

**Involvement of TRPV4 in serotonin-evoked scratching**

Tasuku Akiyama, Margaret Ivanov, Masaki Nagamine, Auva Davoodi, Mirela Iodi Carstens, Akihiko Ikoma, Ferda Cevikbas, Cordula Kempkes, Joerg Buddenkotte, Martin Steinhoff, E Carstens

**Cite this article as:** Tasuku Akiyama, Margaret Ivanov, Masaki Nagamine, Auva Davoodi, Mirela Iodi Carstens, Akihiko Ikoma, Ferda Cevikbas, Cordula Kempkes, Joerg Buddenkotte, Martin Steinhoff, E Carstens, Involvement of TRPV4 in serotonin-evoked scratching, *Journal of Investigative Dermatology* accepted article preview 1 October 2015; doi: [10.1038/jid.2015.388](https://doi.org/10.1038/jid.2015.388).

This is a PDF file of an unedited peer-reviewed manuscript that has been accepted for publication. NPG are providing this early version of the manuscript as a service to our customers. The manuscript will undergo copyediting, typesetting and a proof review before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers apply.

Received 16 July 2015; revised 26 August 2015; accepted 5 September 2015;  
Accepted article preview online 1 October 2015

**Original Article**

Involvement of TRPV4 in serotonin-evoked scratching

Tasuku Akiyama<sup>1,2</sup>, Margaret Ivanov<sup>1</sup>, Masaki Nagamine<sup>1</sup>, Auva Davoodi<sup>1</sup>, Mirela Iodi Carstens<sup>1</sup>, Akihiko Ikoma<sup>3</sup>, Ferda Cevikbas<sup>3</sup>, Cordula Kempkes<sup>3</sup>, Joerg Buddenkotte<sup>3,4</sup>, Martin Steinhoff<sup>3,4</sup>, and E. Carstens<sup>1</sup>

**Affiliations:**

<sup>1</sup>University of California, Davis  
Department of Neurobiology, Physiology and Behavior  
1 Shields Avenue  
Davis, CA 95616, USA.

<sup>2</sup>Temple University  
Department of Dermatology  
Department of Anatomy & Cell Biology  
Temple Itch Center  
3500 Broad St,  
Philadelphia, PA19140, USA.

<sup>3</sup>University of California, San Francisco  
Departments of Dermatology and Surgery

<sup>4</sup>U Dept. of Dermatology and UCD Charles Institute for Translational Dermatology,  
University College Dublin, Dublin, Ireland

**Short title: Role of TRPV4 in serotonin-evoked itch****Abbreviations:**

5-HT: 5-hydroxytryptamine (serotonin)  
CGRP: calcitonin gene related peptide  
DRG: dorsal root ganglion  
ET-1: endothelin-1  
Mrgpr: Mas-related G-protein-coupled receptor  
PARs: protease-activated receptors  
SP: substance P  
TRP: Transient receptor potential  
TRPV4: transient receptor potential vanilloid type-4  
WT: wild-type

**Corresponding Author:**

Tasuku Akiyama, PhD  
Temple University  
Departments of Dermatology and Anatomy & Cell Biology, and Temple Itch Center  
3500 Broad St,

Philadelphia, PA19140, USA.  
Tel.: 1-215-707-9509  
Fax: 1-215-707-9510  
e-mail: [tasuku.akiyama@temple.edu](mailto:tasuku.akiyama@temple.edu)

Prof. E. Carstens  
University of California, Davis  
Department of Neurobiology, Physiology & Behavior  
1 Shields Avenue  
Davis, California 95616  
Tel.: 1-530-752-7767 (lab)  
Fax: 1-530-752-5582  
e-mail: [ecarstens@ucdavis.edu](mailto:ecarstens@ucdavis.edu)

Prof. Martin Steinhoff  
U Dept. of Dermatology and UCD Charles Institute for Translational Dermatology,  
University College Dublin, Dublin, Ireland.  
[Martin.steinhoff@ucd.ie](mailto:Martin.steinhoff@ucd.ie)

Accepted manuscript

**ABSTRACT**

Several thermo-sensitive TRP channels (TRPV1, -3; TRPA1) have been implicated in itch. In contrast, the role of transient receptor potential vanilloid type-4 (TRPV4) in itch is unknown. Therefore, we investigated if TRPV4, a temperature-sensitive cation channel, plays an important role in acute itch in mice. Four different pruritogens including serotonin (5-hydroxytryptamine, 5-HT), histamine, SLIGRL (PAR2/MrgprC11 agonist) and chloroquine (MrgprA3 agonist) were intradermally injected and itch-related scratching behavior was assessed. TRPV4 knockout (TRPV4KO) mice exhibited significantly fewer 5-HT-evoked scratching bouts compared to wild-type (WT) mice. Notably, no differences between TRPV4KO and WT mice were observed in the number of scratch bouts elicited by SLIGRL and histamine. Pretreatment with a TRPV4 antagonist significantly attenuated 5-HT-evoked scratching in vivo. Using calcium imaging in cultured primary murine dorsal root ganglion (DRG) neurons, the response of neurons after 5-HT application, but not other pruritogens, was significantly lower in TRPV4KO compared to WT mice. A TRPV4 antagonist significantly suppressed 5-HT-evoked responses in DRG cells from WT mice. Approximately 90% of 5-HT-sensitive DRG neurons were immunoreactive for an antibody to TRPV4, as assessed by calcium imaging. These results indicate that serotonin-induced itch is linked to TRPV4.

## Introduction

Itch can be elicited by a wide variety of chemical stimuli, including inflammatory mediators: amines, cytokines, proteases, neuropeptides, and Mas-related G-protein-coupled receptor (Mrgpr) agonists (Akiyama and Carstens, 2014). Histamine, an inflammatory mediator, is the best-known itch inducer and is predominantly released by mast cells and basophils, and possibly keratinocytes (Dvorak, 1998; Inami *et al.*, 2013). Histamine H1 and H4 receptors play a role in histamine-evoked itch (Bell *et al.*, 2004). Serotonin (5-HT), another inflammatory mediator, is released by mast cells, melanocytes, and platelets, to evoke itch (Kushnir-Sukhov *et al.*, 2007; Slominski *et al.*, 2003; Turetta *et al.*, 2004). While the intradermal injection of 5-HT elicits robust scratching behaviors in rodents (Nojima and Carstens, 2003; Yamaguchi *et al.*, 1999), either the intradermal injection or the iontophoretic application of 5-HT elicits mild to moderate itch in humans (Hosogi *et al.*, 2006; Weisshaar *et al.*, 2004; Weisshaar *et al.*, 1997). Proteases like trypsins, kallikreins or tryptase exert pruritogenic effects through the activation of protease-activated receptors (PARs). PAR-2 is overexpressed in the skin of atopic dermatitis patients and its tethered ligand, SLIGRL, evokes itch-related behaviors in mice (Akiyama *et al.*, 2009; Steinhoff *et al.*, 2003). Mrgprs have recently been linked to chemically-evoked itch (Han *et al.*, 2013). Chloroquine, an agonist of MrgprA3, as well as bovine adrenal medullary peptide BAM8-22, an agonist of MrgprC11, both elicit itch. It has been reported that SLIGRL, a tethered ligand for PAR-2, in addition acts as an agonist of MrgprC11 (Liu *et al.*, 2011).

Transient receptor potential (TRP) ion channels are involved in sensory physiology including itch and pain as well as vision, taste, olfaction, hearing, touch, and

thermosensation. Recent studies have revealed that several thermo-sensitive TRP channels are implicated in itch (Akiyama and Carstens, 2013). TRPV1 is activated by noxious heat ( $\geq 43$  °C) and is required for itch evoked by histamine and IL-31 (Cevikbas *et al.*, 2014; Imamachi *et al.*, 2009). Rodent TRPA1 has been reported to respond to cold temperatures (below 17-18°C) (Chen *et al.*, 2013a) and is required for itch evoked by chloroquine, IL-31, thymic stromal lymphopoietin, endothelin-1, and bile acids (Cevikbas *et al.*, 2014; Kido-Nakahara *et al.*, 2014; Lieu *et al.*, 2014; Wilson *et al.*, 2011; Wilson *et al.*, 2013). TRPV3 is activated by warm temperatures (range 33-39 °C). Mice harboring a gain-of-function mutation in TRPV3 developed dermatitis accompanied by itch behavior (Yoshioka *et al.*, 2009). TRPV4 is another TRP channel activated by moderately warm temperatures (range 27-34 °C) and is expressed in sensory neurons as well as keratinocytes in the skin. TRPV4 mRNA was upregulated in skin with itching burn scars (Yang *et al.*, 2015) and in photodermatitis (Moore *et al.*, 2013). However, the role of TRPV4 in itch is largely unknown. We tested if TRPV4 is required for certain types of itch in mice and demonstrate that TRPV4 is required for the transmission of serotonin-induced itch, but not of three other tested pruritogens.

## Results

5-HT evoked itch is dependent on TRPV4 *in vivo*. Scratching elicited by 5-HT, but not the other pruritogens (histamine, SLIGRL, chloroquine) was significantly reduced in the rostral back model (Fig. 1a). Interestingly, chloroquine-evoked scratching was significantly enhanced in TRPV4KO mice (Fig. 1a). In the present study, we did not further investigate the mechanisms underlying this enhancement. 5-HT-elicited

scratching in TRPA1KO and TRPV1KO mice was not significantly different compared to that in WT mice (Fig. 1a). In the cheek model, 5-HT predominantly elicited scratching which was significantly diminished in TRPV4KO mice (Fig. 1b). These findings suggest that 5-HT-elicited scratching requires TRPV4 *in vivo*.

To confirm the role of TRPV4 in 5-HT-evoked itch, a pharmacological approach was also used. The TRPV4 antagonist HC067047 significantly inhibited 5-HT-evoked scratching (Fig. 2). 5-HT-evoked scratching was not significantly affected by pretreatment with the H1 histamine receptor antagonist terfenadine, but was significantly inhibited by pretreatment with the 5-HT<sub>2</sub> antagonist, ketanserin (Fig. 2). These results are consistent with previous reports (Akiyama *et al.*, 2012; Yamaguchi *et al.*, 1999).

Calcium imaging. To investigate whether 5-HT-responsive cells express TRPV4, calcium imaging of DRG cells were performed, followed by immunostaining. The specificity of the TRPV4 antibody was confirmed by detecting immunoreactivity of TRPV4 in DRG cells of WT mice (Fig. 3b), but not TRPV4KO mice (Fig. 3c, n=129). To investigate whether 5-HT-responsive cells express TRPV4, calcium imaging of DRG cells were performed, followed by immunostaining. Eighteen percent (206/1131) of all DRG cells from WT mice were immunopositive for the TRPV4 antibody. Of those DRG cells shown by calcium imaging to respond to 5-HT, 93% (15/16) were immunopositive for TRPV4 (Fig. 3a). Eighty one percent of TRPV4-immunoreactive DRG cells were unresponsive to 5-HT.

We compared 5-HT-evoked responses in DRG neurons isolated from TRPV1KO, TRPA1KO, and TRPV4KO mice with those isolated from WT mice. DRG cells isolated from TRPV4KO mice showed a significant decrease in the proportion that were 5-HT-

sensitive (2/112). In contrast, there were no significant differences in the proportions of 5-HT-sensitive DRG neurons isolated from TRPA1KO mice (26/423) and TRPV1KO (9/205) compared with those from WT mice (10/202, Fig. 4). DRG cells isolated from TRPV4KO mice showed no changes in the proportions of histamine-, chloroquine- or SLIGRL-sensitive neurons compared to those from WT mice (Fig. 4).

A pharmacological approach was again taken to confirm whether TRPV4 functions downstream of 5-HT receptor activation in DRG neurons. Responses of DRG neurons to 5-HT were markedly reduced by application of the TRPV4 antagonist (Fig. 5a). Fig. 5b also shows that the TRPV4 antagonist significantly reduced the mean 5-HT-evoked responses. In contrast, controls showed no decline in successive 5-HT-evoked responses (Fig. 5c). The majority of 5-HT-sensitive DRG neurons responded to capsaicin (TRPV1 agonist), hypotonic solution (TRPV4 activator) and mustard oil (TRPA1 agonist) (Fig. 5d), while  $\leq 30\%$  of 5-HT-sensitive DRG cells responded to eugenol or carvacol, agonists of TRPV3/TRPA1. The majority of histamine-sensitive DRG neurons responded to capsaicin (Fig. 5e), while the majority of chloroquine-sensitive DRG neurons responded to mustard oil (Fig. 5f). The majority of SLIGRL-sensitive DRG neurons responded to both capsaicin and mustard oil (Fig. 5g).

## Discussion

Chronic itch is the most frequent symptom in dermatology with a significant impact on patients' quality of life. Defining the mediators, receptors, circuits of receptor interactions and signaling pathways involved in histamine-independent itch may lead to novel therapies for this unmet medical need in dermatological therapy. In contrast to

TRPV1, TRPV3 and TRPA1, the role of TRPV4 in the regulation of itch is currently unknown. Our findings support the hypothesis that TRPV4 mediates itch evoked by 5-HT, but not by histamine, chloroquine or the PAR2/MrgprA3 agonist SLIGRL. This conclusion is supported by the following: (1) 5-HT-evoked scratching was suppressed in mice genetically lacking TRPV4 as well as mice treated with a TRPV4 antagonist. (2) Calcium imaging studies of DRG neurons provided confirming results. (3) Over 90% of 5-HT-sensitive DRG cells expressed TRPV4. Given that 5-HT is upregulated in lesional skin of atopic dermatitis, psoriasis and contact dermatitis (El-Nour *et al.*, 2007; Huang *et al.*, 2004a, b; Nordlind *et al.*, 2008), TRPV4 antagonists may be useful in treating chronic itch associated with these skin conditions.

Additionally, we demonstrate that TRPV4KO mice did not exhibit any reduction in scratching evoked by chloroquine, histamine, or SLIGRL. It was previously reported that histamine-evoked scratching is mediated by TRPV1 and that chloroquine- and SLIGRL-evoked scratching requires TRPA1 (Imamachi *et al.*, 2009; Liu *et al.*, 2011; Wilson *et al.*, 2011). TRPV1 is not required for 5-HT-evoked scratching (Imamachi *et al.*, 2009), consistent with our present data. TRPA1 is required for scratching evoked by a 5-HT<sub>7</sub> agonist, but not a 5-HT<sub>2</sub> agonist (Liu *et al.*, 2013; Morita *et al.*, 2015). Although we did not observe a reduction of 5-HT-evoked scratching in TRPA1KO mice, TRPA1 might be involved in 5-HT-evoked itch. A previous study showed that several TRP channels modulate itch signaling evoked by chloroquine (Than *et al.*, 2013). Itch evoked by 5-HT could be modulated by several TRP channels, including TRPV4 and TRPA1.

TRPV4 expression has been demonstrated in DRG and trigeminal ganglion (TG) neurons, and in cutaneous nerves (Chen *et al.*, 2013b; Girard *et al.*, 2013; Gopinath *et al.*,

2005; Suzuki *et al.*, 2003; Vergnolle *et al.*, 2010; Wang *et al.*, 2015; Yamada *et al.*, 2009). While large DRG and TG neurons express TRPV4 (Chen *et al.*, 2013b; Suzuki *et al.*, 2003), small DRG and TG (peptidergic) neurons expressing calcitonin gene related peptide (CGRP) and/or substance P (SP) also express TRPV4 (Chen *et al.*, 2013b; Moore *et al.*, 2013; Vergnolle *et al.*, 2010). In the present study, we found that the majority of 5-HT-sensitive DRG neurons express TRPV4. Although there is currently no direct evidence that any type of serotonin receptor is coupled with TRPV4, it was reported that a 5-HT-induced  $Ca^{2+}$  response is mediated by TRPV4 in pulmonary arterial smooth muscle cells (Xia *et al.*, 2013). 5-HT<sub>2A</sub> and 5-HT<sub>1B</sub> receptor subtypes are presumably involved in this response (Rodat-Despoix *et al.*, 2008). In mouse DRG neurons, 5-HT sensitized neuronal responses to 4 $\alpha$ PDD, a selective TRPV4 agonist, through phospholipase C and protein kinase C-dependent pathways (Cenac *et al.*, 2010). These findings imply that certain serotonin receptors are coupled with TRPV4.

The present findings confirm that 5-HT-evoked itch is histamine-independent (Akiyama *et al.*, 2012; Imamachi *et al.*, 2009). DRG neurons express 5-HT<sub>1B</sub>, 5-HT<sub>1D</sub>, 5-HT<sub>1F</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>3</sub>, 5-HT<sub>4</sub>, 5-HT<sub>5B</sub> and 5-HT<sub>7</sub> receptor subtypes (Ohta *et al.*, 2006). The 5-HT receptor subtypes involved in itch are currently under investigation. We presently observed that the 5-HT<sub>2</sub> antagonist ketanserin abolished 5-HT-evoked scratching, consistent with a previous study (Yamaguchi *et al.*, 1999). A recent study reported that a 5-HT<sub>3</sub> antagonist inhibited 5-HT-evoked scratching in mice (Ostadhadi *et al.*, 2015). However, previous studies showed that 5-HT<sub>3</sub> antagonists failed to inhibit 5-HT-evoked scratching in mice and rats, and itch in humans (Nojima and Carstens, 2003; Weisshaar *et al.*, 1997; Yamaguchi *et al.*, 1999), suggesting that 5-HT<sub>3</sub> is less likely

involved in 5-HT-evoked itch. A very recent study suggests that 5-HT<sub>7</sub> is involved in 5-HT-evoked itch (Morita *et al.*, 2015). There is no report regarding the effects of the 5-HT<sub>2</sub> antagonist or the 5-HT<sub>7</sub> antagonist on 5-HT-evoked itch in humans.

TRPV4 is largely expressed in skin epidermal keratinocytes and plays a role in epidermal barrier homeostasis (Kida *et al.*, 2012), inflammation and photodermatitis-associated pain (Moore *et al.*, 2013). Keratinocytes also express 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, and 5-HT<sub>7</sub> receptor subtypes (Slominski *et al.*, 2003), suggesting that 5-HT may be able to activate keratinocytes in conjunction with TRPV4. A recent study also reported that 5-HT stimulates IL-6 release from primary human keratinocytes (Kaneko *et al.*, 2009). It is thus possible that 5-HT could activate one or more 5-HT subtypes coupled with TRPV4, as noted above, to evoke release of substances from keratinocytes that could act on anatomically closely associated sensory nerve endings. A recent study revealed that activation of TRPV4 in keratinocytes contributes to release of endothelin-1 (ET-1), another important pruritogen (Moore *et al.*, 2013). However, ET-1 is unlikely to be involved in 5-HT-evoked itch, as ET-1-evoked itch is mediated by TRPA1, but not TRPV4 (Kido-Nakahara *et al.*, 2014).

In summary, we demonstrate that TRPV4 is involved in histamine-independent itch associated with serotonin, and is thus linked to activation of one or more 5-HT receptor subtypes. In contrast, TRPV4 is not linked to other pruritogen receptors for histamine, chloroquine or PAR2/ MrgprA3 agonists. Further studies are demanded to fully explore to which pruritic pathways TRPV4 is linked. Furthermore, it will be significant to clarify the role of TRPV4 in pruritic diseases in humans.

## Materials and Methods

Behavioral tests. Experiments were performed using adult male knockout mice lacking TRPV4 (TRPV4KO), TRPV1 (TRPV1KO), TRPA1 (TRPA1KO), and wild-type (WT; littermates of TRPV4KO mice; C57BL/6 mice) (19-34 g) under a protocol approved by the UC Davis Animal Care and Use Committee. Knockout mice were maintained under specific pathogen-free conditions and were bred by backcrossing with C57BL/6 mice. The fur on the rostral back or the cheek was shaved and mice were habituated to the Plexiglas recording arena one week prior to testing. For intraperitoneal (ip) injections, either terfenadine (30 mg/kg; Sigma-Aldrich, St. Louis, MO) (Akiyama *et al.*, 2012), ketanserin (3 mg/kg; Sigma-Aldrich) (Yamaguchi *et al.*, 1999), HC067047 (10 mg/kg; Tocris Bioscience) (Everaerts *et al.*, 2010), or vehicle (10% DMSO+10% Tween80 in saline) was administered, followed 30 min later by intradermal (id) injection (10  $\mu$ l) of either chloroquine (193 nmol; Sigma-Aldrich), histamine (271 nmol; Sigma-Aldrich), serotonin (47 nmol; Sigma-Aldrich), or SLIGRL (76 nmol; Quality Controlled Biochemicals, Hopkinton, MA). Microinjections were made id in the nape of the neck or cheek using a 30 G needle attached to a Hamilton microsyringe by PE-50 tubing. Immediately after the id injection, mice were placed into the arena and videotaped from above for 30 min. Generally 3–4 mice were injected and videotaped simultaneously. Immediately after commencing videotaping, all investigators left the room. Videotapes were reviewed by investigators blinded to the treatment, and the number of scratch bouts was counted at 5-min intervals. A scratch bout was defined as one or more rapid back-and-forth hind paw motion(s) directed toward and contacting the injection site, and ending with licking or biting of the toes and/or placement of the hind paw on the floor.

Hind paw movements directed away from the injection site (e.g., ear-scratching) and grooming movements were not counted. A wipe was defined as a singular motion of the ipsilateral, but not bilateral, forelimb beginning at the caudal extent of the injected cheek, and proceeding in a rostral direction. The inner aspect of the ankle and/or forelimb typically contacted the cheek with the paw closed. One-way ANOVA followed by the Bonferroni post-test or unpaired t-tests (two-tailed) was used to compare the total number of scratch bouts across pretreatment groups. In all cases  $p < 0.05$  was considered to be significant.

Calcium imaging. Experiments were performed using adult male TRPV4KO mice, TRPA1KO mice, TRPV1KO mice, and WT (C57BL/6) mice (18–34 g) under a protocol approved by the UC Davis Animal Care and Use Committee. The animal was euthanized under sodium pentobarbital anesthesia, and upper- to mid-cervical DRGs were acutely dissected and enzymatically digested at 37°C for 10 min in HBSS (Invitrogen, Carlsbad, CA) containing 20 U/ml papain (Worthington Biochemical, Lakewood, NJ) and 6.7 mg/ml L-cysteine (Sigma, St. Louis, MO), followed by 10 min at 37°C in HBSS containing 3 mg/ml collagenase (Worthington Biochemical). The ganglia were then mechanically triturated using fire-polished glass pipettes. DRG cells were pelleted; suspended in MEM with Earle's balanced salt solution (Gibco, Life Technologies, Carlsbad, CA) containing 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Life Technologies), 1× vitamin (Gibco, Life Technologies), and 10% horse serum (Quad Five, Ryegate, MT); plated on poly-d-lysine-coated glass coverslips; and cultured for 16–24 h.

DRG cells were incubated in Ringer's solution (pH 7.4, 140 mM NaCl, 4 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, and 4.54 mM NaOH) with 10 µM Fura-2

AM and 0.05% of Pluronic F-127 (Invitrogen). Coverslips were mounted on a custom-made aluminum perfusion block and viewed through an inverted microscope (Nikon TS100, Technical Instruments, Burlingame, CA). Fluorescence was excited by UV light at 340 nm and 380 nm alternately, and emitted light was collected via a CoolSNAP camera attached to a Lambda LS lamp and a Lambda optical filter changer (Sutter Instrument, Novato, CA). Ratiometric measurements were made using Simple PCI software (Hamamatsu, Sewickley, PA) every 3 s.

Solutions were delivered by a solenoid-controlled eight-channel perfusion system (ValveLink, AutoMate Scientific, San Francisco, CA). One of the following agents was delivered: chloroquine (300  $\mu\text{M}$ ), histamine (100  $\mu\text{M}$ ), 5-HT (100  $\mu\text{M}$ ) and the PAR-2/MrgprA3 agonist SLIGRL-NH<sub>2</sub> (100  $\mu\text{M}$ ). In some experiments, one of the following agents was delivered after the delivery of pruritogen: capsaicin (1  $\mu\text{M}$ ), eugenol (100  $\mu\text{M}$ ), carvacol (100  $\mu\text{M}$ ), 30% hypotonic solution, and mustard oil (100  $\mu\text{M}$ ). Stimulus duration was 30 s (10 s for capsaicin). In other experiments, 5-HT was delivered three times at 3.5-min intervals. HC067047 (1  $\mu\text{M}$ ) (Everaerts *et al.*, 2010) was delivered beginning 10 s prior to the second 5-HT application for a duration of 2 min. Potassium chloride (144 mM) was always delivered at the end of each experiment. Ratios were normalized to baseline. Cells were judged to be sensitive if the ratio value increased by >10% of the resting level following chemical application. Only cells responsive to high potassium were included for analysis. After the experiment, coverslips were etched with a diamond pen to provide landmarks for alignment with subsequent immunohistofluorescent labeling of the same cells. Fisher's exact test was used to compare the percentages of pruritogen-sensitive cells between groups.

Immunocytochemistry. After calcium imaging, DRG cells in the culture dish were fixed in formalin, followed by 30% sucrose, and then incubated with 5% normal serum. They were immunostained with rabbit anti-TRPV4 antibody (1:300; Abcam, Cambridge MA) at 4°C overnight, followed by incubation with the corresponding secondary antibody conjugated with Alexa Fluor 488 (1:500; Life Technologies, Grand Island, NY) for 2 h. DRG cells were counterstained with DAPI in the mounting medium (Vector Laboratories, Burlingame, CA). Images were captured using a fluorescence microscope (Nikon Eclipse Ti; Technical Instruments). Immunohistofluorescent images were aligned with images captured during calcium imaging to determine the percentages of 5-HT-responsive DRG cells that were double labeled for TRPV4.

#### **Conflict of Interest**

None of the authors declares any conflict of interest.

#### **Acknowledgments**

The work was supported by grants from the National Institutes of Health DE013685 (TO EEC), AR057194 (TO EEC), AR063228 (TO TA), AR059402-01 (TO MS), and SFI senior investigator award (TO MS). The authors thank Dr. Tudor Selescu and Dr. Oliver Bogen for their helpful comments. The TRPV4KO mice were kindly provided by Dr. J. Levine. The TRPV1KO mice and the TRPA1KO mice were kindly provided by Dr. D. Julius.

## References

- Akiyama T, Carstens E (2013) Neural processing of itch. *Neuroscience* 250:697-714.
- Akiyama T, Carstens E (2014) Spinal Coding of Itch and Pain. In: *Itch: Mechanisms and Treatment* (Carstens E, Akiyama T, eds), Boca Raton (FL).
- Akiyama T, Carstens MI, Ikoma A, *et al.* (2012) Mouse model of touch-evoked itch (alloknesis). *J Invest Dermatol* 132:1886-91.
- Akiyama T, Merrill AW, Zanotto K, *et al.* (2009) Scratching behavior and Fos expression in superficial dorsal horn elicited by protease-activated receptor agonists and other itch mediators in mice. *J Pharmacol Exp Ther* 329:945-51.
- Bell JK, McQueen DS, Rees JL (2004) Involvement of histamine H4 and H1 receptors in scratching induced by histamine receptor agonists in Balb C mice. *Br J Pharmacol* 142:374-80.
- Cenac N, Altier C, Motta JP, *et al.* (2010) Potentiation of TRPV4 signalling by histamine and serotonin: an important mechanism for visceral hypersensitivity. *Gut* 59:481-8.
- Cevikbas F, Wang X, Akiyama T, *et al.* (2014) A sensory neuron-expressed IL-31 receptor mediates T helper cell-dependent itch: Involvement of TRPV1 and TRPA1. *J Allergy Clin Immunol* 133:448-60.
- Chen J, Kang D, Xu J, *et al.* (2013a) Species differences and molecular determinant of TRPA1 cold sensitivity. *Nature communications* 4:2501.
- Chen Y, Williams SH, McNulty AL, *et al.* (2013b) Temporomandibular joint pain: a critical role for Trpv4 in the trigeminal ganglion. *Pain* 154:1295-304.
- Dvorak AM (1998) Histamine content and secretion in basophils and mast cells. *Prog Histochem Cytochem* 33:III-IX, 169-320.
- El-Nour H, Lundeberg L, Abdel-Magid N, *et al.* (2007) Serotonergic mechanisms in human allergic contact dermatitis. *Acta Derm Venereol* 87:390-6.
- Everaerts W, Zhen X, Ghosh D, *et al.* (2010) Inhibition of the cation channel TRPV4 improves bladder function in mice and rats with cyclophosphamide-induced cystitis. *Proc Natl Acad Sci U S A* 107:19084-9.
- Girard BM, Merrill L, Malley S, *et al.* (2013) Increased TRPV4 expression in urinary bladder and lumbosacral dorsal root ganglia in mice with chronic overexpression of NGF in urothelium. *Journal of molecular neuroscience : MN* 51:602-14.

- Gopinath P, Wan E, Holdcroft A, *et al.* (2005) Increased capsaicin receptor TRPV1 in skin nerve fibres and related vanilloid receptors TRPV3 and TRPV4 in keratinocytes in human breast pain. *BMC women's health* 5:2.
- Han L, Ma C, Liu Q, *et al.* (2013) A subpopulation of nociceptors specifically linked to itch. *Nat Neurosci* 16:174-82.
- Hosogi M, Schmelz M, Miyachi Y, *et al.* (2006) Bradykinin is a potent pruritogen in atopic dermatitis: a switch from pain to itch. *Pain* 126:16-23.
- Huang J, Li G, Xiang J, *et al.* (2004a) Immunohistochemical study of serotonin in lesions of chronic eczema. *Int J Dermatol* 43:723-6.
- Huang J, Li G, Xiang J, *et al.* (2004b) Immunohistochemical study of serotonin in lesions of psoriasis. *Int J Dermatol* 43:408-11.
- Imamachi N, Park GH, Lee H, *et al.* (2009) TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms. *Proc Natl Acad Sci U S A* 106:11330-5.
- Inami Y, Andoh T, Sasaki A, *et al.* (2013) Topical surfactant-induced pruritus: involvement of histamine released from epidermal keratinocytes. *J Pharmacol Exp Ther* 344:459-66.
- Kaneko K, Travers JB, Matsui MS, *et al.* (2009) cis-Urocanic acid stimulates primary human keratinocytes independently of serotonin or platelet-activating factor receptors. *J Invest Dermatol* 129:2567-73.
- Kida N, Sokabe T, Kashio M, *et al.* (2012) Importance of transient receptor potential vanilloid 4 (TRPV4) in epidermal barrier function in human skin keratinocytes. *Pflugers Arch* 463:715-25.
- Kido-Nakahara M, Buddenkotte J, Kempkes C, *et al.* (2014) Neural peptidase endothelin-converting enzyme 1 regulates endothelin 1-induced pruritus. *J Clin Invest* 124:2683-95.
- Kushnir-Sukhov NM, Brown JM, Wu Y, *et al.* (2007) Human mast cells are capable of serotonin synthesis and release. *J Allergy Clin Immunol* 119:498-9.
- Lieu T, Jayaweera G, Zhao P, *et al.* (2014) The bile acid receptor TGR5 activates the TRPA1 channel to induce itch in mice. *Gastroenterology* 147:1417-28.
- Liu B, Escalera J, Balakrishna S, *et al.* (2013) TRPA1 controls inflammation and pruritogen responses in allergic contact dermatitis. *FASEB J* 27:3549-63.

Liu Q, Weng HJ, Patel KN, *et al.* (2011) The distinct roles of two GPCRs, MrgprC11 and PAR2, in itch and hyperalgesia. *Sci Signal* 4:ra45.

Moore C, Cevikbas F, Pasolli HA, *et al.* (2013) UVB radiation generates sunburn pain and affects skin by activating epidermal TRPV4 ion channels and triggering endothelin-1 signaling. *Proc Natl Acad Sci U S A* 110:E3225-34.

Morita T, McClain SP, Batia LM, *et al.* (2015) HTR7 Mediates Serotonergic Acute and Chronic Itch. *Neuron* 87:124-38.

Nojima H, Carstens E (2003) 5-Hydroxytryptamine (5-HT)<sub>2</sub> receptor involvement in acute 5-HT-evoked scratching but not in allergic pruritus induced by dinitrofluorobenzene in rats. *J Pharmacol Exp Ther* 306:245-52.

Nordlind K, Azmitia EC, Slominski A (2008) The skin as a mirror of the soul: exploring the possible roles of serotonin. *Exp Dermatol* 17:301-11.

Ohta T, Ikemi Y, Murakami M, *et al.* (2006) Potentiation of transient receptor potential V1 functions by the activation of metabotropic 5-HT receptors in rat primary sensory neurons. *J Physiol* 576:809-22.

Ostadhadi S, Kordjazy N, Haj-Mirzaian A, *et al.* (2015) 5-HT<sub>3</sub> receptors antagonists reduce serotonin-induced scratching in mice. *Fundamental & clinical pharmacology*.

Rodat-Despoix L, Crevel H, Marthan R, *et al.* (2008) Heterogeneity in 5-HT-induced contractile and proliferative responses in rat pulmonary arterial bed. *Journal of vascular research* 45:181-92.

Slominski A, Pisarchik A, Zbytek B, *et al.* (2003) Functional activity of serotonergic and melatonergic systems expressed in the skin. *J Cell Physiol* 196:144-53.

Steinhoff M, Neisius U, Ikoma A, *et al.* (2003) Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. *J Neurosci* 23:6176-80.

Suzuki M, Watanabe Y, Oyama Y, *et al.* (2003) Localization of mechanosensitive channel TRPV4 in mouse skin. *Neurosci Lett* 353:189-92.

Than JY, Li L, Hasan R, *et al.* (2013) Excitation and modulation of TRPA1, TRPV1, and TRPM8 channel-expressing sensory neurons by the pruritogen chloroquine. *J Biol Chem* 288:12818-27.

Turetta L, Donella-Deana A, Folda A, *et al.* (2004) Characterisation of the serotonin efflux induced by cytosolic Ca<sup>2+</sup> and Na<sup>+</sup> concentration increase in human platelets. *Cell Physiol Biochem* 14:377-86.

- Vergnolle N, Cenac N, Altier C, *et al.* (2010) A role for transient receptor potential vanilloid 4 in tonic-induced neurogenic inflammation. *Br J Pharmacol* 159:1161-73.
- Wang J, Wang XW, Zhang Y, *et al.* (2015) Ca<sup>2+</sup> influx mediates the TRPV4-NO pathway in neuropathic hyperalgesia following chronic compression of the dorsal root ganglion. *Neurosci Lett* 588:159-65.
- Weisshaar E, Dunker N, Rohl FW, *et al.* (2004) Antipruritic effects of two different 5-HT<sub>3</sub> receptor antagonists and an antihistamine in haemodialysis patients. *Exp Dermatol* 13:298-304.
- Weisshaar E, Ziethen B, Gollnick H (1997) Can a serotonin type 3 (5-HT<sub>3</sub>) receptor antagonist reduce experimentally-induced itch? *Inflamm Res* 46:412-6.
- Wilson SR, Gerhold KA, Bifulck-Fisher A, *et al.* (2011) TRPA1 is required for histamine-independent, Mas-related G protein-coupled receptor-mediated itch. *Nat Neurosci* 14:595-602.
- Wilson SR, The L, Batia LM, *et al.* (2013) The epithelial cell-derived atopic dermatitis cytokine TSLP activates neurons to induce itch. *Cell* 155:285-95.
- Xia Y, Fu Z, Hu J, *et al.* (2013) TRPV4 channel contributes to serotonin-induced pulmonary vasoconstriction and the enhanced vascular reactivity in chronic hypoxic pulmonary hypertension. *American journal of physiology Cell physiology* 305:C704-15.
- Yamada T, Ugawa S, Ueda T, *et al.* (2009) Differential localizations of the transient receptor potential channels TRPV4 and TRPV1 in the mouse urinary bladder. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* 57:277-87.
- Yamaguchi T, Nagasawa T, Satoh M, *et al.* (1999) Itch-associated response induced by intradermal serotonin through 5-HT<sub>2</sub> receptors in mice. *Neurosci Res* 35:77-83.
- Yang YS, Cho SI, Choi MG, *et al.* (2015) Increased expression of three types of transient receptor potential channels (TRPA1, TRPV4 and TRPV3) in burn scars with post-burn pruritus. *Acta Derm Venereol* 95:20-4.
- Yoshioka T, Imura K, Asakawa M, *et al.* (2009) Impact of the Gly573Ser substitution in TRPV3 on the development of allergic and pruritic dermatitis in mice. *J Invest Dermatol* 129:714-22.

## Figures and Figure Legends

**Fig. 1. *TRPV4KO mice are insensitive to 5-HT-mediated itch.*** (a) Saline, histamine (50  $\mu\text{g}/10 \mu\text{l}$ ), chloroquine (CQ, MrgprA3 agonist, 100  $\mu\text{g}/10 \mu\text{l}$ ), SLIGRL-NH2 (PAR-2/MrgprC11 agonist, 50  $\mu\text{g}/10 \mu\text{l}$ ), or 5-HT (10  $\mu\text{g}/10 \mu\text{l}$ ) were injected intradermally in the rostral back. Pruritogen-elicited scratch bouts were counted in WT (open bars) and TRPV4KO mice (black bars) over a 30 min period. 5-HT-elicited scratching was significantly diminished in TRPV4 KO mice, but not TRPV1KO mice or TRPA1KO mice. Error bars are S.E.M. \*  $p < 0.05$ , significant difference from WT group (unpaired t-test or 1-way analysis of variance followed by the Bonferroni test,  $n = 6/\text{group}$ ). (b) 5-HT (10  $\mu\text{g}/10 \mu\text{l}$ ) was injected intradermally into the cheek. Hindlimb scratch bouts and ipsilateral forelimb wipes directed to the cheek were counted over 30 min. \*  $p < 0.05$ , significant difference from WT group (unpaired t-test,  $n = 4/\text{group}$ ).

**Fig. 2. *TRPV4 antagonist inhibits 5-HT-evoked scratching.*** Terfenadine (histamine H1 receptor antagonist), ketanserin (5-HT<sub>2</sub> receptor antagonist), HC067047 (TRPV4 antagonist) or vehicle were administered ip 30 min prior to an intradermal injection of 5-HT. 5-HT-evoked scratching was significantly diminished in mice treated with ketanserin and HC067047. Error bars are S.E.M. \*  $p < 0.05$ , significant difference from WT group (1-way analysis of variance followed by the Bonferroni test,  $n = 6/\text{group}$ ).

**Fig. 3. *TRPV4 is expressed in 5-HT-sensitive DRG cells.*** (a) Cultured DRG cells from naïve WT mice were exposed to 5-HT (100  $\mu\text{M}$ ) followed by high potassium solution (HiK). Responses were measured by Fura-2 ratiometric calcium imaging, followed by

immunostaining with a TRPV4 antibody. Bottom panels show, from left to right, pre-5-HT, post 5-HT and post HiK responses, and TRPV4 immunostaining. 5-HT-sensitive DRG cells expressed TRPV4 (yellow circles). Scale bar = 50  $\mu$ m. (b) Cultured DRG cells from naïve WT mice were immunostained with a TRPV4 antibody (green). Blue indicates nuclei (DAPI). Scale bar = 50  $\mu$ m. (c) As in b for TRPV4KO mice.

**Fig. 4. Incidence of 5-HT-sensitive DRG cells was decreased in TRPV4KO mice.**

Graph plots percentages of cultured DRG cells from wild type (WT) mice (open bars) and TRPV4KO mice (black bars) that responded to 5-HT (100  $\mu$ M), histamine (HIS, 100  $\mu$ M), chloroquine (CQ, 300  $\mu$ M) or SLIGRL-NH<sub>2</sub> (100  $\mu$ M). The percentage of 5-HT-sensitive cells was significantly lower in DRG cells from TRPV4KO mice (\*  $p < 0.05$ , Fisher's exact test) relative to WT DRG cells ( $n=112-423$ ). In contrast, the percentages of cells responsive to histamine, chloroquine and SLIGRL were similar for DRG cells from WT compared to TRPV4KO mice ( $n=124-266$ ).

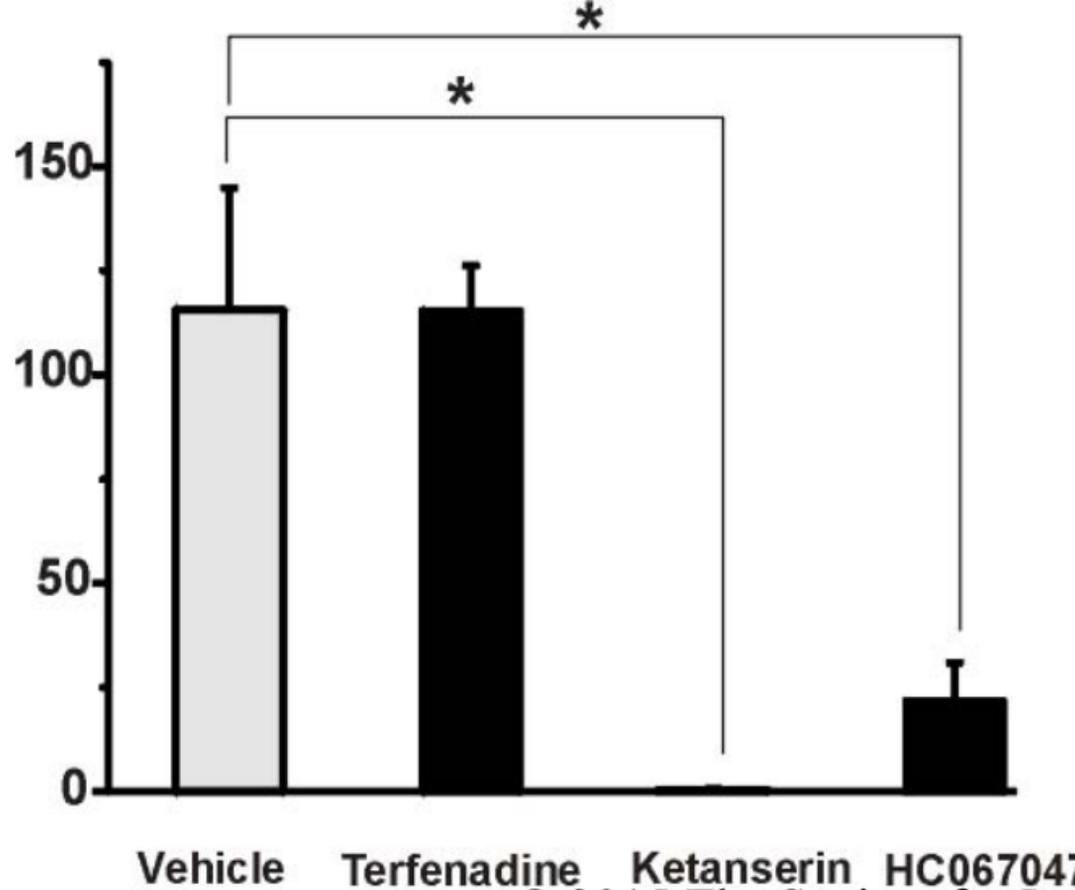
**Fig. 5. TRPV4 antagonist inhibited 5-HT-elicited responses of DRG cells.**

(a) Cultured DRG cells isolated from naïve WT mice were exposed to 5-HT (100  $\mu$ M) three times. Responses were measured by Fura-2 ratiometric calcium imaging. Application of the TRPV4 antagonist, HC 067047 (1  $\mu$ M), reduced the response to 5-HT (middle trace). (b) Overall percent reduction by TRPV4 antagonist. \*  $p < 0.05$ , significant difference from 1<sup>st</sup> responses (1-way analysis of variance followed by the Bonferroni test,  $n = 8$ ) (c) Vehicle control showing no reduction in successive 5-HT-

evoked responses ( $n = 11$ ). (d) Percentages of cultured 5-HT-sensitive DRG cells from WT mice that responded to capsaicin (TRPV1 agonist), eugenol (TRPV3 agonist), carvacol (TRPV3/TRPA1 agonist), hypotonic solution (TRPV4 activator) or mustard oil (TRPA1 agonist). Most 5-HT-sensitive cells responded to hypotonic solution.  $n=13-17$ . (e) Same as in (d) for histamine-sensitive DRG neurons. Most 5-HT-sensitive cells responded to capsaicin.  $n=16$ . (f) Same as in (d) for chloroquine-sensitive DRG neurons. Most chloroquine-sensitive cells responded to mustard oil.  $n=26$ . (g) Same as in (d) for SLIGRL-sensitive DRG neurons. Most SLIGRL-sensitive cells responded to capsaicin and mustard oil.  $n=11$ .

Accepted manuscript

Scratch bouts/ 30 min

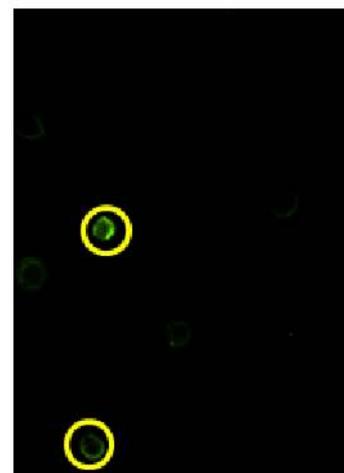
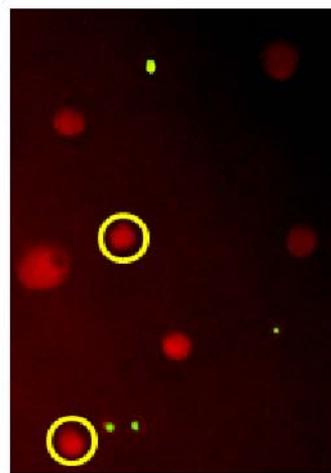
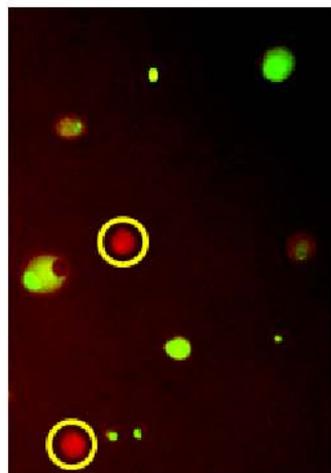
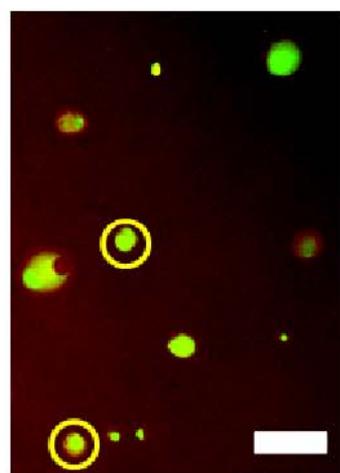
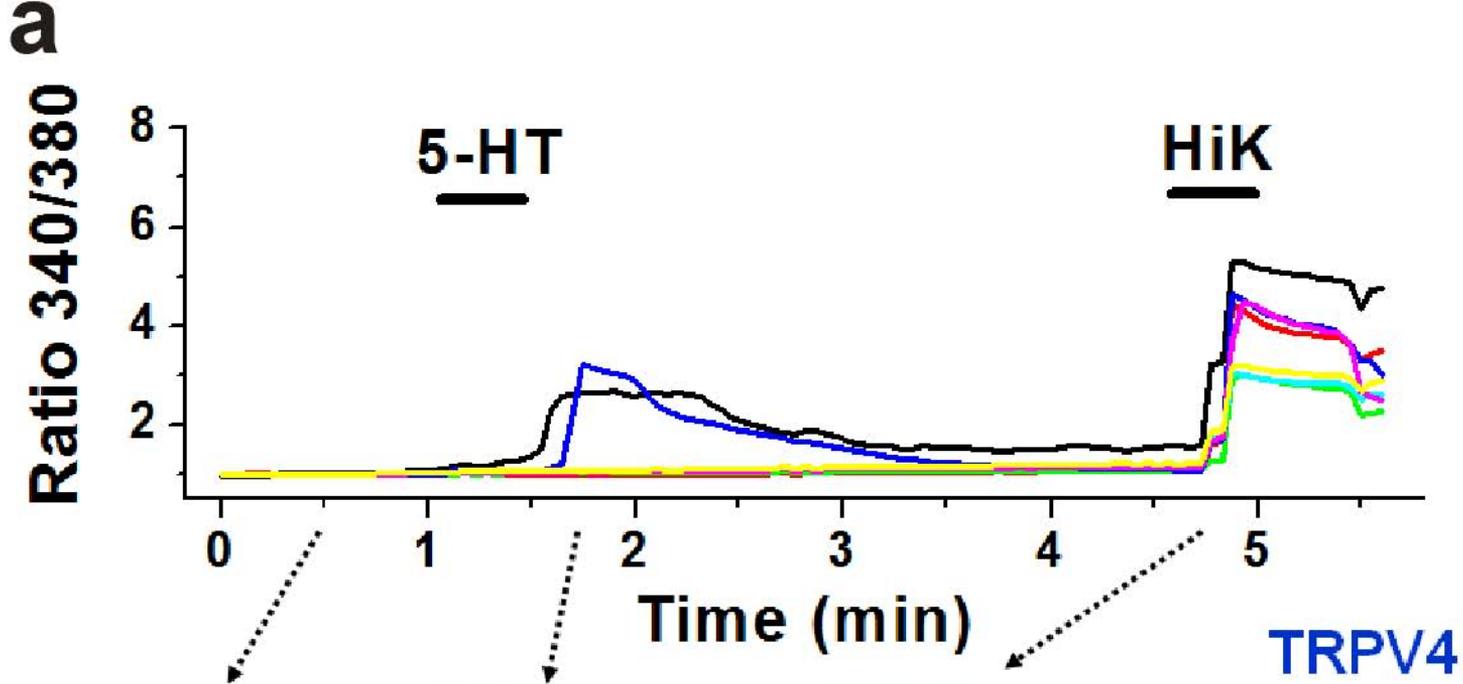


Vehicle

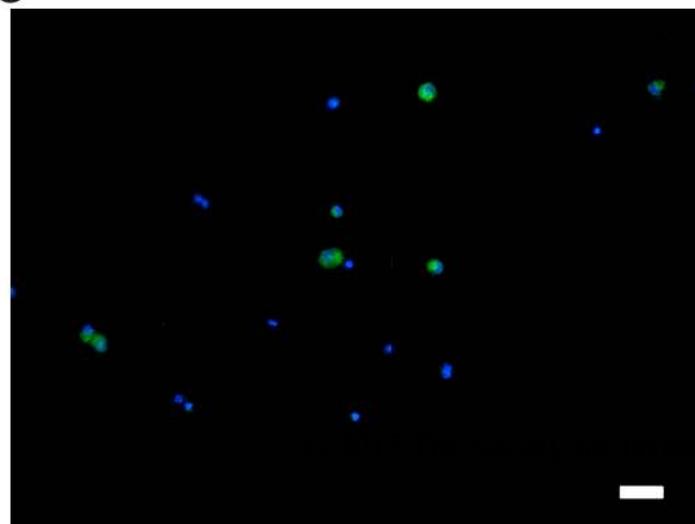
Terfenadine  
H1R

Ketanserin  
5-HT2R

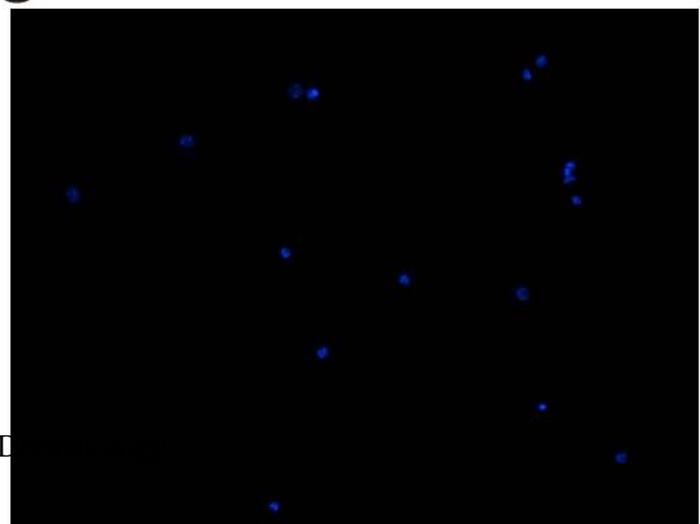
HC067047  
TRPV4



**b**



**c**



igative D

