ORIGINAL ARTICLE

See related commentary on pg 7

Involvement of TRPV4 in Serotonin-Evoked Scratching

Tasuku Akiyama[1,2], Margaret Ivanov[1], Masaki Nagamine[1], Auva Davoodi[1], Mirela I. Carstens[1], Akihiko Ikoma[3], Ferda Cevikbas[3], Cordula Kempkes[3], Joerg Buddenkotte[3,4], Martin Steinhoff[3,4] and E. Carstens[1]

Several thermosensitive transient receptor potential channels (transient receptor potential vanilloid type-1, -3; transient receptor potential cation channel, subfamily A, member 1) 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 (protease-activated receptors 2/mas-related G-protein-coupled receptor C11 agonist), and chloroquine (mas-related G-protein-coupled receptor A3 agonist), were intradermally injected into mice and itch-related scratching behavior was assessed. TRPV4 knockout mice exhibited significantly fewer 5-HT-evoked scratching bouts compared with wild-type mice. Notably, no differences between TRPV4 knockout and wild-type 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 neurons, the response of neurons after 5-HT application, but not other pruritogens, was significantly lower in TRPV4 knockout compared with wild-type mice. A TRPV4 antagonist significantly suppressed 5-HT-evoked responses in dorsal root ganglion cells from wild-type mice. Approximately 90% of 5-HT-sensitive dorsal root ganglion neurons were immunoreactive for an antibody to TRPV4, as assessed by calcium imaging. These results indicate that 5-HT-induced itch is linked to TRPV4.

Journal of Investigative Dermatology (2016) **136,** 154-160; doi:10.1038/JID.2015.388

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-hydroxytryptamine [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). Whereas 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., 1997, 2004). Proteases such as 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, and bovine adrenal medullary peptide 8-22, 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 thermosensitive TRP channels are implicated in itch (Akiyama and Carstens, 2013). Transient receptor potential vanilloid type-1 (TRPV1) is activated by noxious heat (≥ 43 °C) and is required for itch evoked by histamine and IL-31 (Cevikbas et al., 2014; Imamachi et al., 2009). Rodent transient receptor potential cation channel, subfamily A, member 1

---

[1] *Department of Neurobiology, Physiology and Behavior, University of California, Davis, California, USA;* [2] *Department of Dermatology, Department of Anatomy & Cell Biology, Temple Itch Center, Temple University, Philadelphia, Pennsylvania, USA;* [3] *Departments of Dermatology and Surgery, University of California, San Francisco, USA; and* [4] *U Dept. of Dermatology and UCD Charles Institute for Translational Dermatology, University College Dublin, Dublin, Ireland*

Correspondence: Tasuku Akiyama, Departments of Dermatology and Anatomy & Cell Biology and Temple Itch Center, Temple University, 3500 Broad St, Philadelphia, Pennsylvania 19140, USA. E-mail: tasuku.akiyama@temple.edu *or Earl Carstens, Department of Neurobiology, Physiology & Behavior, University of California, Davis, 1 Shields Avenue Davis, California 95616, USA. E-mail:* eecarstens@ucdavis.edu *or Martin Steinhoff, U Dept. of Dermatology and UCD Charles Institute for Translational Dermatology, University College Dublin, Dublin, Ireland. E-mail:* Martin.steinhoff@ucd.ie

Abbreviations: DRG, dorsal root ganglion; 5-HT, 5-hydroxytryptamine (serotonin); Mrgpr, Mas-related G-protein-coupled receptor; PAR, protease-activated receptor; TRP, transient receptor potential; TRPV1, transient receptor potential vanilloid type-1; TRPV4, transient receptor potential vanilloid type-4; TRPV1KO, TRPV1 knockout; TRPV4KO, TRPV4 knockout; WT, wild-type

Received 16 July 2015; revised 26 August 2015; accepted 5 September 2015; accepted manuscript published online 1 October 2015

(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, 2013). Transient receptor potential vanilloid type-3 is activated by warm temperatures (33–39 °C). Mice harboring a gain-of-function mutation in transient receptor potential vanilloid type-3 developed dermatitis accompanied by itch behavior (Yoshioka et al., 2009). TRPV4 is another TRP channel activated by moderately warm temperatures (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 with 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, thus, demonstrated that TRPV4 is required for the transmission of 5-HT-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, and chloroquine) was significantly reduced in the rostral back model (Figure 1a). Interestingly, chloroquine-evoked scratching was significantly enhanced in TRPV4 knockout (TRPV4KO) mice (Figure 1a). In this study, we did not further investigate the mechanisms underlying this enhancement. 5-HT-elicited scratching in TRPA1KO and TRPV1 knockout (TRPV1KO) mice was not significantly different compared with that in wild-type (WT) mice (Figure 1a). In the cheek model, 5-HT predominantly elicited scratching that was significantly diminished in TRPV4KO mice (Figure 1b). These findings suggest that 5-HT-elicted 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 (Figure 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-HT2 antagonist, ketanserin (Figure 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 dorsal root ganglion (DRG) cells was performed, followed by immunostaining. The specificity of the TRPV4 antibody was confirmed by detecting immunoreactivity of TRPV4 in DRG cells of WT mice (Figure 3b), but not TRPV4KO mice (Figure 3c, n = 129). To investigate whether 5-HT-responsive cells express TRPV4, calcium imaging of DRG cells was performed, followed by immunostaining. Eighteen percent (206 of 1,131) of all DRG cells from WT mice were immunopositive for the TRPV4 antibody. Of the DRG cells shown by calcium imaging to respond to 5-HT, 93% (15 of 16) were immunopositive for TRPV4 (Figure 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 of those that were 5-HT sensitive (2 of 112). In contrast, there were no significant differences in the proportions of 5-HT-sensitive DRG neurons isolated from TRPA1KO mice (26 of 423) and TRPV1KO (9 of 205) compared with those from WT mice (10 of 202; Figure 4). DRG cells isolated from TRPV4KO mice showed no changes in the proportions of histamine-, chloroquine-, or SLIGRL-sensitive neurons compared with those from WT mice (Figure 4).

A pharmacological approach was again taken to confirm whether TRPV4 functions downstream of 5-HT receptor

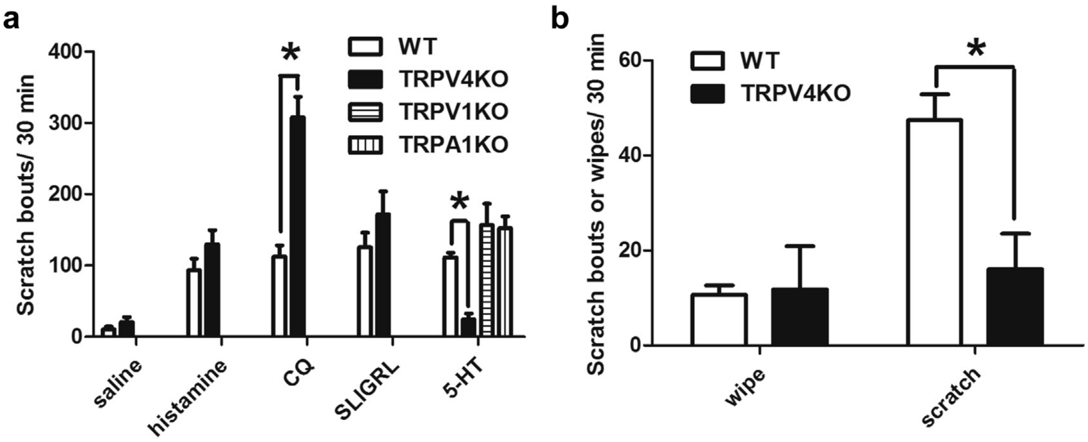

Figure 1. TRPV4KO mice are insensitive to 5-HT-mediated itch. (**a**) Saline, histamine (50 μg/10 μl), chloroquine (CQ, MrgprA3 agonist, 100 μg/10 μl), SLIGRL-NH2 (PAR-2/MrgprC11 agonist, 50 μg/10 μl), or 5-HT (10 μg/10 μl) were injected intradermally in the rostral back. Pruritogen-elicited scratch bouts were counted in WT (open bars) and TRPV4KO mice (black bars) over a 30-minute period. 5-HT-elicited scratching was significantly diminished in TRPV4KO mice, but not in TRPV1KO or TRPA1KO mice. Error bars are SEM. *$P < 0.05$, significant difference from the WT group (unpaired *t*-test or one-way analysis of variance followed by the Bonferroni test, $n = 6$ per group). (**b**) 5-HT (10 μg/10 μl) was injected intradermally into the cheek. Hindlimb scratch bouts and ipsilateral forelimb wipes directed to the cheek were counted over 30 minutes. *$P < 0.05$, significant difference from the WT group (unpaired *t*-test, $n = 4$ per group). 5-HT, 5-hydroxytryptamine (serotonin); PAR, protease-activated receptor; TRPV1KO, TRPV1 knockout; TRPV4KO, TRPV4 knockout; TRPA1KO, TRPA1 knockout; WT, wild-type.

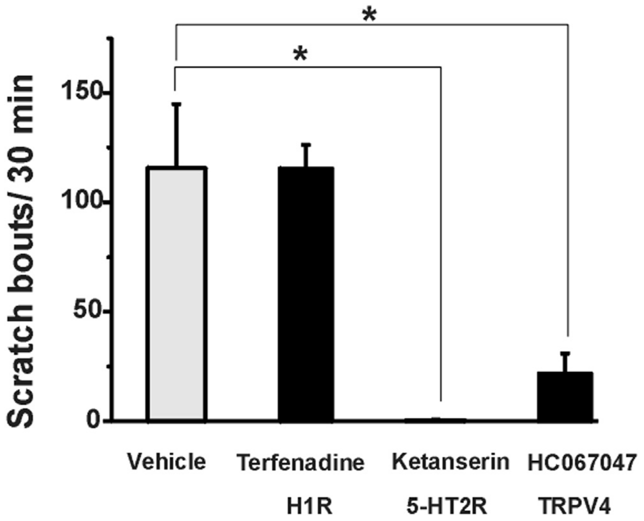

Figure 2. TRPV4 antagonist inhibits 5-HT-evoked scratching. Terfenadine (histamine H1 receptor antagonist), ketanserin (5-HT2 receptor antagonist), HC067047 (TRPV4 antagonist), or vehicle were administered ip 30 minutes before an intradermal injection of 5-HT. 5-HT-evoked scratching was significantly diminished in mice treated with ketanserin and HC067047. Error bars are SEM. *$P < 0.05$, significant difference from the WT group (one-way analysis of variance followed by the Bonferroni test, $n = 6$ per group). 5-HT, 5-hydroxytryptamine (serotonin); ip, intraperitoneal; TRPV4, transient receptor potential vanilloid type-4; WT, wild-type.

activation in DRG neurons. Responses of DRG neurons to 5-HT were markedly reduced by the application of the TRPV4 antagonist (Figure 5a). Figure 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 (Figure 5c). The majority of 5-HT-sensitive DRG neurons responded to capsaicin (TRPV1 agonist), hypotonic solution (TRPV4 activator), and mustard oil (TRPA1 agonist; Figure 5d), whereas $\leq$30% of 5-HT-sensitive DRG cells responded to eugenol or carvacrol, agonists of transient receptor potential vanilloid type-3 and/or TRPA1. The majority of histamine-sensitive DRG neurons responded to capsaicin (Figure 5e), whereas the majority of chloroquine-sensitive DRG neurons responded to mustard oil (Figure 5f). The majority of SLIGRL-sensitive DRG neurons responded to both capsaicin and mustard oil (Figure 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, transient receptor potential vanilloid type-3, 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: (i) 5-HT-evoked scratching was suppressed in mice genetically lacking TRPV4 as well as mice treated with a TRPV4 antagonist. (ii) Calcium imaging studies of DRG neurons provided confirming results. (iii) More than 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, 2004b; Nordlind et al., 2008), TRPV4 antagonists may be useful in treating chronic itch associated with these skin conditions.

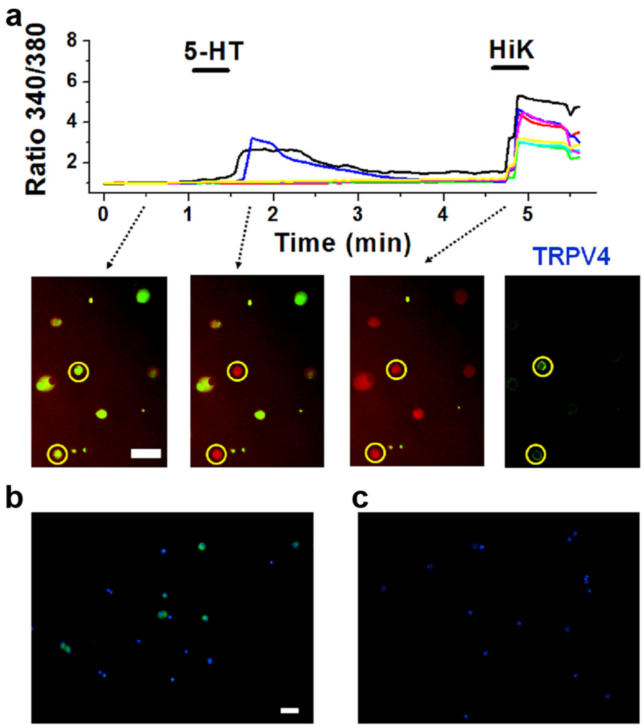

Figure 3. TRPV4 is expressed in 5-HT-sensitive DRG cells. (**a**) Cultured DRG cells from naive WT mice were exposed to 5-HT (100 μM) followed by high potassium solution (HiK). Responses were measured by Fura-2 ratiometric calcium imaging, followed by immunostaining with a TRPV4 antibody. Bottom panels 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 μm. (**b**) Cultured DRG cells from naive WT mice were immunostained with a TRPV4 antibody (green). Blue indicates nuclei (DAPI). Scale bar = 50 μm. (**c**) As in (**b**) for TRPV4KO mice. DAPI, 4′,6-diamidino-2-phenylindole; DRG, dorsal root ganglion; 5-HT, 5-hydroxytryptamine (serotonin); TRPV4, transient receptor potential vanilloid type-4; TRPV4KO, TRPV4 knockout; WT, wild-type.

In addition, 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$_7$ agonist, but not a 5-HT$_2$ 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 neurons, and in cutaneous nerves (Chen

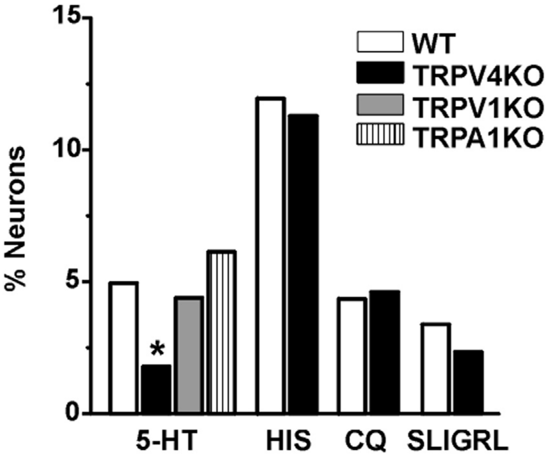

Figure 4. Incidence of 5-HT-sensitive DRG cells was decreased in TRPV4KO mice. The graph plots percentages of cultured DRG cells from wild-type (WT) (open bars) and TRPV4KO mice (black bars) that responded to 5-HT (100 μM), histamine (HIS, 100 μM), chloroquine (CQ, 300 μM), or SLIGRL-NH2 (100 μM). The 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 with TRPV4KO mice (n = 124−266). DRG, dorsal root ganglion; 5-HT, 5-hydroxytryptamine (serotonin); TRPV4KO, TRPV4 knockout.

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). Whereas large DRG and trigeminal ganglion neurons express TRPV4 (Chen et al., 2013b; Suzuki et al., 2003), small DRG and trigeminal ganglion (peptidergic) neurons expressing calcitonin gene-related peptide and/or substance P also express TRPV4 (Chen et al., 2013b; Moore et al., 2013; Vergnolle et al., 2010). In this 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 5-HT 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$_{2A}$ and 5-HT$_{1B}$ receptor subtypes are presumably involved in this response (Rodat-Despoix et al., 2008). In mouse DRG neurons, 5-HT sensitized neuronal responses to 4α-Phorbol 12,13-didecanoate, a selective TRPV4 agonist, through phospholipase C and protein kinase C-dependent pathways (Cenac et al., 2010). These findings imply that certain 5-HT 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$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1F}$, 5-HT$_{2A}$, 5-HT$_{3}$, 5-HT$_{4}$, 5-HT$_{5B}$, and 5-HT$_{7}$ receptor subtypes (Ohta et al., 2006). The 5-HT receptor subtypes involved in itch are currently under investigation. We presently observed

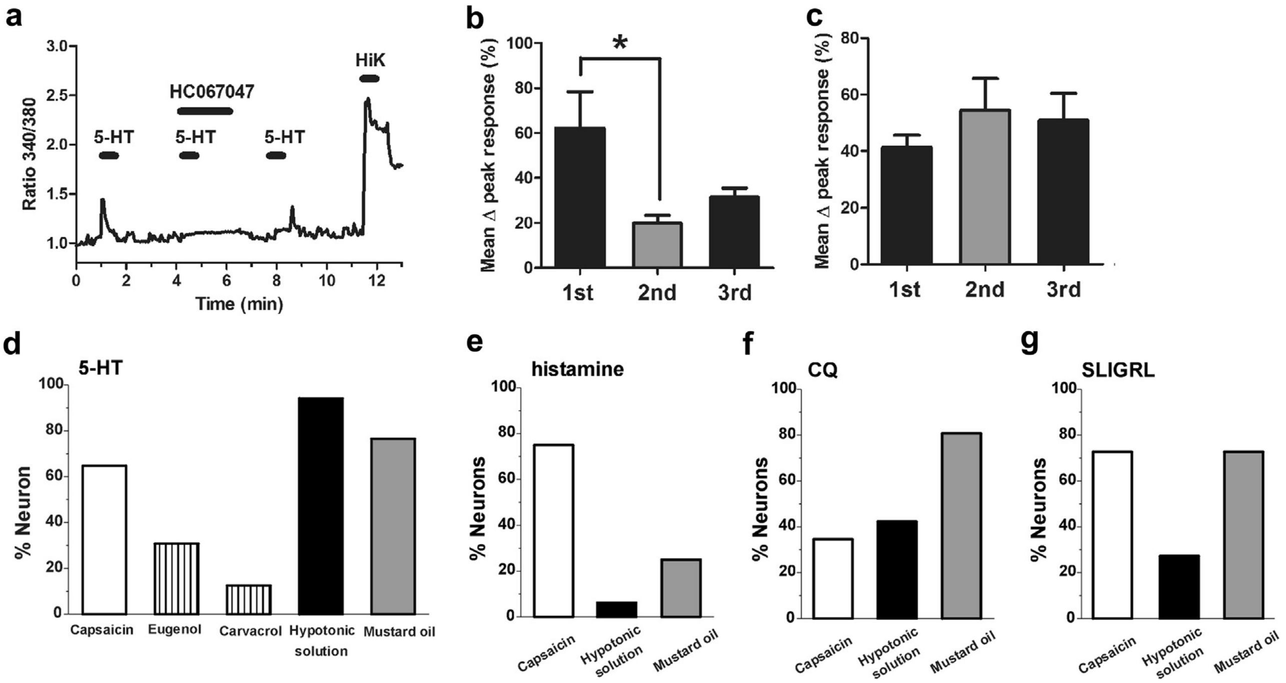

Figure 5. TRPV4 antagonist inhibited 5-HT-elicited responses of DRG cells. (**a**) Cultured DRG cells isolated from naive WT mice were exposed to 5-HT (100 μM) three times. Responses were measured by Fura-2 ratiometric calcium imaging. Application of the TRPV4 antagonist, HC067047 (1 μM), reduced the response to 5-HT (middle trace). (**b**) Overall percent reduction by TRPV4 antagonist. $*P < 0.05$, significant difference from first responses (one-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), carvacrol (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). DRG, dorsal root ganglion; 5-HT, 5-hydroxytryptamine (serotonin); TRPA1, transient receptor potential cation channel, subfamily A, member 1; TRPV1, transient receptor potential vanilloid type-1; TRPV3, transient receptor potential vanilloid type-3; TRPV4, transient receptor potential vanilloid type-4; TRPV4KO, TRPV4 knockout; WT, wild-type.

that the 5-HT_2 antagonist ketanserin abolished 5-HT-evoked scratching, consistent with a previous study (Yamaguchi et al., 1999). A recent study reported that a 5-HT_3 antagonist inhibited 5-HT-evoked scratching in mice (Ostadhadi et al., 2015). However, previous studies showed that 5-HT_3 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_3 is less likely involved in 5-HT-evoked itch. A very recent study suggests that 5-HT_7 is involved in 5-HT-evoked itch (Morita et al., 2015). There is no report regarding the effects of the 5-HT_2 antagonist or the 5-HT_7 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_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_7 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 the activation of TRPV4 in keratinocytes contributes to the release of endothelin-1, another important pruitogen (Moore et al., 2013). However, endothelin-1 is unlikely to be involved in 5-HT-evoked itch, as endothelin-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 5-HT, and is thus linked to the 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; Grant et al., 2007; Liedtke et al., 2003), TRPV1 (TRPV1KO), TRPA1 (TRPA1KO), and 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 1 week before testing. For intraperitoneal 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, Bristol, UK; Everaerts et al., 2010), or vehicle (10% DMSO + 10% Tween80 in saline) was administered, followed 30 minutes later by intradermal injection (10 μl) of either chloroquine (193 nmol; Sigma-Aldrich), histamine (271 nmol; Sigma-Aldrich), 5-HT (47 nmol; Sigma-Aldrich), or SLIGRL (76 nmol; Quality Controlled Biochemicals, Hopkinton, MA). Microinjections were made intradermal 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 intradermal injection, mice were placed into the arena and videotaped from above for 30 minutes. 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 the licking or biting of the toes and/or the 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 analysis of variance followed by the Bonferroni posttest 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, TRPA1KO, TRPV1KO, 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 minutes in Hanks' Balanced Salt Solution (HBSS; Invitrogen, Carlsbad, CA) containing 20 U/ml papain (Worthington Biochemical, Lakewood, NJ) and 6.7 mg/ml L-cysteine (Sigma-Aldrich), and then again for 10 minutes 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 hours.

DRG cells were incubated in Ringer's solution (pH 7.4, 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 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 the 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 SimplePCI software (Hamamatsu, Sewickley, PA) every 3 seconds.

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 μM), histamine (100 μM), 5-HT (100 μM), and the PAR-2/MrgprA3 agonist SLIGRL-NH2 (100 μM). In some experiments, one of the following agents was delivered after the delivery of prutiogen: capsaicin (1 μM), eugenol (100 μM), carvacrol (100 μM), 30%

hypotonic solution, and mustard oil (100 µM). Stimulus duration was 30 seconds (10 seconds for capsaicin). In other experiments, 5-HT was delivered three times at 3.5-minute intervals. HC067047 (1 µM; Everaerts et al., 2010) was delivered beginning 10 seconds before the second 5-HT application for a duration of 2 minutes. 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 greater than 10% of the resting level after 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 immunohistofluoresent 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 a 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 hours. DRG cells were counterstained with 4′,6-diamidino-2-phenylindole 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

The authors state no 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 via Dr. N. Bunnett. The original source of the mice was Dr. W. Liedtke. The TRPV1KO mice and the TRPA1KO mice were kindly provided by Dr. D. Julius.

REFERENCES

Akiyama T, Carstens E. Neural processing of itch. Neuroscience 2013;250: 697–714.

Akiyama T, Carstens E. Spinal coding of itch and pain. In: Carstens E, Akiyama T, editors. Itch: mechanisms and treatment. Boca Raton, FL: CRC Press/Taylor & Francis; 2014.

Akiyama T, Carstens MI, Ikoma A, Cevikbas F, Steinhoff M, Carstens E. Mouse model of touch-evoked itch (alloknesis). J Invest Dermatol 2012;132: 1886–91.

Akiyama T, Merrill AW, Zanotto K, Carstens MI, Carstens E. 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 2009;329:945–51.

Bell JK, McQueen DS, Rees JL. Involvement of histamine H4 and H1 receptors in scratching induced by histamine receptor agonists in BalbC mice. Br J Pharmacol 2004;142:374–80.

Cenac N, Altier C, Motta JP, d'Aldebert E, Galeano S, Zamponi GW, et al. Potentiation of TRPV4 signalling by histamine and serotonin: an important mechanism for visceral hypersensitivity. Gut 2010;59:481–8.

Cevikbas F, Wang X, Akiyama T, Kempkes C, Savinko T, Antal A, et al. A sensory neuron-expressed IL-31 receptor mediates T helper cell-dependent itch: Involvement of TRPV1 and TRPA1. J Allergy Clin Immunol 2014;133:448–60.

Chen J, Kang D, Xu J, Lake M, Hogan JO, Sun C, et al. Species differences and molecular determinant of TRPA1 cold sensitivity. Nat Commun 2013a;4: 2501.

Chen Y, Williams SH, McNulty AL, Hong JH, Lee SH, Rothfusz NE, et al. Temporomandibular joint pain: a critical role for Trpv4 in the trigeminal ganglion. Pain 2013b;154:1295–304.

Dvorak AM. Histamine content and secretion in basophils and mast cells. Prog Histochem Cytochem 1998;33:III–IX, 169–320.

El-Nour H, Lundeberg L, Abdel-Magid N, Lonne-Rahm SB, Azmitia EC, Nordlind K. Serotonergic mechanisms in human allergic contact dermatitis. Acta Derm Venereol 2007;87:390–6.

Everaerts W, Zhen X, Ghosh D, Vriens J, Gevaert T, Gilbert JP. Inhibition of the cation channel TRPV4 improves bladder function in mice and rats with cyclophosphamide-induced cystitis. Proc Natl Acad Sci USA 2010;107: 19084–9.

Girard BM, Merrill L, Malley S, Vizzard MA. Increased TRPV4 expression in urinary bladder and lumbosacral dorsal root ganglia in mice with chronic overexpression of NGF in urothelium. J Mol Neurosci 2013;51:602–14.

Gopinath P, Wan E, Holdcroft A, Facer P, Davis JB, Smith GD. Increased capsaicin receptor TRPV1 in skin nerve fibres and related vanilloid receptors TRPV3 and TRPV4 in keratinocytes in human breast pain. BMC Womens Health 2005;5:2.

Grant AD, Cottrell GS, Amadesi S, Trevisani M, Nicoletti P, Materazzi S, et al. Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice. J Physiol 2007;578:715–33.

Han L, Ma C, Liu Q, Weng HJ, Cui Y, Tang Z, et al. A subpopulation of nociceptors specifically linked to itch. Nat Neurosci 2013;16:174–82.

Hosogi M, Schmelz M, Miyachi Y, Ikoma A. Bradykinin is a potent pruritogen in atopic dermatitis: a switch from pain to itch. Pain 2006;126:16–23.

Huang J, Li G, Xiang J, Yin D, Chi R. Immunohistochemical study of serotonin in lesions of chronic eczema. Int J Dermatol 2004a;43:723–6.

Huang J, Li G, Xiang J, Yin D, Chi R. Immunohistochemical study of serotonin in lesions of psoriasis. Int J Dermatol 2004b;43:408–11.

Imamachi N, Park GH, Lee H, Anderson DJ, Simon MI, Basbaum AI, et al. TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms. Proc Natl Acad Sci USA 2009;106: 11330–5.

Inami Y, Andoh T, Sasaki A, Kuraishi Y. Topical surfactant-induced pruritus: involvement of histamine released from epidermal keratinocytes. J Pharmacol Exp Ther 2013;344:459–66.

Kaneko K, Travers JB, Matsui MS, Young AR, Norval M, Walker SL. cis-Urocanic acid stimulates primary human keratinocytes independently of serotonin or platelet-activating factor receptors. J Invest Dermatol 2009;129: 2567–73.

Kida N, Sokabe T, Kashio M, Haruna K, Mizuno Y, Suga Y, et al. Importance of transient receptor potential vanilloid 4 (TRPV4) in epidermal barrier function in human skin keratinocytes. Pflugers Arch 2012;463:715–25.

Kido-Nakahara M, Buddenkotte J, Kempkes C, Ikoma A, Cevikbas F, Akiyama T, et al. Neural peptidase endothelin-converting enzyme 1 regulates endothelin 1-induced pruritus. J Clin Invest 2014;124:2683–95.

Kushnir-Sukhov NM, Brown JM, Wu Y, Kirshenbaum A, Metcalfe DD. Human mast cells are capable of serotonin synthesis and release. J Allergy Clin Immunol 2007;119:498–9.

Liedtke W, Friedman JM. Abnormal osmotic regulation in trpv4-/- mice. Proc Natl Acad Sci U S A 2003;100:13698–703.

Lieu T, Jayaweera G, Zhao P, Poole DP, Jensen D, Grace MI, et al. The bile acid receptor TGR5 activates the TRPA1 channel to induce itch in mice. Gastroenterology 2014;147:1417–28.

Liu B, Escalera J, Balakrishna S, Fan L, Caceres AI, Robinson E, et al. TRPA1 controls inflammation and pruritogen responses in allergic contact dermatitis. FASEB J 2013;27:3549–63.

Liu Q, Weng HJ, Patel KN, Tang Z, Bai H, Steinhoff M, et al. The distinct roles of two GPCRs, MrgprC11 and PAR2, in itch and hyperalgesia. Sci Signal 2011;4:ra45.

Moore C, Cevikbas F, Pasolli HA, Chen Y, Kong W, Kempkes C, et al. UVB radiation generates sunburn pain and affects skin by activating epidermal TRPV4 ion channels and triggering endothelin-1 signaling. Proc Natl Acad Sci USA 2013;110:E3225–34.

Morita T, McClain SP, Batia LM, Pellegrino M, Wilson SR, Kienzler MA, et al. HTR7 mediates serotonergic acute and chronic itch. Neuron 2015;87: 124–38.

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

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

Ohta T, Ikemi Y, Murakami M, Imagawa T, Otsuguro K, Ito S. Potentiation of transient receptor potential V1 functions by the activation of metabotropic 5-HT receptors in rat primary sensory neurons. J Physiol 2006;576:809–22.

Ostadhadi S, Kordjazy N, Haj-Mirzaian A, Mansouri P, Dehpour AR. 5-HT3 receptors antagonists reduce serotonin-induced scratching in mice. Fundam Clin Pharmacol 2015;29:310–5.

Rodat-Despoix L, Crevel H, Marthan R, Savineau JP, Guibert C. Heterogeneity in 5-HT-induced contractile and proliferative responses in rat pulmonary arterial bed. J Vasc Res 2008;45:181–92.

Slominski A, Pisarchik A, Zbytek B, Tobin DJ, Kauser S, Wortsman J. Functional activity of serotoninergic and melatoninergic systems expressed in the skin. J Cell Physiol 2003;196:144–53.

Steinhoff M, Neisius U, Ikoma A, Fartasch M, Heyer G, Skov PS, et al. Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. J Neurosci 2003;23:6176–80.

Suzuki M, Watanabe Y, Oyama Y, Mizuno A, Kusano E, Hirao A, et al. Localization of mechanosensitive channel TRPV4 in mouse skin. Neurosci Lett 2003;353:189–92.

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;288:12818–27.

Turetta L, Donella-Deana A, Folda A, Bulato C, Deana R. Characterisation of the serotonin efflux induced by cytosolic Ca2+ and Na+ concentration increase in human platelets. Cell Physiol Biochem 2004;14:377–86.

Vergnolle N, Cenac N, Altier C, Cellars L, Chapman K, Zamponi GW, et al. A role for transient receptor potential vanilloid 4 in tonicity-induced neurogenic inflammation. Br J Pharmacol 2010;159:1161–73.

Wang J, Wang XW, Zhang Y, Yin CP, Yue SW. Ca(2+) influx mediates the TRPV4-NO pathway in neuropathic hyperalgesia following chronic compression of the dorsal root ganglion. Neurosci Lett 2015;588: 159–65.

Weisshaar E, Dunker N, Rohl FW, Gollnick H. Antipruritic effects of two different 5-HT3 receptor antagonists and an antihistamine in haemodialysis patients. Exp Dermatol 2004;13:298–304.

Weisshaar E, Ziethen B, Gollnick H. Can a serotonin type 3 (5-HT3) receptor antagonist reduce experimentally-induced itch? Inflamm Res 1997;46: 412–6.

Wilson SR, Gerhold KA, Bifolck-Fisher A, Liu Q, Patel KN, Dong X, et al. TRPA1 is required for histamine-independent, Mas-related G protein-coupled receptor-mediated itch. Nat Neurosci 2011;14:595–602.

Wilson SR, The L, Batia LM, Beattie K, Katibah GE, McClain SP, et al. The epithelial cell-derived atopic dermatitis cytokine TSLP activates neurons to induce itch. Cell 2013;155:285–95.

Xia Y, Fu Z, Hu J, Huang C, Paudel O, Cai S, et al. TRPV4 channel contributes to serotonin-induced pulmonary vasoconstriction and the enhanced vascular reactivity in chronic hypoxic pulmonary hypertension. Am J Physiol Cell Physiol 2013;305:C704–15.

Yamada T, Ugawa S, Ueda T, Ishida Y, Kajita K, Shimada S. Differential localizations of the transient receptor potential channels TRPV4 and TRPV1 in the mouse urinary bladder. J Histochem Cytochem 2009;57: 277–87.

Yamaguchi T, Nagasawa T, Satoh M, Kuraishi Y. Itch-associated response induced by intradermal serotonin through 5-HT2 receptors in mice. Neurosci Res 1999;35:77–83.

Yang YS, Cho SI, Choi MG, Choi YH, Kwak IS, Park CW, et al. Increased expression of three types of transient receptor potential channels (TRPA1, TRPV4 and TRPV3) in burn scars with post-burn pruritus. Acta Derm Venereol 2015;95:20–4.

Yoshioka T, Imura K, Asakawa M, Suzuki M, Oshima I, Hirasawa T, et al. Impact of the Gly573Ser substitution in TRPV3 on the development of allergic and pruritic dermatitis in mice. J Invest Dermatol 2009;129: 714–22.