phosphoinositide-binding protein, functions as a regulatory subunit of TRPV1. Cell 2008;133(3):475-485.

[19] Larsen EG, Cho TS, McBride ML, Feng J, Manivannan B. TMEM184B promotes pruriceptive neuron specification to allow itch sensitivity. bioRxiv doi :101101/20200125919902 2020.

[20] Leuner K, Kraus M, Woelfle U, Beschmann H, Harteneck C, Boehncke W-H, Schempp CM, Müller WE. Reduced TRPC channel expression in psoriatic keratinocytes is associated with impaired differentiation and enhanced proliferation. PLoS One 2011;6(2):e14716-e14716.

[21] Li X, Eadara S, Jeon S, Liu Y, Muwanga G, Qu L, Caterina MJ, Meffert MK. Combined single-molecule fluorescence in situ hybridization and immunohistochemistry analysis in intact murine dorsal root ganglia and sciatic nerve. STAR Protoc 2021;2(2):100555.

[22] Li Z, He SQ, Xu Q, Yang F, Tiwari V, Liu Q, Tang Z, Han L, Chu YX, Wang Y, Hin N, Tsukamoto T, Slusher B, Guan X, Wei F, Raja SN, Dong X, Guan Y. Activation of MrgC receptor inhibits N-type calcium channels in small-diameter primary sensory neurons in mice. Pain 2014;155(8):1613-1621.

[23] Liu B, Escalera J, Balakrishna S, Fan L, Caceres AI, Robinson E, Sui A, McKay MC, McAlexander MA, Herrick CA, Jordt SE. TRPA1 controls inflammation and pruritogen responses in allergic contact dermatitis. FASEB J 2013;27(9):3549-3563.

[24] Liu Q, Sikand P, Ma C, Tang Z, Han L, Li Z, Sun S, LaMotte RH, Dong X. Mechanisms of itch evoked by beta-alanine. J Neurosci 2012;32(42):14532-14537.

[25] Liu Q, Tang Z, Surdenikova L, Kim S, Patel KN, Kim A, Ru F, Guan Y, Weng H-J, Geng Y, Undem BJ, Kollarik M, Chen Z-F, Anderson DJ, Dong X. Sensory Neuron-Specific

GPCR Mrgprs Are Itch Receptors Mediating Chloroquine-Induced Pruritus. Cell 2009;139(7):1353-1365.

[26] Liu T, Ji RR. New insights into the mechanisms of itch: are pain and itch controlled by distinct mechanisms? Pflugers Arch 2013;465(12):1671-1685.

[27] Liu X, Wang D, Wen Y, Zeng L, Li Y, Tao T, Zhao Z, Tao A. Spinal GRPR and NPRA contribute to chronic itch in a murine model of allergic contact dermatitis. Journal of Investigative Dermatology 2020.

[28] McNeil BD, Pundir P, Meeker S, Han L, Undem BJ, Kulka M, Dong X. Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. Nature 2015;519(7542):237-241.

[29] Meixiong J, Anderson M, Limjunyawong N, Sabbagh MF, Hu E, Mack MR, Oetjen LK, Wang F, Kim BS, Dong X. Activation of Mast-Cell-Expressed Mas-Related G-Protein-Coupled Receptors Drives Non-histaminergic Itch. Immunity 2019;50(5):1163-1171 e1165.

[30] Mishra SK, Hoon MA. The cells and circuitry for itch responses in mice. Science 2013;340(6135):968-971.

[31] Mollanazar NK, Smith PK, Yosipovitch G. Mediators of Chronic Pruritus in Atopic Dermatitis: Getting the Itch Out? Clin Rev Allergy Immunol 2016;51(3):263-292.

[32] Nguyen MQ, von Buchholtz LJ, Reker AN, Ryba NJ, Davidson S. Single-nucleus transcriptomic analysis of human dorsal root ganglion neurons. Elife 2021;10.

[33] Pan H, Fatima M, Li A, Lee H, Cai W, Horwitz L, Hor CC, Zaher N, Cin M, Slade H, Huang T, Xu XZS, Duan B. Identification of a Spinal Circuit for Mechanical and Persistent Spontaneous Itch. Neuron 2019;103(6):1135-1149 e1136.

[34] Peppiatt-Wildman CM, Albert AP, Saleh SN, Large WA. Endothelin-1 activates a Ca2+-permeable cation channel with TRPC3 and TRPC7 properties in rabbit coronary artery myocytes. J Physiol 2007;580(Pt.3):755-764.

[35] Pinho-Ribeiro FA, Verri WA, Jr., Chiu IM. Nociceptor Sensory Neuron-Immune Interactions in Pain and Inflammation. Trends Immunol 2017;38(1):5-19.

[36] Qu L, Fan N, Ma C, Wang T, Han L, Fu K, Wang Y, Shimada SG, Dong X, LaMotte RH. Enhanced excitability of MRGPRA3- and MRGPRD-positive nociceptors in a model of inflammatory itch and pain. Brain 2014;137(Pt 4):1039-1050.

[37] Qu L, Fu K, Yang J, Shimada SG, LaMotte RH. CXCR3 chemokine receptor signaling mediates itch in experimental allergic contact dermatitis. Pain 2015;156(9):1737-1746.

[38] Qu L, Li Y, Pan X, Zhang P, LaMotte RH, Ma C. Transient Receptor Potential Canonical 3 (TRPC3) Is Required for IgG Immune Complex-Induced Excitation of the Rat Dorsal Root Ganglion Neurons. The Journal of Neuroscience 2012;32(28):9554.

[39] Ru F, Sun H, Jurcakova D, Herbstsomer RA, Meixong J, Dong X, Undem BJ. Mechanisms of pruritogen-induced activation of itch nerves in isolated mouse skin. J Physiol 2017;595(11):3651-3666.

[40] Sharif B, Ase AR, Ribeiro-da-Silva A, Seguela P. Differential Coding of Itch and Pain by a Subpopulation of Primary Afferent Neurons. Neuron 2020.

[41] Sharif B, Ase AR, Ribeiro-da-Silva A, Seguela P. Differential Coding of Itch and Pain by a Subpopulation of Primary Afferent Neurons. Neuron 2020;106(6):940-951 e944.

[42] Shiratori-Hayashi M, Hasegawa A, Toyonaga H, Andoh T, Nakahara T, Kido-Nakahara M, Furue M, Kuraishi Y, Inoue K, Dong X, Tsuda M. Role of P2X3 receptors in scratching behavior in mouse models. J Allergy Clin Immunol 2019;143(3):1252-1254 e1258.

[43] Shiratori-Hayashi M, Yamaguchi C, Eguchi K, Shiraishi Y, Kohno K, Mikoshiba K, Inoue K, Nishida M, Tsuda M. Astrocytic STAT3 activation and chronic itch require IP3R1/TRPC-dependent Ca(2+) signals in mice. J Allergy Clin Immunol 2021;147(4):1341-1353.

[44] Shuba YM. Beyond Neuronal Heat Sensing: Diversity of TRPV1 Heat-Capsaicin Receptor-Channel Functions. Front Cell Neurosci 2020;14:612480.

[45] Silverberg JI. Comorbidities and the impact of atopic dermatitis. Ann Allergy Asthma Immunol 2019;123(2):144-151.

[46] Song T, Zheng Y-M, Vincent PA, Cai D, Rosenberg P, Wang Y-X. Canonical transient receptor potential 3 channels activate NF-κB to mediate allergic airway disease via PKC-α/IκB-α and calcineurin/IκB-β pathways. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2016;30(1):214-229.

[47] Sudi SB, Tanaka T, Oda S, Nishiyama K, Nishimura A, Sunggip C, Mangmool S, Numaga-Tomita T, Nishida M. TRPC3-Nox2 axis mediates nutritional deficiency-induced cardiomyocyte atrophy. Sci Rep 2019;9(1):9785.

[48] Than JY, Li L, Hasan R, Zhang X. Excitation and modulation of TRPA1, TRPV1, and TRPM8 channel-expressing sensory neurons by the pruritogen chloroquine. J Biol Chem 2013;288(18):12818-12827.

[49] Urban N, Schaefer M. Direct Activation of TRPC3 Channels by the Antimalarial Agent Artemisinin. Cells 2020;9(1).

[50] Usoskin D, Furlan A, Islam S, Abdo H, Lonnerberg P, Lou D, Hjerling-Leffler J, Haeggstrom J, Kharchenko O, Kharchenko PV, Linnarsson S, Ernfors P. Unbiased

classification of sensory neuron types by large-scale single-cell RNA sequencing. Nat Neurosci 2015;18(1):145-153.

[51] Vohra PK, Thompson MA, Sathish V, Kiel A, Jerde C, Pabelick CM, Singh BB, Prakash YS. TRPC3 regulates release of brain-derived neurotrophic factor from human airway smooth muscle. Biochimica et biophysica acta 2013;1833(12):2953-2960.

[52] Wang L, Li J, Zhang J, He Q, Weng X, Huang Y, Guan M, Qiu C. Inhibition of TRPC3 downregulates airway hyperresponsiveness, remodeling of OVA-sensitized mouse. Biochemical and Biophysical Research Communications 2017;484(1):209-217.

[53] Wilson SR, Gerhold KA, Bifolck-Fisher A, Liu Q, Patel KN, Dong X, Bautista DM. TRPA1 is required for histamine-independent, Mas-related G protein-coupled receptor-mediated itch. Nature neuroscience 2011;14(5):595-602.

[54] Wilson SR, Nelson AM, Batia L, Morita T, Estandian D, Owens DM, Lumpkin EA, Bautista DM. The ion channel TRPA1 is required for chronic itch. J Neurosci 2013;33(22):9283-9294.

[55] Xu X, Lozinskaya I, Costtell M, Lin Z, Ball JA, Bernard R, Behm DJ, Marino JP, Schnackenberg CG. Characterization of small molecule TRPC3 and TRPC6 agonist and antagonists. Biophys J 2013;104:454a.

[56] Zhang S, Edwards TN, Chaudhri VK, Wu J, Cohen JA, Hirai T, Rittenhouse N, Schmitz EG, Zhou PY, McNeil BD, Yang Y, Koerber HR, Sumpter TL, Poholek AC, Davis BM, Albers KM, Singh H, Kaplan DH. Nonpeptidergic neurons suppress mast cells via glutamate to maintain skin homeostasis. Cell 2021;184(8):2151-2166 e2116.

[57] Zhao J, Munanairi A, Liu X-Y, Zhang J, Hu L, Hu M, Bu D, Liu L, Xie Z, Kim BS, Yang Y, Chen Z-F. PAR2 mediates itch via TRPV3 signaling in keratinocytes. Journal of Investigative Dermatology 2020.

Figure legends

Figure 1. TRPC3 is expressed in a subset of mouse TG neurons. (A) RNAScope in situ hybridization (ISH) of *Trpc3* mRNA expression in a subset of TG neurons of wildtype mice. *Trpc3* mRNA signal (red) was colocalized with NeuN, a pan neuronal marker (green). n = 3 mice; scale bar, 100 µm. (B) Size frequency distribution of *Trpc3* positive neurons. (C) RNAScope images showing the absence of *Trpc*3 mRNA signal in wildtype TG sections stained with a negative probe (n = 3 mice). (D) Fluorescent images for the co-expression of *Trpc3* mRNA signal with IB4, CGRP, TH, and GS. Scale bar, 100 µm. (E) Coexpression characterization of *Trpc3* positive neurons (n = 3 mice).

Figure 2. TRPC3 is colocalized with itch related receptors and present in itch sensory neurons. (A-E) Representative image of the coexpression of *Trpc3 mRNA* with *Trpa1 mRNA* (A), P2X3 (B), *Sst mRNA* (C), *Nppb mRNA* (D), and GFP signals (MrgprA3$^{GFP\text{-}Cre}$ mouse; E) in mouse TG neurons (n = 3 mice), along with quantitative analysis of percentage overlap. Scale bar, 50 µm.

Figure 3. TRPC3 agonism evokes Ca^{2+} responses in mouse TG neurons. (A) Representative fluorescence images of mouse TG neurons loaded with Fura-2 at baseline and after stimulation with GSK1702934A (GSK; 100 µM) and KCl (50 mM). Arrows indicate TG neurons responsive to a given treatment. Scale bar: 100 µm. (B-F) Representative traces of Ca^{2+} responses evoked by vehicle (veh; 0.1% ethanol; B), 0.1 (C), 1 (D), 10 (E), 100 µM (F) of GSK (1 min) and KCl (50 mM, 20 s) in TG neurons from wildtype mice. Black bars above the traces indicate

the timing of chemical application. (G) Summary graph showing that GSK evoked dose-dependent Ca^{2+} responses in TG neurons from *Trpc3*$^{+/+}$ mice. This effect was diminished in TG neurons from global *Trpc3*$^{-/-}$ mice. $^{*}p < 0.05$ vs vehicle; $^{\#}p < 0.05$ vs wildtype 100 µM, Chi-square test. The numbers of responsive cells vs total tested are given in parentheses. (H) Summary graph showing the overlap of GSK-, chloroquine (CQ; 1 mM)-, allyl isothiocyanate (AITC, 100 µM)-, and capsaicin (Cap; 1 µM)- responsive TG neurons from wildtype mice. The numbers of responsive cells vs total tested are given in parentheses.

Figure 4. Activation of TRPC3 elicits acute histamine-independent itch in naïve mice. (A) Schematic of cheek injection, behavioral assessments. (B, C) The total scratching (B) and wiping bouts (C) were elicited in response to subcutaneous (s.c.) injection of vehicle (20% HPCD, 10 µl), and GSK at the different doses (10 µl in 20% HPCD) into the cheek of wildtype mice. n = 8 -10 mice per group; $^{**}p < 0.01$, $^{***}p < 0.001$ vs vehicle; one-way ANOVA with Tukey's test. (D-E) Time course of scratching (D) and wiping (E) responses before and after s.c injection of GSK at different doses (10 µl) or vehicle (20% HPCD, 10 µl). n = 8 -10 mice per group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs vehicle; two-way ANOVA for repeated measures followed by Bonferroni's post hoc test. (E) Summary graph showing the effects of H1R blocker cetirizine (i.p.; 30 mg/kg, 100 µl) or vehicle (saline, 100 µl) on scratching responses induced by s.c. injection of GSK (20 µg, 10 µl in 20% HPCD) into the cheek of naïve mice. n = 8 mice per group. $P > 0.05$, unpaired Student's t test. (F-G) Summary graph showing the effects of global deletion of *Trpc3* on scratching (F) and wiping (G) behaviors evoked by s.c. injection of GSK (20 µg,10 µl in 20% HPCD) into mouse cheek. n = 8 mice per group; $^{**}p < 0.001$ vs *Trpc3*$^{+/+}$; unpaired Student's *t* test.

Figure 5. Genetic deletion of *Trpc3* attenuates acute itch induced by histamine-independent

pruritogens. (A-D) The total number of scratches in 30 min induced by i.d. nape injection of 50 µl of pruritogens, including ET-1 (25 ng; A), SLIGRL-NH_2 (100 µg; B), histamine (100 µg; C) and α-Me-5-HT (20 µg; D) in wildtype and global *Trpc3*$^{-/-}$ mice. n = 8 -12 mice per group; *p < 0.05, vs *Trpc3*$^{+/+}$ mice; Unpaired Student's *t* test. (E, F) Summary of scratching responses evoked by ET-1(E; 25 ng; 50 µl) and SLIGRL-NH_2 (F; 100 ug; 50 µl) in wildtype and *PirtCre::Trpc3*$^{fl/fl}$ mice. n = 9 -12 mice per group; *p < 0.05 vs *Trpc3*$^{fl/fl}$ mice; unpaired Student's *t* test.

Figure 6. TRPC3 mediates allergic itch in the CHS model. (A) Experimental diagram showing the induction of CHS on mouse ear, drug delivery and behavioral procedures. (B-D) Measurements of spontaneous scratching bouts on the ear of wildtype, global *Trpc3*$^{-/-}$ mice (B), sensory neuron specific *Trpc3* knockout mice (*PirtCre::Trpc3*$^{fl/fl}$; C) and keratinocyte specific Trpc3 knockout mice (*K14Cre::Trpc3*$^{fl/fl}$; D) subjected to vehicle control (Ctrl group) and SADBE challenge (CHS group). The number of spontaneous scratching events was counted over a 30 min period. n = 8 -11 mice per group; **p < 0.01 vs Ctrl; # p < 0.05 vs *Trpc3*$^{+/+}$, two-way ANOVA with Bonferroni's post hoc test. (E) Spontaneous scratching bouts in mice treated with Pyr3 (i.p.; 0.1 mg/kg, 100 µl) compared to those treated with vehicle (veh; 0.1% DMSO, 100 µl) on day 3 after CHS induction. n = 8 mice per group; **p < 0.01 vs Ctrl; unpaired Student's *t* test.

Figure 7. TRPC3 does not contribute to cutaneous inflammation in the CHS model. (A) Time course of ear thickness in wildtype and global *Trpc3*$^{-/-}$ mice treated with vehicle (Ctrl) and SADBE (CHS). n = 6 - 8 mice per group; **p < 0.01, ##p < 0.01, vs Ctrl, two-way ANOVA for repeated measures followed by Bonferroni's post hoc test. (B) Representative images of H&E-stained ear sections taken from wildtype and global *Trpc3*$^{-/-}$ mice treated with vehicle (Ctrl) or SADBE (CHS) on day 3 after the 3rd challenge. Scale bar, 200 µm. (C) Quantification of

epidermal thickness. n = 4 mice per group; *p < 0.05, two-way ANOVA with Bonferroni's post hoc test. (C) qRT-PCR analysis of the mRNA expression levels of *il6*, *cxcl1*, *il1b* and *cxcl10* in the ear of wildtype and global *Trpc3*$^{-/-}$ mice on day 3 after challenge. n = 3 - 4 mice per group. *p < 0.05, two-way ANOVA with Bonferroni's post hoc test. (E) Representative flow cytometry plots of vehicle (Ctrl)- and SADBE (CHS)-treated ear skin of *Trpc3*$^{+/+}$ and *Trpc3*$^{-/-}$ mice. Numbers indicate the percentage of positive cells within boxes. (F) Quantification of the proportion of CD45^{+} leukocytes per biopsy from vehicle (Ctrl)- and SADBE (CHS)-treated ear skin of wildtype and *Trpc3*$^{-/-}$ mice. n = 5 - 6 mice per group. **p < 0.01, two-way ANOVA with Bonferroni's post hoc test.

Figure 8. CHS upregulates the expression and function of TRPC3 in mouse TG neurons. (A) qRT-PCR analysis of *Trpc3* mRNA expression normalized to that of *Actb* in in the TG of control (Ctrl; n = 3 mice) and CHS mice (n = 4 mice) one day after the 3rd SADBE challenge. *p < 0.01 vs Ctrl; unpaired Student's t test. (B-C) Representative traces of Ca^{2+} responses evoked by GSK (100 µM, 1 min) in TG neurons from control (Ctrl; B) and CHS (C) mice. Black bars above the traces indicate the timing of chemical application. (D) The percentage of TG neurons that responded to GSK was significantly larger in CHS mice than that in control mice. *p < 0.05 vs Ctrl, Chi-square test. The numbers of responsive cells vs total tested are given in parentheses. (E) The percentage of GSK responsive neurons that also responded to CQ or Cap was significantly increased in CHS mice compared to that in control animals. *p < 0.05, **p < 0.01 vs control, Chi-square test. The numbers of responsive cells vs total tested are given in parentheses.

Fig. 1

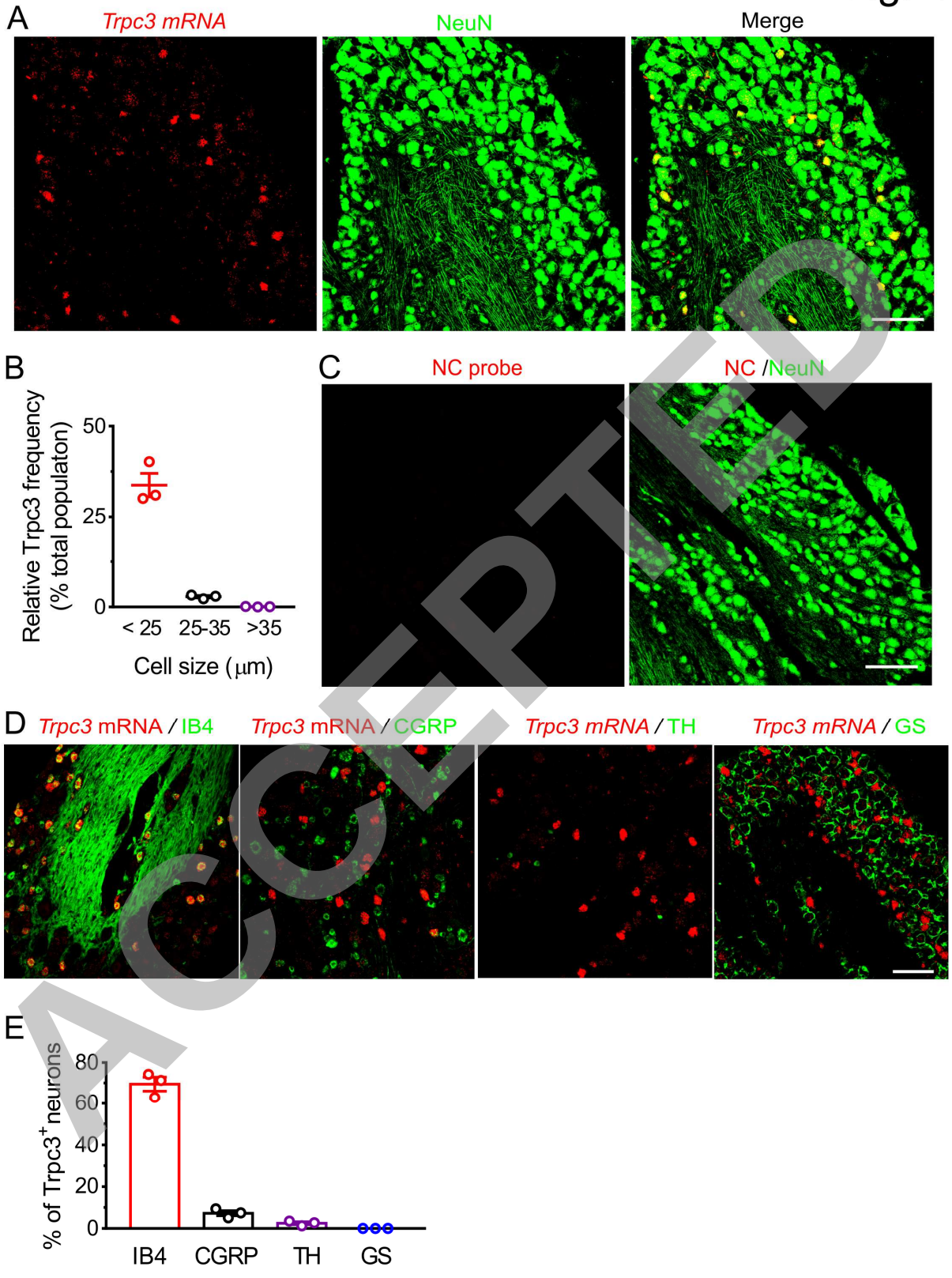

Fig. 2

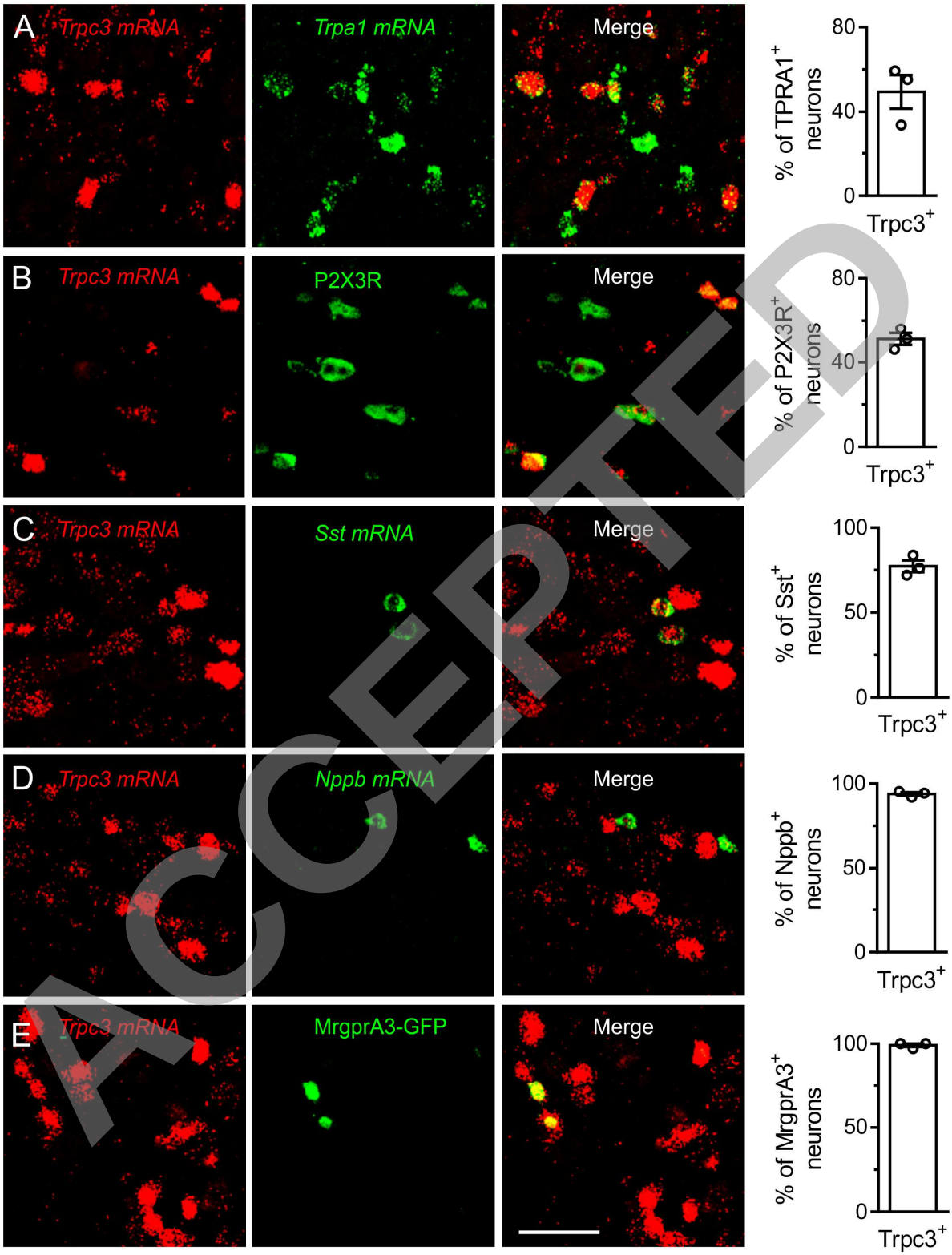

Fig. 3

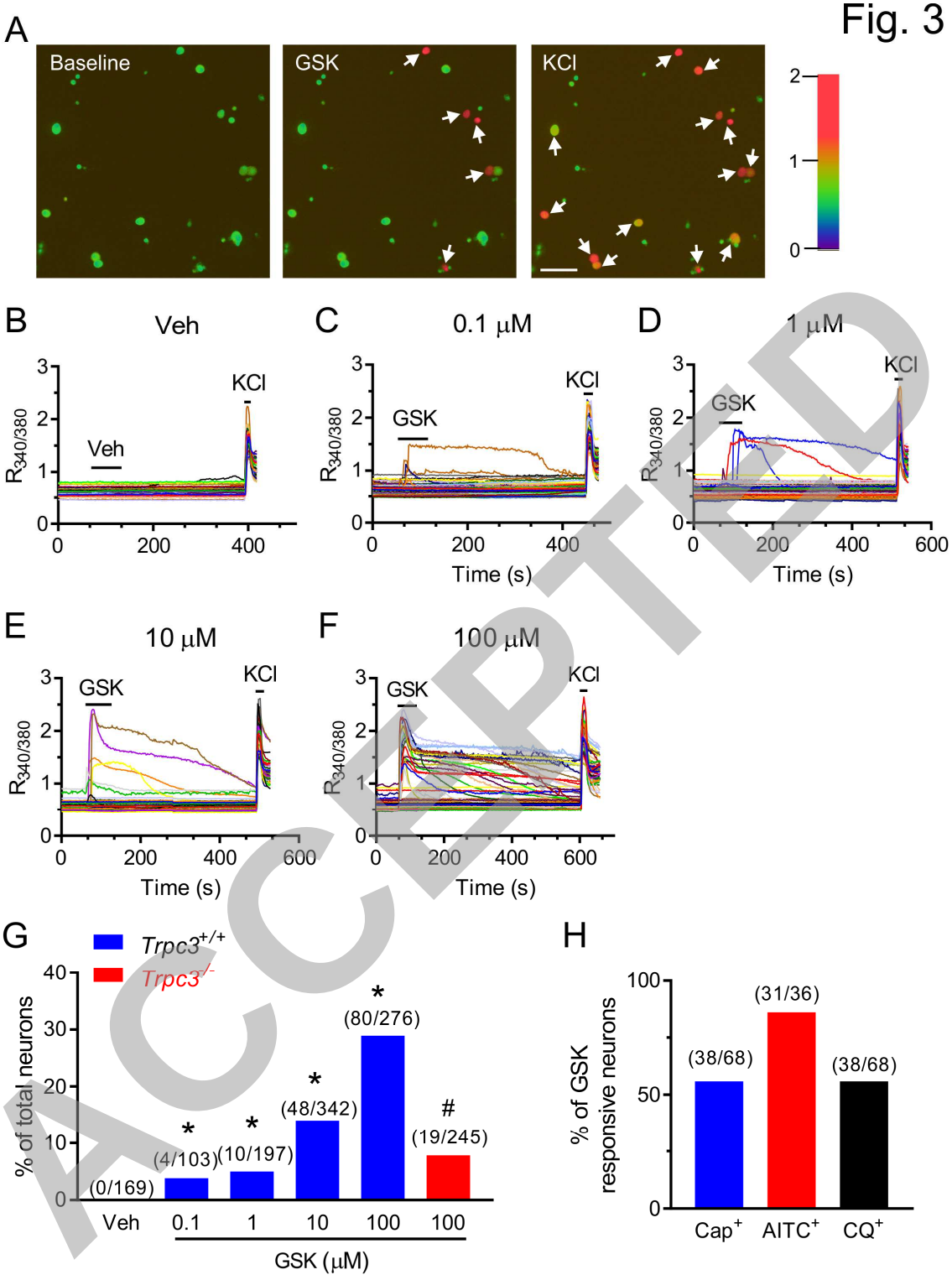

Fig. 4

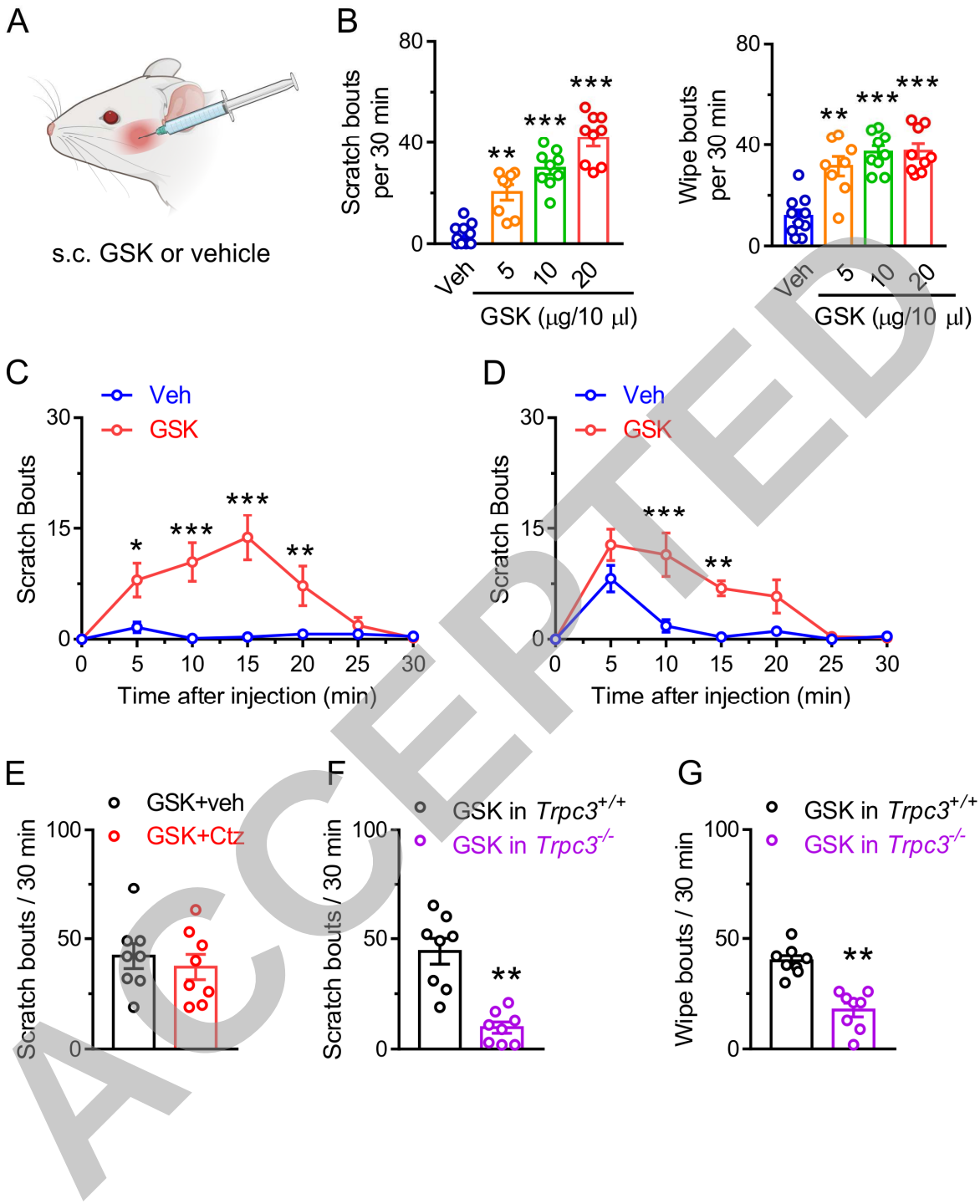

Fig. 5

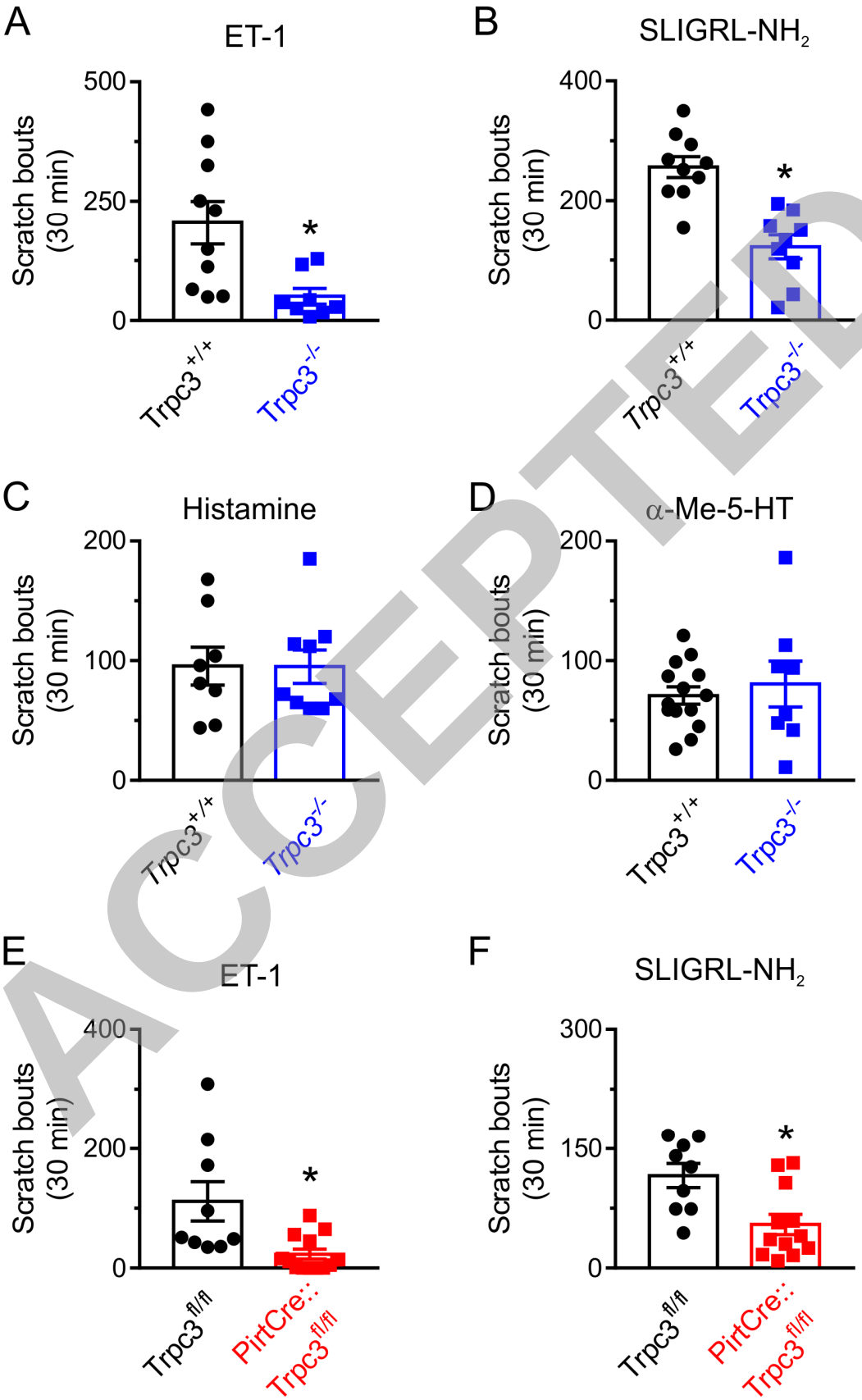

Fig. 6

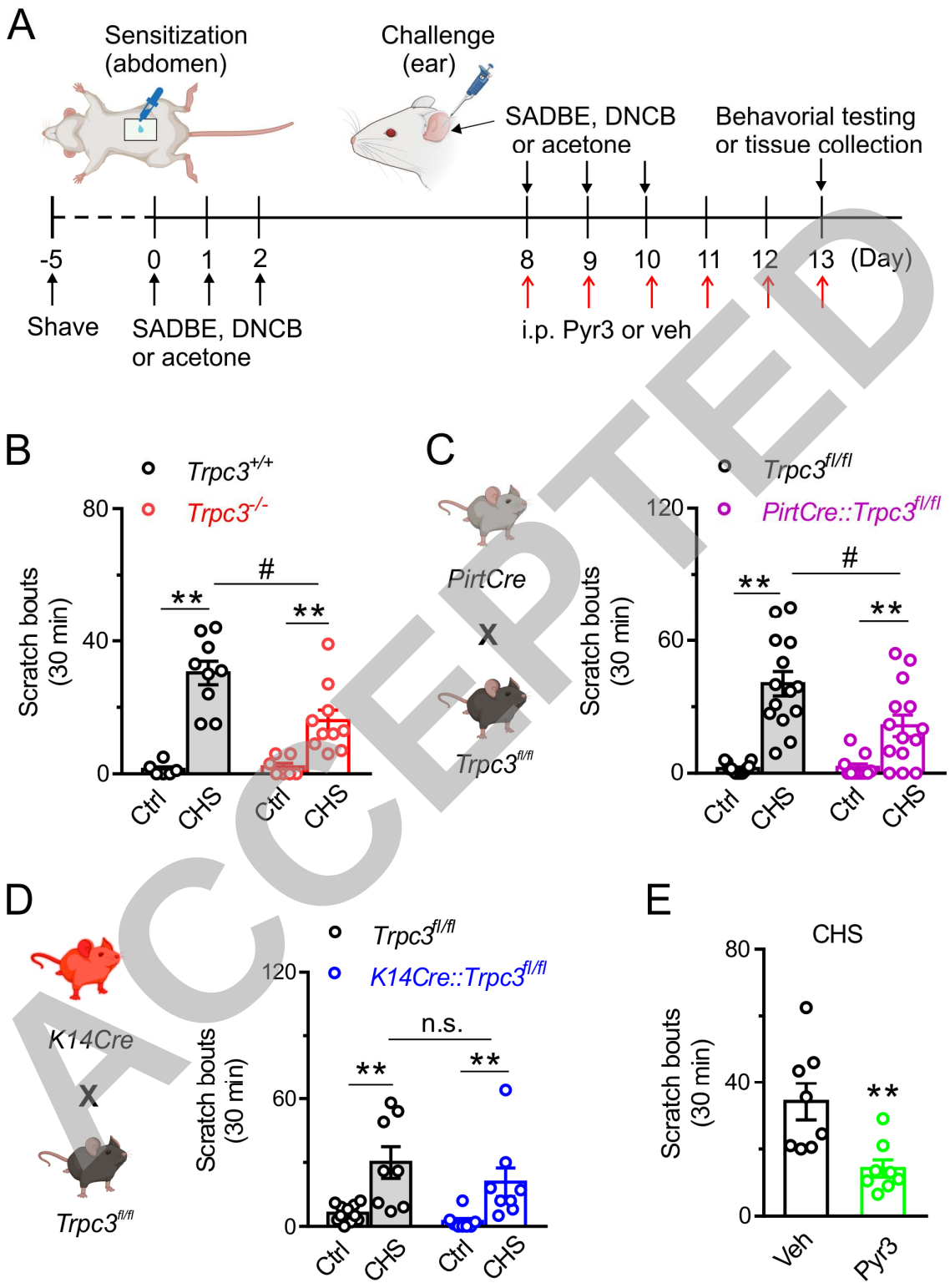

Fig. 7

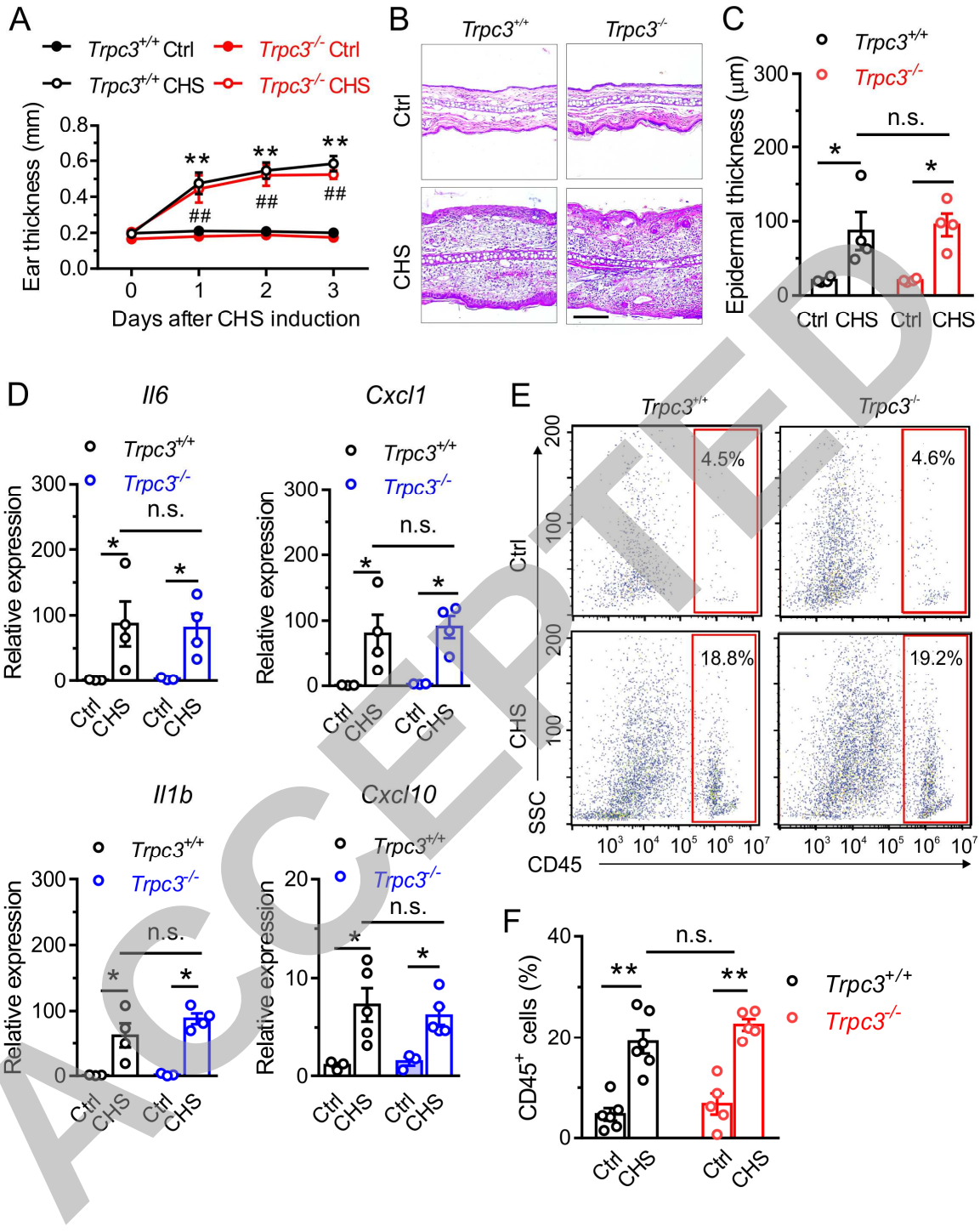

Fig. 8