Title
The role of the ion channel, TRPA1, in itch transduction in the mammalian peripheral nervous system.

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The role of the ion channel, TRPA1, in itch transduction in the mammalian peripheral nervous system.

By
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A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Molecular and Cell Biology in the Graduate Division of the University of California, Berkeley

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Abstract
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Itch is defined as an unpleasant sensation that evokes a desire to scratch. In contrast to acute itch that is transient, chronic itch is a persistent, debilitating condition for which there are few treatment options. Chronic itch accompanies a number of skin diseases and systemic conditions, as well as a variety of neurological disorders. However, little is known about the molecules and cell types that mediate acute or chronic itch in primary sensory neurons and skin.

We have established an essential role for the ion channel, TRPA1, in multiple forms of both acute and chronic itch. We have shown that TRPA1 is required for neuronal activation and itch behaviors in mice downstream of acute exogenous and endogenous itch compounds. Similarly, TRPA1 is required for itch behavior and itch-evoked expressional changes in both sensory neurons and skin in a mouse model of dry skin.

We have also identified a novel itch-causing compound: Thymic Stromal Lymphopoietin (TSLP). Numerous studies suggest that the epithelially-derived cytokine TSLP acts as a master switch that triggers both the initiation and maintenance of the chronic itch disease atopic dermatitis. Our work demonstrates that TSLP activates sensory neurons directly and leads to acute itch behaviors. TRPA1 is required for both TSLP-evoked neuronal activation and itch behaviors. Taken together, our work shows that TRPA1 is a master regulator of itch signaling.
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The sensation of itch is detected by the somatosensory system. Itch signals are transduced by primary afferent somatosensory neurons innervating the skin. The cell bodies of primary somatosensory neurons reside in the dorsal root ganglia (DRG) and trigeminal ganglia (TG). These peripheral neurons are pseudo-bipolar, sending long afferent projections to the skin and viscera where detection of stimuli occurs. DRG send efferent projections to the dorsal horn of the spinal cord and TG send efferent projections directly to the brain stem. These primary afferents then form synapses on spinal cord interneurons that project to sites in the central nervous system, namely the brain stem and thalamus. The molecules required for itch stimulus transduction in the skin remain largely enigmatic.

(The remainder of this section is a reproduction of a chapter by the name Role of Transient Receptor Potential Channels in Acute and Chronic Itch published in Itch: Mechanisms and Treatment in 2014. For this chapter, I made figures and wrote the manuscript with D.M.B)

Members of the Transient Receptor Potential (TRP) family have emerged as key players in itch transduction in the periphery. TRP channels are tetrameric cation selective ion channels that are found in diverse species, from flies to humans. The founding member of the TRP channel superfamily is the Drosophila TRP ion channel, a transduction channel required for light-evoked excitation of photoreceptors. In phototransduction, activation of the PLC pathway leads to the opening of TRP and its homolog TRP-L; flies lacking these channels display no light-evoked transduction currents and are blind. Since then, over 27 members have been identified in a variety of cell types and tissues (Fig. 1).

TRP channels are divided into seven subgroups based on protein homology rather than function; TRPC, TRPV, TRPM, TRPA, TRPN, TRPP, TRPML. Generally, TRP channels function as polymodal cellular sensors involved in a wide variety of cellular processes. Many TRPs have been found to participate in sensory transduction pathways, including thermosensation, mechanosensation, taste perception, perception of pungent compounds, pheromone sensing and osmolarity regulation. A number of excellent reviews describe the vast roles of TRP channels and will not be discussed.

Here we discuss the role of four TRP channels that have been proposed to play a role in itch transduction: TRPV1, TRPA1, TRPM8 and TRPV3. Historically these four channels have been implicated in the transduction of noxious thermal, chemical and/or mechanical stimuli and more recent studies have implicated these channels in the transduction of itch.

**TRPV1**
TRPV1 was first identified as a receptor for capsaicin, the active ingredient in hot chili peppers that elicits a burning, heat sensation when eaten. TRPV1 is a ligand-activated,
Figure 1: Transient receptor potential (TRP) ion channel family.

TRP channels are tetrameric cation selective channels with six transmembrane spanning helices. Distinct TRP channels have (left) intracellular N- and C-termini that vary dramatically in size and contain a variety of protein interaction and modulatory domains (not shown). The putative pore domain is depicted in red. TRP channels are divided into seven subgroups based on protein homology rather than function: TRPC, TRPV, TRPM, TRPA, TRPN, TRPP, and TRPML (right). Members of six of these subgroups have been identified in a variety of tissue types in humans. This chapter will focus on members of the TRPV, TRPA, and TRPM families and their roles in pruriception.

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non-selective cation channel \textsuperscript{10}. This channel is highly expressed a subset of temperature-sensitive somatosensory neurons that have cell bodies in the dorsal root and trigeminal sensory ganglia, and project afferents to innervate target organs in the periphery, such as the skin \textsuperscript{10,11}.

Capsaicin activates TRPV1 by binding to an intracellular region of the channel in between the second and third transmembrane domain \textsuperscript{12}. TRPV1 is also activated by heat, with a threshold of activation of approximately 43°C and a coefficient of temperature dependence (Q10) of \textasciitilde 40 \textsuperscript{10}. A number of endogenous ligands also activate TRPV1; protons, anandamide, lipoxygenase products, and N-arachidonoyl dopamine have all been proposed to modulate TRPV1 activity \textit{in vivo} \textsuperscript{13-15}. Activation of TRPV1 permits the influx of cations into the peripheral nerve terminal to promote depolarization and action potential propagation to the central nervous system.

The localization of TRPV1 in heat-sensitive neurons and the ability of TRPV1 to be directly activated by heat supported a model where this channel functions as an \textit{in vivo} thermoreceptor. Indeed, TRPV1-deficient mice display decreased sensitivity to noxious heat in acute behavioral assays \textsuperscript{16,17}. TRPV1 also plays a key role in thermal hypersensitivity following injury or inflammation. WT mice display thermal hyperalgesia and allodynia, the increased pain sensitivity to both noxious, and previously innocuous stimuli, respectively, in injury or inflammatory models. Mice lacking TRPV1 fail to develop hyperalgesia and allodynia in these models, demonstrating the importance of TRPV1 in pain hypersensitivity \textsuperscript{16,17}.

How does inflammation change TRPV1 activity to promote thermal hypersensitivity? Many of the inflammatory mediators responsible for pain hypersensitivity, including NGF, ATP, chemokines, and prostaglandins, all activate G-protein coupled receptors (GPCRs) that signal via phospholipase C (PLC) \textsuperscript{18}. PLC signaling in turn modulates TRPV1 activity, such that the open probability of the channel is shifted to body temperature; thus TRPV1 promotes neuronal excitability in the absence of heat \textsuperscript{12}. Indeed, most TRP channels are activated or modulated downstream of GPCRs\textsuperscript{5}. As most pruritogens activate GPCRs, and trigger itch via activation of somatosensory afferents, TRP channels, including TRPV1, were attractive candidate itch transducers\textsuperscript{4}.

The idea of a link between thermal pain and itch has been known for centuries. First, patients with chronic itch conditions have long reported that scalding heat helps to alleviate their pruritus. Second, topical application of the TRPV1 agonist, capsaicin, has been used to treat itch associated with many skin conditions. In 1850 the first formal report of the use of capsaicin to treat itch, and pain, appeared in an 1850 publication recommending the use of a hot pepper extract on burning or itching extremities \textsuperscript{19}. Today, topical capsaicin formulations are used widely to manage pain and beneficial effects of capsaicin have been reported in chronic, localized pruritic disorders,
particularly those of neuropathic origin, such as notalgia paresthetica, brachioradial pruritus, prurigo nodularis, aquagenic pruritus and pruritus associated with chronic kidney disease\textsuperscript{20-24}. Consistent with these treatments in humans, neonatal capsaicin treatment decreases allergy-associated scratching in mice\textsuperscript{25}.

The antinociceptive and antipruritic effects of topical capsaicin are thought to manifest through the defunctionalization of TRPV1 positive primary afferents, mediated by direct desensitization of TRPV1, or voltage gated sodium channels in the short term, and nerve terminal retraction due to excitotoxic terminal damage induced by excessive calcium and inhibition of mitochondrial respiration in the long term\textsuperscript{26}. Indeed, immunohistochemical studies using antibodies to nerve terminal proteins, like PGP 9.5, show that capsaicin application induces localized loss of nociceptive nerve fiber terminals in the epidermis and dermis\textsuperscript{27}. Therefore, the use of capsaicin to treat pruritus implicates either TRPV1 or TRPV1 containing primary afferents in pruriception.

A number of studies suggested that TRPV1 mediates histamine signal transduction in primary sensory neurons (Fig 2). First, many histamine sensitive fibers are also capsaicin sensitive. Second, histamine sensitizes primary afferent fibers to heat stimuli. These experiments strongly suggested that the histamine receptor and TRPV1 are coexpressed in sensory afferents\textsuperscript{28-30}. Third, histamine-evoked calcium transients in rat dorsal root ganglia neurons are inhibited by TRPV1 antagonists, capsazepine and SC0030\textsuperscript{31}. These authors proposed that activation of the H1 histamine receptor leads to activation of TRPV1 via an intracellular second messenger pathway involving phospholipase A2 and lipooxygenase. Fourth, trypsin-evoked itch behaviors are attenuated in both capsazepine treated and TRPV1- deficient mice\textsuperscript{32}. Fifth, patients with allergic rhinitis display an increased itch response to TRPV1 stimulation from seasonal allergen exposure\textsuperscript{33}. Finally, TRPV1 antagonists inhibit pruritus in atopic dermatitis and the commonly prescribed anti pruritic, tacrolimus, has been suggested to work in part by inhibiting/desensitizing TRPV1\textsuperscript{34,35}.

A definitive role for TRPV1 in histamine-evoked itch behavior was finally established in 2009 when it was shown that TRPV1-deficient mice displayed significantly attenuated itch behavior in response to injection of histamine. This study also found that histamine-evoked scratching behavior is attenuated in PLC\(\beta\)3-deficient mice\textsuperscript{36}. As such, a model emerged where activation of histamine H1 G\(_q\)-coupled GPCRs signals via PLC\(\beta\)3 to open TRPV1 (through an unknown mechanism) in murine primary afferents. However, while itch behavior was attenuated in TRPV1 deficient mice, the behavior was not completely ablated and in addition to Histamine receptor 1, Histamine receptors 3 and 4 are also expressed in primary sensory neurons\textsuperscript{37}. Similarly, not all histamine-sensitive DRGs are capsaicin sensitive\textsuperscript{31}. This suggests that other channels, potentially downstream of other histamine receptors, may be involved in transducing histamine-evoked itch signals.
III. TRPV1-expressing afferents and acute itch.
While a role for TRPV1 in histamine-evoked itch is well established, many pruritogens act independently of TRPV1. How do other pruritogens promote excitability in sensory neurons? A hint came from the original TRPV1-directed studies. Two potent pruritogens, serotonin and endothelin-1 were shown to evoke robust scratching in TRPV1-deficient mice. However, itch behavior was significantly attenuated in mice treated with intrathecal capsaicin to ablate TRPV1-positive afferents. Intrathecal capsaicin injections in mice, similar to the topical capsaicin applications in humans discussed above, promotes receptor defunctionalization, as well as excitotoxic neuronal ablation; as such, this treatment results in mice lacking TRPV1-positive neurons. Taken together, these data suggest that other channels, expressed in TRPV1-positive afferents are required for histamine-independent itch. Consistent with this idea, two drugs that evoked anti-histamine resistant itch, imiquimod and chloroquine, trigger itch-evoked scratching in TRPV1-deficient mice, but not TRPV1-ablated mice. Additionally, blocking synaptic transmission by deletion of the glutamate transporter, VGLUT2, selectively in TRPV1-positive neurons fibers attenuates pain and itch behaviors.

IV. TRPA1 and histamine-independent itch
The TRPA1 ion channel is highly expressed in a subset of TRPV1-positive neurons and plays a key role in multiple types of histamine-independent itch. TRPA1 is an irritant receptor that is TRPA1 is robustly activated by a wide variety of exogenous irritants that cause pain and inflammation. Environmental chemicals that target TRPA1 include allyl isothiocyanate (AITC), cinnamaldehyde and allicin, which are the pungent compounds found in mustard, cinnamon and garlic extracts, respectively. TRPA1 is also a target of endogenous inflammatory agents. TRPA1 is activated by prostaglandins, such as 15d-PGJ2, PGA2, and Δ12-PGJ2, that are modulated by PLC-coupled receptors mediating inflammation, such as the bradykinin receptor. Many studies also suggest that TRPA1 can be activated directly by reactive oxygen species including hydrogen peroxide and the lipid peroxidation products 4-HNE, 4-ONE, and 4-HHE. Data from TRPA1-deficient mice has shown that TRPA1 is required both for acute behavioral responses to AITC and for prolonged mechanical and thermal hypersensitivity following AITC exposure. TRPA1 is also required for inflammatory responses to formalin and the α,β-unsaturated aldehyde acrolein, an airway irritant present in tear gas, vehicle exhaust, and smoke. These studies show that TRPA1 acts as a general mediator of inflammation that can be activated by a host of endogenous and exogenous irritants.

There is now a growing body of evidence that suggests a significant role for TRPA1 in histamine-independent itch (Fig. 2). Members of the novel Mas-related G-Protein coupled receptor (Mrgpr) family, MrgprA3 and MrgprC11, are activated by two different histamine-independent pruritogens, chloroquine (CQ) and BAM8-22 (BAM), respectively. TRPA1 is required for both CQ and BAM-evoked calcium signals and action potential firing in somatosensory neurons, as well as CQ and BAM-evoked scratching in mice.
Figure 2: TRPA1 and TRPV1 play key roles in acute itch stimuli transduction.

TRPV1 and TRPA1 are calcium-permeable cation channels that can be activated downstream of numerous pruritogen GPCRs. (left) Fibers expressing TRPV1 are responsible for transmitting histamine-dependent itch; TRPV1 is activated downstream of the histamine receptor via the PLC pathway. (right) Fibers expressing TRPV1 and TRPA1 transmit histamine-independent itch signals; TRPA1 is activated downstream of the Mas-related G protein-coupled receptors, MrgprA3 and MrgprC11, as well as the protease activated receptor 2 (PAR2). While TRPV1 is present in these neurons, it is not required for itch transduction.

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Similarly, endothelin 1-evoked scratching in mice is attenuated in the presence of the TRPA1 inhibitor, AP18. TRPA1 has since been shown to be specifically required for scratching in an oxidative stress model of itch. In this model, RTX ablation also reduces scratching, suggesting a role for C fibers. Genetic or pharmacological blockade of TRPA1 activity, but not TRPV1, decreases oxidative stress-evoked scratching. In line with this, we have recently found that both acute TSLP-evoked scratching as well as chronic dry skin-evoked scratching (in the acetone ether water model of dry skin, AEW) are significantly attenuated in TRPA1-deficient mice (unpublished data). Taken together, these data demonstrate that TRPA1 is a downstream transduction channel onto which multiple histamine-independent acute and chronic itch pathways converge.

V. TRPV3 and keratinocyte-mediated itch pathways.
Keratinocytes, the epithelial cells that make up the stratified epidermis of the skin, play a key role in itch by secreting a variety of mediators that target sensory neurons and immune cells. TRPV3 is expressed in keratinocytes and has been proposed to play a role in promoting itch signaling and behaviors.

TRPV3 was originally identified as a heat sensitive ion channel in keratinocytes. Mouse TRPV3 is preferentially activated by innocuous, warm, temperature with a threshold of ~33°C. Warmth-evoked currents in cultured keratinocytes display biophysical properties that match those of TRPV3 currents in heterologous cells. Consistent with a role in warm sensing, mice lacking TRPV3 display deficits in responses to innocuous and noxious heat, and keratinocyte-specific TRPV3 knock-in mice display increased avoidance of noxious heat, in the absence of functional TRPV1 channels. While it is unclear how TRPV3-dependent signaling in keratinocytes promotes sensory neuron transduction, one model suggests that ATP is the signaling molecule linking keratinocytes and neurons. Like most TRP channels, TRPV3 is a polymodal sensor that can be activated by the natural plant product, camphor, and by nitrates that lead to nitric oxide production in the skin.

The characterization of rodent strains that spontaneously develop atopic dermatitis-like lesions first implicated TRPV3 in pruritus. Sequencing revealed that these mice had gain of function mutations in TRPV3 (Gly573Ser) that were found to be sufficient to drive AD-like skin alterations. Likewise, gain of function mutations in TRPV3 in humans have been linked to Omsted syndrome, a condition that results in severe chronic itch. Consistent with a role for TRPV3 in chronic itch, TRPV3-deficient mice do not develop chronic itch in a dry skin model of chronic itch and overexpression of TRPV3 in keratinocytes is sufficient to promote secretion of the pruritogen, prostaglandin E2. However, how prostaglandin, or other keratinocyte-released mediators, promote pruritus remains unknown.
VI. TRPM8 in inhibition of itch

Unlike other TRP channels that promote itch signaling, TRPM8, the cold and menthol receptor, is hypothesized to inhibit itch signal transmission. Phenomenologically, cooling is known to soothe and relieve itch sensations. In humans, cooling of the skin inhibits atopic dermatitis-evoked itch in patients. Menthol, icilin, and cooling have been also been shown to inhibit histamine and lichenification-evoked itch in humans. Likewise, skin cooling attenuates spinal neuron responses to subcutaneous histamine injection.

TRPM8 is activated by a variety of natural plants products that induce cooling sensations, including menthol, menthone, and eucalyptol. It is also activated by cold, with an activation temperature of ~25°C. TRPM8 is expressed in a subset of primary afferent sensory neurons. Importantly, sensory neurons isolated from TRPM8-deficient mice have attenuated responses to menthol, icilin, and cold. Behavioral studies also reveal severe deficits in cold-evoked responses in these knockout mice, as measured by acetone evaporative cooling, cold plate, and two-choice temperature assays. However, TRPM8-deficient mice show normal cold-evoked behavior in response to noxious cold (<10°C), suggesting that other channels may also play a role in cold temperature detection.

The requirement of TRPM8 in menthol and cold sensitivity in vivo suggests that TRPM8, or TRPM8-positive neurons, may mediate the attenuation of itch by cold, and cold mimetics. However, future studies are required to test this directly. It will be of particular interest to determine whether these neurons are linked to inhibitory itch interneurons in the spinal cord.

CONCLUSIONS AND FUTURE PROSPECTS

Research over the last decade shows a critical role of TRP channels in acute itch transduction. As such, TRP channel antagonists may be useful for the selective attenuation of itch. However, most studies have focused on acute, rather than chronic itch. Little is known about the molecules that mediate chronic itch in primary sensory neurons and skin. Whether TRP channel signaling contributes to chronic itch is unknown and represents a major question in itch biology.

The TRP channels discussed here have dual roles in itch, and in other somatosensory pathways and modalities, like pain. A question that emerges from these studies is how any one channel can drive distinct itch or pain behaviors in response to differing stimuli. Multiple models have been proposed to account for this dual role of TRP channels. One is based on population coding, where a TRP agonist would evoke excitation of both itch-specific and pain- or temperature-specific fibers, and computation in the CNS would determine which signal is transmitted. Alternatively, the spatial contrast theory of itch,
The recent discovery of itch-specific spinal cord neurons suggests that central circuits may generate the specificity observed in itch signaling\textsuperscript{79,80}. However, the relationship between itch and pain remains a pressing question in somatosensation.
CHAPTER I: TRPA1 is required for histamine-independent, Mas-related G protein-coupled receptor-mediated itch.

This chapter is a reproduction of the paper by the same name published in Nature Neuroscience May 2011. My contributions were to Figure 1, 2a-c, 3a-c, 4, 5a-c, and S1. For this paper I performed cellular imaging, PCR, and mouse behavioral experiments and made figures and wrote the manuscript with K.G. and D.M.B.

SUMMARY
Itch, the unpleasant sensation that evokes a desire to scratch, accompanies numerous skin and nervous system disorders. In many cases, pathological itch is insensitive to antihistamine treatment. Recent studies have identified members of the Mas-related GPCR (Mrgpr) family that are activated by mast cell mediators and promote histamine-independent itch. MrgprA3 and MrgprC11 act as receptors for the pruritogens chloroquine and BAM8–22, respectively. However, the signaling pathways and transduction channels activated downstream of these pruritogens are largely unknown. We found that TRPA1 is the downstream target of both MrgprA3 and MrgprC11, in cultured sensory neurons and heterologous cells. TRPA1 is required for Mrgpr-mediated signaling, as sensory neurons from TRPA1-deficient mice exhibited profoundly diminished responses to chloroquine and BAM8–22. Likewise, TRPA1-deficient mice displayed little to no scratching in response to these pruritogens. Our findings demonstrate that TRPA1 is an essential component of the signaling pathways that promote histamine-independent itch.

INTRODUCTION
Acute pruritis, or itch, serves an important protective function by warning against harmful agents in the environment such as insects, toxic plants or other irritants. Itch also promotes scratching, which aids in clearing pruritogens and attenuates itch sensations. In contrast, pruritus can also be a debilitating condition that accompanies numerous skin, systemic, and nervous system disorders. While many forms of itch are mediated by histamine signaling, there are clearly other key neural pathways. For example, a side effect of the antimalarial drug chloroquine (CQ) is antihistamine-resistant, intolerable itch. Likewise, spicules from the plant Mucuna pruriens produce intense itch via a histamine-independent pathway. Moreover, immune cells release a variety of pruritogens that mediate allergy-evoked itch, psoriasis and eczema, and anti-histamines are not effective in treating the full spectrum of allergic disorders. Finally, most pathophysiological itch conditions are insensitive to antihistamine treatment and therapeutic targets have yet to be identified.

While the molecular and cellular mechanisms of itch have yet to be fully elucidated, recent studies have begun to delineate the basic characteristics of the itch circuitry. There is now evidence implicating dedicated neuronal pathways for itch, separate from pain. Mice lacking gastrin-releasing peptide receptor (GRPR)-positive cells in dorsal horn of the spinal cord display reduced itch behaviors, but normal pain behaviors. Distinct subsets of primary afferent neurons mediating itch have also been identified. Approximately 5–20% of primary afferent C-fibers are activated by endogenous itch-
producing compounds released by non-neuronal cells in the skin (e.g., mast cells), as well as by exogenous pruritogens, such as chloroquine⁴,³⁶,⁵³.

Itch-sensitive C-fibers can be divided into multiple subgroups based on pruritogen-sensitivity. A subset of primary afferent C-fibers that express the capsaicin receptor, TRPV1, can be divided into three groups based on receptor expression and pruritogen sensitivity. The first group expresses the 5-hydroxytryptamine receptor 3 and the H1 histamine receptor, and mediates itch-evoked responses to serotonin and histamine³⁶. A second group expresses Mas-related GPCR A3 (MrgprA3) that mediates itch-evoked responses to CQ. The third group expresses both MrgprA3 and MrgprC11, the receptor for the endogenous pruritogen, BAM8–22 (BAM)⁵³. MrgprA3 and MrgprC11 are members of the newly identified, sensory neuron-specific Mas-related G protein-coupled receptor family. While the function of most Mrgprs remain unknown, MrgprA3 and MrgprC11 have been shown to play key roles in histamine-independent pruritus. MrgprC11 is targeted by mast cell pruritogens released during allergic inflammation⁹³. MrgprA3 is activated by the antimalarial drug CQ, which causes acute itch in rodents and intolerable itch in some patients.

The signaling mechanisms by which pruritogen-evoked activation of MrgprA3 and MrgprC11 leads to neuronal excitation remain unknown. MrgprA3 and MrgprC11 are expressed in a subset of TRPV1 positive afferents. In addition, MrgprA3-evoked excitation is inhibited by ruthenium red, a blocker of TRPA1 and TRPV1 channels⁵³. While TRPV1-expressing afferents mediate responses to a variety of pruritogens, mice lacking functional TRPV1 channels display reduced responses to histamine, but normal responses to serotonin and endothelin-1³⁶. These data imply that other ion channels are also activated by pruritogens in TRPV1-expressing afferents. These findings suggest that both TRPV1 and TRPA1 are candidate transduction channels in the Mrgpr-pruritic pathways.

The irritant receptor TRPA1 is highly expressed in a subset of TRPV1-positive neurons. TRPA1 is activated by a number of pain producing compounds such as isothiocyanates, the pungent compounds present in mustard oil and other Brassica plants, cinnamon oil, and cannabinoids. Additionally, TRPA1 is activated downstream of G protein-coupled receptors, including the pro-algesic bradykinin receptor⁴³,⁴⁴. Histamine, serotonin, chloroquine and BAM8–22 all evoke itch by acting on G protein-coupled receptors⁵³,⁹⁴,⁹⁵. Thus, TRPA1 is a key candidate transduction channel for itch.

Here we show that TRPA1 is an essential player in the transduction of Mrgpr-mediated itch. Cultured sensory neurons from TRPA1-deficient mice exhibit profoundly diminished responses to both chloroquine and BAM8–22. The functional coupling between MrgprA3 and TRPA1 is attenuated by disruption of Gβγ signaling, while coupling between MrgprC11 and TRPA1 requires PLC signaling. TRPA1 is required for Mrgpr-evoked itch in vivo, as mice lacking TRPA1 do not display the chloroquine- or BAM8–22-evoked itch behaviors typical of wild type animals. Our findings support an emerging role for TRP channels in the transduction of pruritic stimuli.
RESULTS
BAM8–22 and CQ activate TRPA1 and TRPV1-expressing neurons
The endogenous pruritogen BAM8–22 and the pruritic antimalarial drug chloroquine activate a subset of TRPV1-positive neurons. To determine whether these pruritogens activate the subset of TRPV1-positive neurons that also express TRPA1, we used ratiometric calcium (Ca²⁺) imaging to examine overlap between BAM- and CQ-sensitivity, and sensitivity to the TRPA1 agonist, allyl isothiocyanate (mustard oil; Fig. 1). We found that 9.8±1.2% of dorsal root ganglia (DRG) neurons and 16.1±2.3% of trigeminal (TG) neurons (Fig. 1A–C; n≥1050 neurons) showed robust increases in intracellular Ca²⁺ following CQ (1 mM) application, while only 6.2±1.2% of DRG and 5.4±0.9% of TG neurons were responsive to both CQ and BAM (100 μM; Fig. 1A–C; n≥390 neurons). Subsequent exposure to mustard oil (MO; 200 μM) or capsaicin (Cap; 1 μM) produced further increases in Ca²⁺ levels in all CQ- and BAM-positive cells (Fig. 1B–C). These results suggest that BAM and CQ activate a subset of TRPV1-positive sensory neurons that also express the ion channel TRPA1. To further test this, we used PCR to correlate TRPA1 gene expression with CQ and BAM sensitivity in individual sensory neurons, as determined by calcium imaging. Cells were subjected to RT-PCR using MrgprA3, MrgprC11 and TRPA1-specific primers. As previously reported, BAM- and CQ-sensitive neurons showed amplification of MrgprA3 and MrgprC11, respectively (Fig. 1D; Supplementary Figure 1). Likewise, all BAM-sensitive neurons also expressed MrgprA3 (7 of 7) consistent with previous studies, and our imaging data (Fig. 1B). In addition, the TRPA1 was amplified from all CQ-sensitive neurons (CQ⁺; n=7) and BAM-responsive (BAM⁺; n=7) neurons (Fig. 1D; Supplementary Fig. 1). In contrast, BAM-, CQ-, and MO-insensitive cells did not display MrgprA3, MrgprC11 or TRPA1 expression (BAM⁻ and CQ⁻; Fig. 1D; Supplementary Fig. 1). These results clearly show that CQ activates a subset of sensory neurons that express TRPA1 and TRPV1; BAM, in turn, activates a subset of CQ-sensitive cells.

Histamine and other phospholipase C (PLC)-coupled receptor agonists promote the release of Ca²⁺ from intracellular stores and subsequent activation of TRP channels. Consistent with a previous study showing that the BAM receptor, MrgprC11, couples to PLC, BAM application evokes Ca²⁺ release from intracellular stores in the absence of extracellular Ca₂⁺ (Ca²⁺EXT; Fig. 1E). Subsequent addition of Ca²⁺EXT triggers a rise in intracellular Ca²⁺ due to influx (Fig. 1E). Unlike BAM, CQ application in the absence of Ca²⁺EXT fails to mobilize Ca²⁺ release from stores. However, CQ application in extracellular Ca²⁺ triggers influx across the plasma membrane (Fig. 1F). This demonstrates that both BAM and CQ trigger the influx of Ca²⁺ through transduction channels in the plasma membrane. TRPV1 and TRPA1 are likely candidate transducers because they are expressed in CQ- and BAM-sensitive cells (Fig. 1) and are inhibited by ruthenium red, which abolishes CQ-evoked signaling. We thus asked whether BAM- and CQ-evoked excitation is attenuated by pharmacological or genetic knockdown of TRPV1 or TRPA1 channels.
Figure 1 Chloroquine and BAM activate a subset of TRPA1-positive sensory neurons

(a) BAM-evoked (100 µM, yellow arrowheads) and chloroquine-evoked (CQ, 1 mM, white arrows) responses in cultured DRG neurons (representative Fura-2 ratiometric images). Scale bar represents 10 µm. (b) Representative BAM- and chloroquine-responsive cell. Fura-2 ratio in response to BAM (100 µM), chloroquine (1 mM), mustard oil (MO, 200 µM), and capsaicin (Cap, 1 µM). (c) Representative chloroquine-sensitive, BAM-insensitive cell. Fura-2 ratio in response to BAM (100 µM), chloroquine (1 mM), mustard oil (200 µM) and capsaicin (1 µM). (d) PCR analysis of Mrgpra3, Mrgprc11 and Trpa1 expression in chloroquine- and BAM-sensitive neurons compared with expression in chloroquine-, BAM- and mustard oil–insensitive large-diameter sensory neurons. Mrgprc11 and Trpa1 were amplified in BAM-sensitive (BAM+), but not BAM-insensitive (BAM−), cells. Mrgpra3 and Trpa1 were amplified in chloroquine-sensitive (CQ+), but not chloroquine-insensitive (CQ−) cells. MrgprA3, MrgprC11 and Trpa1 were all amplified from DRG cDNA. Note the presence of control Gapdh in all samples. None of these genes were amplified in negative controls (CON, no reverse transcription). (e) Representative trace showing Ca2+ response to BAM (100 µM) in the absence (1 mM EGTA) and presence (2 mM Ca2+) of extracellular calcium. (f) Representative response to chloroquine (1 mM) in the absence (1 mM EGTA) and presence (2 mM Ca2+) of extracellular calcium.
Supplementary Figure 1: PCR analysis of MrgprA3, MrgprC11 and TRPA1 expression in CQ–positive, BAM–positive and CQ/BAM/MO–negative, large diameter sensory neurons.

Full–length agarose gels used in Figure 1. BAM- (BM) and CQ- (CQ) responding cells, and large-diameter, nonresponsive cells (NR), were selected using calcium imaging. Two to three cell samples were combined for RT-PCR using primers against MrgprA3 (A3), MrgprC11 (C11), TRPA1 (A1) and GAPDH (G). Samples were loaded as described below and are listed as sample name, followed by primer set. Fermentas 1kb Plus DNA Ladder was used in ladder lanes (L). No RT control samples were used as negative controls. Whole trigeminal ganglia were used as positive controls (POS). Blank, unlabeled lanes were not loaded (B). Regions used in Figure 1d are indicated by red boxes.

**Gel a.**

**Gel b.**

**Gel c.**

**Gel d**
TRPV1 is not required for BAM or CQ signaling

We first compared BAM- and CQ-evoked Ca\(^{2+}\) signals in neurons isolated from TRPV1-deficient mice to those isolated from wild type littermates (Fig. 2A-C). Cultured neurons isolated from TRPV1-deficient mice showed a decrease in the proportion of BAM-sensitive neurons (Fig. 2A,C) but no change in the magnitude of the Ca\(^{2+}\) signal in the responsive cells, as compared to wild type (WT peak=1.38±0.11; V1\(^{-/-}\) peak=1.52±0.16; p=0.59). Similar results were observed in wild type neurons treated with the TRPV1 antagonist, capsazepine (Fig. 2C). In contrast, no significant differences in the amplitude (WT peak=1.57±0.18; V1\(^{-/-}\) peak= 1.62±0.21) or prevalence (Fig. 2A,C) of CQ-evoked signals were observed. Wild type neurons treated with capsazepine displayed normal CQ-evoked signals (Fig. 2C).

To further probe the role of TRPV1 in CQ and BAM signaling, we next performed current-clamp recording of CQ- and BAM-evoked action potential firing in wild type and TRPV1-deficient neurons (Fig. 2D). No significant differences in action potential firing were observed between wild type and TRPV1-deficient neurons following application of BAM (WT: 39.1±10.5; Trpv1\(^{-/-}\): 46.0±15.0; p=0.73; Fig. 2D) or CQ (WT: 8.0±1.8; Trpv1\(^{-/-}\): 7.2±1.6; p=0.74; Fig. 2D). Taken together, these results demonstrate that functional TRPV1 channels are not required for BAM- or CQ-evoked excitation.

TRPA1 is required for BAM and CQ-evoked neuronal excitation

We next asked whether deficiencies in TRPA1 would alter neuronal CQ and BAM sensitivity. Unlike TRPV1-deficient neurons that display a partial attenuation of BAM responses (Fig. 2), BAM-evoked Ca\(^{2+}\) signaling is ablated in TRPA1-deficient neurons (Fig. 3A,C). Similarly, pharmacological inhibition of TRPA1 with the selective antagonist HC-030031 (HC; 100 µM\(^{98-100}\) significantly decreased neuronal sensitivity to BAM (Fig. 3C; Trpa1\(^{+/+}\): 6.18±1.49; Trpa1\(^{-/-}\): 0.57±0.36; HC-treated: 1.12±0.71).

We also examined the role of TRPA1 in CQ-evoked neuronal activation. CQ-evoked Ca\(^{2+}\) signals were significantly attenuated in TRPA1-deficient neurons (Fig. 3B-C) as compared to wild type neurons (Fig. 3B). Consistent with previous studies, MO-evoked responses were also attenuated in TRPA1-deficient neurons (Fig. 3A-B). Likewise, pharmacological inhibition of TRPA1 with HC-030031 (HC; 100 µM) significantly decreased neuronal sensitivity to CQ (Fig. 3C). Importantly, the prevalence of capsaicin-responsive cells was similar in wild type, mutant, and HC-treated neurons (Trpa1\(^{+/+}\): 52.1±5.17%; Trpa1\(^{-/-}\): 56.7±6.9%; HC-treated: 55.5±6.1%).

Finally, we used current-clamp recording to probe the role of TRPA1 in CQ- and BAM-evoked neuronal excitation. CQ- and BAM-evoked action potential firing was compared in DRG neurons treated with vehicle versus HC-030031 (Fig. 3D). TRPA1 inhibition significantly attenuated CQ-evoked action potential firing (CQ+ vehicle=6.2±1.2; 100 µM HC-030031=0.25±0.1; p=0.003; Fig. 3D) and BAM-evoked firing (BAM+ vehicle=21.3±4.2; 100 µM HC-030031=2.40±0.98; p=0.002; Fig. 3D). Together, our
Figure 2: TRPV1 is not required for chloroquine- or BAM-evoked excitation of neurons.

(a) Cultured sensory neurons isolated from wild-type and TRPV1-deficient mice were exposed to BAM (100 µM), followed by mustard oil (200 µM) and capsaicin (1 µM), and analyzed by Fura-2 ratiometric calcium imaging (representative responses). (b) Cultured sensory neurons isolated from wild-type and TRPV1-deficient mice were exposed to chloroquine (1 µM), followed by mustard oil (200 µM) and capsaicin (1 µM), and analyzed by Fura-2 ratiometric calcium imaging (representative responses). (c) The prevalence of chloroquine sensitivity was similar in wild-type (black), TRPV1-deficient (gray) and capsazepine-treated (CPZ, 20 µM, white) neurons. In contrast, the prevalence of BAM sensitivity was reduced in TRPV1-deficient (gray, P < 0.01, one-way ANOVA) and CPZ-treated neurons (white, P < 0.05, one-way ANOVA) relative to wild-type neurons (black). The prevalence of histamine (HIS) sensitivity was also reduced in TRPV1-deficient (gray, P < 0.05, one-way ANOVA) and CPZ-treated neurons (white, P < 0.05, one-way ANOVA; n = 3 animals per genotype, n ≥ 500 neurons per genotype) relative to wild-type neurons (black). Error bars represent s.e.m. (NS, not significant, P > 0.5; *P < 0.05, **P < 0.01, ***P < 0.001). (d) TRPV1 is not required for chloroquine- or BAM-evoked action potential firing. Representative current-clamp recording showing that wild-type and TRPV1-deficient neurons fired similar numbers of action potentials in response to BAM (100 µM) and chloroquine (1 mM). No responses to capsaicin (1 µM) were observed in TRPV1-deficient neurons (n = 5–13 cells per genotype).
Figure 3: TRPA1 is required for chloroquine- and BAM-evoked excitation of neurons.

(a) Cultured sensory neurons isolated from wild-type and TRPA1-deficient mice were exposed to BAM (100 µM), followed by mustard oil (200 µM) and capsaicin (1 µM), and responses were measured by Fura-2 ratiometric calcium imaging (representative responses). (b) Cultured sensory neurons isolated from wild-type and TRPA1-deficient mice were exposed to chloroquine (1 µM), followed by mustard oil (200 µM) and capsaicin (1 µM), and responses were measured by Fura-2 ratiometric calcium imaging (representative responses). (c) The prevalence of chloroquine sensitivity was significantly reduced in TRPA1-deficient (gray, P < 0.01, one-way ANOVA) and HC-03001–treated neurons (white, 100 µM, P < 0.001, one-way ANOVA) relative to wild-type neurons (black). Similarly, the prevalence of BAM sensitivity was reduced in TRPA1-deficient (gray, P < 0.001, one-way ANOVA) and HC-030031–treated neurons (white, P < 0.001, one-way ANOVA) relative to wild type (black). In contrast, the prevalence of histamine sensitivity was similar in wild-type (black), TRPA1-deficient (gray, P = 0.73, one-way ANOVA) and HC-030031-treated neurons (white, P = 0.61, one-way ANOVA, n = 3 animals per genotype, n ≥ 500 neurons per genotype). NS, not significant, P > 0.5; **P < 0.01, ***P < 0.001. Error bars represent s.e.m. (d) TRPA1 is required for chloroquine-evoked action potential firing. Representative current-clamp recording showed that HC-03001 (100 µM) blocked chloroquine-evoked action potential firing (n ≥ 5 cells per compound).
results clearly show that functional TRPA1 channels are required for CQ and BAM-evoked neuronal excitability.

While TRPA1 is required for CQ and BAM signaling, it does not mediate all forms of itch. Neurons isolated from TRPA1-deficient animals (Fig. 3C) or treated with HC-030031 (100 µM; Fig. 3C) display normal histamine-evoked responses. These findings are consistent with previous studies showing that TRPV1, but not TRPA1, is required for histamine signaling in sensory neurons\(^{31,36,101}\).

**MrgprA3 and MrgprC11 functionally couple to TRPA1**

The GPCRs MrgprA3 and MrgprC11 are required for CQ and BAM signaling in sensory neurons, respectively\(^{53}\). In addition to being activated directly by endogenous and exogenous irritants, TRPA1 is a receptor-operated channel that can be activated by bradykinin, or other GPCR-coupled inflammatory mediators\(^{48,51}\). We therefore asked whether CQ or BAM could activate heterologous TRPA1 channels expressed in the CQ- and BAM-insensitive neuroblastoma cell line, NG108. CQ and BAM fail to trigger Ca\(^{2+}\) influx into TRPA1-transfected cells (Fig. 4A). However, these cells responded robustly to application of MO (200 µM; Fig. 4A), confirming the presence of functional TRPA1 channels. CQ-evoked Ca\(^{2+}\) signals were not observed in NG108 cells transfected with Mrgpra3 alone (Fig. 4A-B). Consistent with our findings in TRPV1-deficient neurons (Fig. 2), CQ failed to trigger Ca\(^{2+}\) signals in cells expressing Mrgpra3 and TRPV1 (Fig. 4B). In contrast, NG108 cells transfected with both TRPA1 and Mrgpra3 (A1/A3) displayed robust increases in intracellular Ca\(^{2+}\) following CQ application (Fig. 4A-B); these responses were attenuated by HC-030031 (100 µM; not shown). Thus, both MrgprA3 and TRPA1 receptors are required to confer CQ-sensitivity to NG108 cells.

BAM-evoked Ca\(^{2+}\) signals were observed in NG108 cells transfected with Mrgprc11 alone, but not cells transfected with TRPA1, TRPV1 or vector alone (Fig. 4C). This is consistent with our data showing that MrgprC11 activation leads to Ca\(^{2+}\)-release from stores (Fig. 1E), and previous studies linking MrgprC11 and PLC\(^{102}\). Co-transfection of TRPV1 with Mrgprc11 caused an increase in the amplitude of the BAM response (30.1% increase; \(p=0.005\) Fig. 4C, middle). However, co-expression of TRPA1 with Mrgprc11 led to an even more robust increase in intracellular Ca\(^{2+}\), 81% higher than with Mrgprc11 alone (\(p=0.0001\); Fig. 4C). These data suggest that both TRPV1 and TRPA1 couple to MrgprC11, consistent with our findings that both channels contribute to BAM-evoked Ca\(^{2+}\) responses in neurons (Figs. 2C and 3C).

**MrgprA3 and MrgprC11 couple to TRPA1 via distinct mechanisms**

We next examined the mechanisms by which MrgprA3 preferentially activates TRPA1, but not TRPV1. Because many TRP channels are activated or modulated by PLC-coupled receptors\(^{103}\), and many pruritogens and members of the Mrgpr family activate PLC signaling\(^{97}\), we first tested the role of PLC in CQ-evoked signaling. The PLC inhibitor, U73122, had no effect on the amplitude (Fig. 5A) or prevalence (Fig. 5B).
Figure 4: MrgprA3 and MrgprC11 couple to TRPA1 in neuronal cell lines.

(a) Chloroquine-evoked (1 mM) calcium response in NG108 cells transfected with both Trpa1 and Mrgpra3 (bottom), Mrgpra3 alone (top), or Trpa1 alone (middle). TRPA1 expression was assessed by application of mustard oil (100 µM). Scale bar represents 10 µm. (b) Chloroquine-evoked Fura-2 ratiometric responses (average traces) in NG108 cells transfected with Mrgpra3 (left), Mrgpra3 and Trpv1 (middle), or with Mrgpra3 and Trpa1². Ionomycin (1 µM) treatment indicated that the Mrgpra3-transfected cells were healthy and loaded with Fura-2. Capsaicin (1 µM) and mustard oil (200 µM) were used to activate TRPV1 and TRPA1 channels, respectively. MrgprA3 expression was assessed by GFP fluorescence (data not shown). (c) BAM-evoked Fura-2 ratiometric responses (average traces) in NG108 cells transfected with Mrgprc11 (1.58 ± 0.16, left), Mrgprc11 and Trpv1 (2.1 ± 0.3, middle), or with Mrgprc11 and Trpa1 (2.8 ± 0.3, right). Values are shown as peak ± s.e.m. (Mrgprc11 alone versus Mrgprc11 + Trpv1, P = 0.005; Mrgprc11 alone versus Mrgprc11 + Trpa1, P = 0.0001; Mrgprc11 + Trpa1 versus Mrgprc11 + Trpv1, P = 0.004). Capsaicin (1 µM) and mustard oil (200 µM) were used to activate TRPV1 and TRPA1 channels, respectively. MrgprC11 expression was assessed by GFP fluorescence (data not shown).
of CQ-evoked Ca$^{2+}$ signals in cultured neurons or A1/A3 NG108 cells (not shown). BAM activation of MrgprC11 has been previously demonstrated to act through PLC$^{97}$. Consistent with these findings, U73122 significantly reduced both the amplitude of BAM-evoked Ca$^{2+}$ signals in cultured neurons (Fig. 5B) and the prevalence of BAM-sensitive neurons (Fig. 5C). Likewise, U73122 significantly attenuated histamine signaling in neurons (Fig. 5C). These data show that while PLC signaling is required for BAM- and histamine-evoked signaling, it is not required for MrgprA3 mediated activation of TRPA1.

GPCR signaling leads to the dissociation of both G$\alpha$ and G$\beta\gamma$ subunits. In addition, G$\beta\gamma$ signaling has been shown to directly open ion channels$^{104}$. We thus asked whether G$\beta\gamma$ signaling is required for MrgprA3-evoked activation of TRPA1. Pre-treatment of neurons with gallein, a small molecule inhibitor of G$\beta\gamma$, dramatically reduced both the amplitude of CQ-evoked Ca$^{2+}$ signals (Fig. 5A), and the number of CQ-sensitive cells (vehicle: 17.6±1.1%; gallein: 4.6±1.1%; Fig. 5C). Gallein does not act directly on TRPA1, as mustard oil-evoked activation of TRPA1 is not altered by this inhibitor (not shown). Likewise, gallein has no effect on histamine-evoked signaling in neurons (Fig. 5C). We also probed the role of G$\beta\gamma$ in CQ-evoked neuronal excitation using current-clamp recording. Gallein significantly attenuated membrane depolarization and action potential firing caused by CQ application (vehicle: 17.00±13.24; 100 µM gallein: 1.33±1.53; Fig. 5D). Finally, we explored the role of G$\beta\gamma$ in the coupling between MrgprA3 and TRPA1 in heterologous cells. Co-expression of phosducin (Pdc), a G$\beta\gamma$ chelating peptide$^{105}$, or treatment with gallein, significantly attenuates CQ responses in NG108 cells (Fig. 5E; control=82.34±9.61; phosducin=58.21±11.61; p=0.003; and data not shown). These experiments suggest that G$\beta\gamma\beta\beta$ signaling is required for MrgprA3 coupling to TRPA1.

G$\beta\gamma$ signaling has also been shown to open channels via PLC$^{106,107}$. Thus, we asked whether Gbg signaling is also required for the PLC-dependent coupling between MrgprC11 and TRPA1. Pre-treatment of neurons with gallein had no significant effect on the amplitude of BAM-evoked Ca$^{2+}$ signals in neurons (Fig. 5C), or the fraction of BAM-sensitive cells (Fig. 5C; vehicle (VEH): 7.06±1.94%; U73122 (U7): 0.98±0.95%; gallein: 6.54±3.46%). Similarly, overexpression of Pdc in TRPA1/Mrgprc11 NG108 cells fails to attenuate BAM-evoked responses (Fig. 5E; control=82.37±6.55; phosducin=81.95±6.11; p=0.887). These experiments provide evidence that PLC signaling through G$\alpha_q$ is required for MrgprC11 evoked neuronal activation, and may explain why MrgprC11 can couple to both TRPA1 and TRPV1, similar to the bradykinin receptor$^{44,51}$.

**TRPA1 is required for CQ- and BAM-evoked itch**

Given the requirement for TRPA1 in the cellular actions of CQ and BAM, we asked whether TRPA1-deficient mice also exhibit behavioral deficits in CQ- and BAM-evoked itch. We examined scratching following injection of these pruritogens into the nape of the neck. CQ and BAM evoked robust scratching behaviors in wild type mice. (Fig. 6A). The time spent scratching was significantly attenuated in TRPA1-deficient littermates.
Figure 5: MrgprA3 and MrgprC11 utilize distinct signaling pathways to activate TRPA1.

(a) Chloroquine-evoked (1 mM) calcium signals (representative traces) in cultured sensory neurons following pre-treatment (5 min) with vehicle (left), the Gβγ inhibitor gallein (middle, 100 µM) or the PLC inhibitor U73122 (right, 1 µM) as measured by Fura-2 ratiometric calcium imaging. (b) BAM-evoked (100 µM) calcium signals (representative traces) in cultured sensory neurons following pre-treatment (5 min) with vehicle (left), gallein (middle, 100 µM) or U73122 (right, 1 µM) as measured by Fura-2 ratiometric calcium imaging. (c) Quantification of the percentage of chloroquine-, BAM- and HIS-sensitive neurons following treatment with vehicle (VEH, black), gallein (GAL, white) or U73122 (U7, gray). (d) Gallein inhibited chloroquine-evoked action potential firing. Representative current-clamp recording showed that gallein (100 µM) blocked chloroquine-evoked action potential firing. (n = 3–5 cells per compound). (e) Quantification of percentage of chloroquine-sensitive NG108 cells expressing Mrgpra3 and Trpa1 and BAM-sensitive NG108 cells expressing MrgprC11 and Trpa1 co-transfected with the phosducin (PH) or control vector (CN) (n = 3 transfections, with ≥1,200 cells per treatment). NS, not significant, P > 0.05; **P < 0.01, ***P < 0.001; one way ANOVA. All error bars represent s.e.m.
(Fig. 6A) to levels similar to vehicle injection (Fig. 6A). In contrast, no differences between wild type mice and TRPV1-deficient littermates were observed for either CQ or BAM injection (Fig. 6A). These results suggest that while TRPV1 partially contributes to the cellular responses to BAM in culture, the residual BAM-sensitivity in the TRPV1-deficient neurons drives BAM-evoked itch behaviors and requires functional TRPA1 channels.

To distinguish between CQ- and BAM-evoked itch and pain behavior, we used the “cheek” model of itch, where an irritant is injected into the cheek, rather than the neck. Injection of CQ or BAM evokes robust scratching of the cheek with the hindlimb (Fig. 6B-C). In contrast, injection of an irritant, such as mustard oil (1 mM), evokes wiping of the cheek with one of the forelimbs (Fig. 6B). Standard grooming behaviors always involve rubbing the head or face with both forelimbs (not shown). Wiping was never observed following injection of CQ or BAM. Thus we used this model to better examine the in vivo role of TRPA1 in CQ- and BAM-evoked itch. Using the cheek assay, CQ and BAM evoked prolonged periods of scratching in wild type mice. No significant differences were observed between Trpa1+/+ mice and Trpv1+/+ mice (BAM: A1-WT=49.2 s; V1-WT=50.5 s; p=0.90; one-way ANOVA; CQ: A1-WT=111 s; V1-WT=104.8 s; p=0.94; one-way ANOVA), thus data from these animals were combined (Fig. 6C). Similarly, no significant differences in BAM- or CQ-evoked scratching were observed between wild type and TRPV1-deficient mice (Fig. 6C). In contrast, this scratching behavior was never observed in TRPA1-deficient mice (Fig. 6C). TRPA1-deficient mice were not generally incapable of scratching, or insensitive to all pruritogens, as cheek injection of alpha-methyl-serotonin (2 µM) evoked robust scratching (WT=48.3±10.8 s; Trpa1−/−=51.0±10.4 s; p=0.87; n=11/genotype). These experiments demonstrate that TRPA1 is required for both CQ and BAM-evoked itch.

DISCUSSION

Itch is mediated by both histamine-dependent and independent pathways. Chronic itch associated with skin and systemic diseases is insensitive to antihistamine treatment, and even allergic itch is only marginally inhibited by histamine receptor antagonists. However, little is known about the mechanisms underlying histamine-independent itch. The GPCRs MrgprA3 and MrgprC11 are receptors for CQ and BAM8–22, respectively, two pruritogens that elicit robust antihistamine-insensitive itch53,111. Our results clearly demonstrate that TRPA1 is activated downstream of both MrgprA3 and MrgprC11, and is the primary transduction channel mediating CQ- and BAM-evoked signaling and itch behaviors.

Most Mrgprs are orphan GPCRs and their underlying mechanisms of signal transduction are largely unknown. However, MrgprC11 has been shown to couple to the Gαq/11 pathway and activate PLC in heterologous cells97. Consistent with these findings, we show that MrgprC11-evoked excitation requires functional PLC signaling in neurons. Most TRP channels are activated or modulated by PLC, making them likely downstream targets of MrgprC11. Indeed, MrgprC11-positive BAM-sensitive neurons express both TRPA1 and TRPV1. Thus, it is not surprising that BAM activates both TRPA1 and
Figure 6: TRPA1-deficient mice are insensitive to chloroquine- and BAM-mediated itch.

(a) Itch-evoked scratching was measured in wild-type (WT, black), TRPV1-deficient (Trpv1\(^{-/-}\), dark gray) and TRPA1-deficient (Trpa1\(^{-/-}\), light gray) mice following subcutaneous injection of chloroquine (200 mg per 50 µl, 8 mM) or BAM (60 µg per 10 µl, 3.5 mM) into the nape of the neck. The total time spent scratching was quantified for 20 min after injection. Injection of vehicle (phosphate-buffered saline, 50 µl) elicited some scratching in wild-type mice (VEH, white). (b) In the cheek model of itch, subcutaneous injection of a pruritogen into the cheek (chloroquine, 200 µg per 10 µl, 40 mM) elicited scratching of the cheek with the hindpaw (left). In contrast, injection of an irritant, mustard oil (1 mM), evoked wiping with one of the forelimbs (right). (c) Itch-evoked scratching was measured in wild-type (black), Trpv1\(^{-/-}\) (dark gray) and Trpa1\(^{-/-}\) (light gray) mice following chloroquine (200 µg per 10 µl, 40 mM) or BAM (60 µg per 10 µl, 3.5 mM) injection in the cheek. The total time spent scratching was quantified for 20 min after injection. Injection of vehicle (phosphate-buffered saline, 10 µl) did not elicit scratching or wiping (VEH, white). NS, not significant, P > 0.05; **P < 0.01; ***P < 0.001, one-way ANOVA. All error bars represent s.e.m. (n ≥ 8 mice per genotype).
TRPV1 in heterologous cells or that both channels contribute to BAM-evoked calcium signals in neurons. It is surprising, however, that TRPA1, but not TRPV1, is required for BAM-evoked itch behaviors. This finding is similar to bradykinin-evoked signaling whereby PLC activation robustly activates TRPA1, and weakly activates TRPV1 to promote calcium influx; because calcium also activates TRPA1\textsuperscript{112,113}, calcium permeation through TRPV1 opens additional TRPA1 channels, leading ultimately to mechanical and thermal hypersensitivity. Similar to BAM, loss of TRPV1 or TRPA1 leads to diminished bradykinin-evoked calcium signaling \textit{in vitro}, but only the loss of TRPA1 leads to attenuation of inflammatory behavioral responses. Thus TRPA1 plays a dominant role in both bradykinin and BAM signaling \textit{in vivo}.

Unlike BAM, pharmacological inhibition of PLC does not alter CQ-evoked activation of TRPA1 in sensory neurons or transfected cell lines. These findings are consistent with a previous study showing that CQ-evoked itch is normal in mice lacking PLCb\textsuperscript{36}. In addition, CQ-evoked signaling does not require functional TRPV1 channels in neurons, and MrgprA3 fails to couple to TRPV1 in heterologous cells. What signaling pathway mediates the functional coupling of MrgprA3 to TRPA1, but not TRPV1? In somatosensation, Gbg is required for morphine-evoked analgesia and directly activates N- and P/Q-type calcium channels in cultured dorsal root ganglia neurons\textsuperscript{114,115}. Here we show that Gbg may be yet another signaling molecule capable of modulating the activity of a TRP channel. Gallein, a small molecule inhibitor of Gbg and the Gbg chelating peptide of phosducin specifically attenuate CQ-evoked signaling, with no effects on histamine or BAM signaling. Taken together, these data indicate that Gbg is a likely candidate for mediating the specific coupling of MrgprA3 and TRPA1. Gbg modulates several ion channels via direct binding, including members of the G protein-coupled inwardly-rectifying potassium channel and voltage-gated calcium channel families\textsuperscript{104}. Future studies will elucidate whether Gbg opens TRPA1 channels directly, or via another signaling intermediate.

Our findings support the hypothesis that TRP channels are key mediators of both pain and itch. Previous studies have shown that TRPV1 is a primary transducer of histamine-evoked itch\textsuperscript{36,101}. However, only a subset of TRPV1-positive neurons express histamine receptors and transduce itch. Likewise, only a subset of TRPA1-positive neurons co-express MrgprA3 and respond to CQ, and an even smaller subset of these cells also express MrgprC11 and respond to BAM. The molecularly distinct subsets of TRPA1-positive neurons that transduce BAM and CQ itch signals support the labeled line theory of itch, whereby distinct pruritogens use a dedicated pathway to transduce itch signals. In contrast, the identification of TRPA1 as a key transducer of itch and pain also supports the spatial contrast theory of itch, whereby itch is triggered by the activation of a small number of pain fibers within a receptive field, and pain is initiated when a larger cohort of cells are activated\textsuperscript{116}. Like TRPA1 and TRPV1, MrgprC11 has been proposed to play a role not only in itch, but also in hyperalgesia\textsuperscript{117}. In addition, several studies describe the inhibition of itch by painful chemical or mechanical stimuli\textsuperscript{4,77,118}. Strong support of both itch theories has led to a modified “selectivity” theory of itch\textsuperscript{4}, that incorporates aspects of both itch models. The recent discovery of itch specific spinal
Our results reveal a novel role for TRPA1 in CQ-evoked itch. A major side effect of the MrgprA3 agonist and anti-malarial drug, CQ, is intolerable itch. CQ is cheap, easy to administer, and highly effective in both treating and preventing malaria. Indeed, the demand for CQ is on the rise, as recent studies have shown a decrease in CQ-resistant Plasmodium falciparum\textsuperscript{119}. However, CQ-evoked itch, which is especially prevalent among dark-skinned Africans (up to 70%), is a major cause of poor compliance or treatment defaulting\textsuperscript{81}. Differences in pruritic response to CQ may result from polymorphic differences in the Mrgpr signaling pathway or in TRPA1, as in Familial Episodic Pain Syndrome, recently linked to gain of function mutations in TRPA1\textsuperscript{120}. In such cases, improved therapeutics employing inhibition of MrgprA3 or TRPA1 aimed at alleviating chloroquine-induced itch may enable CQ to remain a useful and relevant treatment in Africa.

Aside from CQ, chronic itch results from skin diseases and systemic conditions, such as eczema, cirrhosis and some cancers, diabetes, as well as neurological disorders including multiple sclerosis, post-herpetic neuralgia and perhaps the most prevalent, allergic inflammation. Mast cell-neuronal interactions are known to play key roles in all of these pruritic conditions. Mast cells are in close association to peripheral nerves and release a variety of pruritic factors that act on sensory neurons. MrgprA4 and MrgprC11 are both activated by neuropeptide FF, a pruritogen released from mast cells during allergy-induced mast cell degranulation\textsuperscript{53,93}. These findings show that endogenous pruritogens target members of the Mrgpr family and demonstrate an essential role for MrgprC11, and therefore TRPA1, in allergic mast cell-mediated inflammation.

Perhaps most importantly, our findings demonstrate that TRPA1 is a downstream transduction channel onto which multiple histamine-independent itch pathways converge. BAM and CQ lead to TRPA1 excitation via two distinct signaling pathways. Our behavioral studies show a dramatic loss of itch-evoked behaviors in TRPA1-deficient animals in response to both of these pruritogens. As such, TRPA1 antagonists may be useful for the selective attenuation of antihistamine-insensitive itch, a problem that is especially relevant to pathological itch conditions. Whether MrgprA3, MrgprC11, and TRPA1 signaling contribute to chronic forms of itch is unknown. Mrgpr and TRPA1-deficient mice now provide a genetic model with which to assess the mechanisms of intractable itch.

**EXPERIMENTAL PROCEDURES**

**Neuronal cell culture**

For all experiments shown, trigeminal or dorsal root ganglion neurons were isolated from P0–P14 mouse pups. However, all results were also confirmed using neuronal cultures from adult mice. Preparation of neurons and ratiometric calcium imaging were
carried out as previously described\textsuperscript{51}. Briefly, neurons from sensory ganglia were dissected and incubated for 10 minutes in 1.4 mg/ml Collagenase P (Roche) in Hanks Calcium-Free Balanced Salt Solution. Neurons were then incubated in 0.25\% Standard Trypsin, Versene-EDTA solution (STV) for 3 minutes with gentle agitation. Cells were washed then triturated and plated in media (MEM Eagle’s with Earle’s BSS medium, supplemented with 10\% horse serum, MEM vitamins, penicillin/streptomycin, and L-glutamine) Neurons were plated onto glass coverslips and used within 20 hours. All media and cell culture supplements were purchased from the UCSF Cell Culture Facility.

\textit{NG108 cell culture}

NG108 cells were cultured on poly-D-lysine-coated chamber slides (Nalgene-Nunc). Cells were transfected with Lipofectamine 2000 (Invitrogen) using 150 ng human \textit{TRPA1}, 150 ng human \textit{TRPV1}, 500 ng human \textit{HRH1}, 500 ng mouse \textit{Mrgprc11}, and/or 500 ng mouse \textit{Mgrpra3} plasmids. 16 hours after transfection, cells were replated onto glass coverslides and used for calcium imaging.

\textit{Calcium Imaging}

For calcium imaging experiments, cells were loaded for 1 hour with 10 \textmu M Fura-2 AM (Invitrogen), supplemented with 0.01\% Pluronic F-127 (Invitrogen), in a physiological Ringer solution containing (in mM) 140 NaCl, 5 KCl, 10 HEPES, 2 CaCl\textsubscript{2}, 2 MgCl\textsubscript{2}, 10 D-(+)-glucose, pH 7.4. All chemicals were purchased from Sigma. Acquired images were displayed as the ratio of 340 nm to 380 nm and aligned using MetaMorph software. Cells were identified as neurons by eliciting depolarization with high potassium solution (75 mM) at the end of each experiment. Neurons were deemed to be sensitive to an agonist if the average ratio during the 10 s after agonist application was \geq15\% above baseline. Image analysis and statistics were performed using custom routines in Matlab and Igor Pro (WaveMetrics). Statistical significance was assessed by one-way analysis of variance (ANOVA), followed by Tukey’s HSD. All graphs displaying Fura-2 ratios have been normalized to the baseline ratio: \textit{Ratio F\textsubscript{340}/F\textsubscript{380}= (Ratio)/(Ratio\textsubscript{t=0}).}

\textit{Electrophysiology}

Primary mouse DRG neurons were assessed for CQ- and BAM-sensitivity using calcium imaging as described above. Cells displaying a >15\% change in Fura-2 ratio following a 15 second application of CQ (1 mM) or BAM (100 \textmu M) were chosen for whole-cell current-clamp recordings. Current clamp recordings were performed as previously described (Fujita et al, 2008). Electrode resistance ranged between 2–6M\textOmega. Internal solution contained (in mM): 140 mM KCl, 5 mM EGTA, 10 mM HEPES (pH 7.4 with KOH). The pipette potential was canceled before seal formation. Liquid junction potentials were <5 mV and were not corrected. Experiments were carried out only on cells with a series resistance of under 30M\textOmega. Resting membrane potential averaged \textpm 55\%\pm8.2 mV with a firing threshold of \textpm 44.5\%\pm7.0 mV. Data were collected at 5 kHz and filtered at 2 kHz (Axopatch 200B, PClamp software).
Mice and Behavior
Mice (20–35 g) were housed with 12 hr light-dark cycle at 21°C. For assessing chloroquine-evoked itch behaviors, mice received a subcutaneous injection into the cheek (10 µL) or neck (50 µL), with one of three solutions: 1) Ca²⁺ and Mg²⁺-free Phosphate buffered saline (PBS); 2) 10 µg BAM dissolved in PBS; or 3) 200 µg chloroquine dissolved in PBS. Mice were videotaped for 25 minutes following injection. The amount of time each mouse spent scratching, and the number of scratch bouts, were quantified over a 20-minute period. One bout of scratching was defined as an episode in which a mouse lifted its paw and scratched continuously for any length of time, until the paw was returned to the floor. Behavioral scoring was performed while blind to genotype and to the solution injected. All experiments were performed under the policies and recommendations of the International Association for the Study of Pain and approved by the University of California, Berkeley Animal Care and Use Committee.

PCR
RNA was isolated from individual sensory neurons. Cells were first examined for chloroquine or BAM8–22 sensitivity by calcium imaging, 3–4 cells in each category were aspirated into a large–diameter glass electrode filled with lysis buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 5 U⁻¹ RNasin (Promega)) and were flash frozen. Reverse transcription was performed using murine Moloney leukemia virus and avian reverse transcriptases at 37 °C for 1 h. The product was diluted 1:10 and used as the template for PCR experiments. Primers for PCR were:

TRPA1
5’–GATGCCTTCAGCACCCCCATTGCTTTCTTAATC–3’
5’–CTAAAAAGTCGGGTGGCTAATAGAACA–3’

MrgC11
5’–GCCTCTTGCGTGGTATTCTGTT–3’
5’–GGGACCTATGCTTTCTATGCTG–3’

MrgA3
5’–CGACAATGACACCCCCAAAACACAA–3’
5’–GGGAGCCAAGGAGCCAGAAC–3’

GAPDH
5’–CCATGACAACCCTTGCGCATTG–3’
5’–CCTGGCCTACCCACCTCCCTTG–3’.

Statistical analysis
Values are reported as the mean ± s.e.m. For comparison between two groups, a one–way ANOVA followed by a Tukey–Kramer post hoc test was used. To analyze a variable between two or more groups over multiple measurements, a two–way ANOVA was used.
CHAPTER II: TRPA1 is required for chronic itch.

This chapter is a reproduction of the paper by the same name published in The Journal of Neuroscience May 2013. My contributions were to Figure 1a-c, 2a-d, 3, 4a-b, 5a-b, and 6. For this paper I performed mouse behavioral experiments, tissue RNA isolation, microarray analysis and made figures and wrote the manuscript with D.M.B.

SUMMARY

Chronic itch is a debilitating condition that affects one in 10 people. Little is known about the molecules that mediate chronic itch in primary sensory neurons and skin. We demonstrate that the ion channel TRPA1 is required for chronic itch. Using a mouse model of chronic itch, we show that scratching evoked by impaired skin barrier is abolished in TRPA1-deficient animals. This model recapitulates many of the pathophysiological hallmarks of chronic itch that are observed in prevalent human diseases such as atopic dermatitis and psoriasis, including robust scratching, extensive epidermal hyperplasia, and dramatic changes in gene expression in sensory neurons and skin. Remarkably, TRPA1 is required for both transduction of chronic itch signals to the CNS and for the dramatic skin changes triggered by dry-skin-evoked itch and scratching. These data suggest that TRPA1 regulates both itch transduction and pathophysiological changes in the skin that promote chronic itch.

INTRODUCTION

Chronic itch is a widespread and debilitating condition that results from a variety of pathological conditions such as eczema, kidney failure, cirrhosis, nervous system disorders, and some cancers. Although a number of factors have been identified that mediate acute itch transduction in primary sensory afferents and the CNS, several major obstacles persist that cloud our understanding of chronic itch. First, very little is known about the fundamental basis for chronic itch perception, including the molecules that underlie chronic itch and promote itch sensations, in both primary sensory neurons and the skin. Second, the signaling pathways in the skin and the peripheral nervous system that initiate and sustain chronic itch conditions remain unknown. Finally, the contribution of the itch-scratch cycle, in which repetitive scratching worsens itch symptoms, to the progression of chronic itch has not been examined thoroughly. Therefore, there is an urgent need to better understand the cellular and molecular mechanisms underlying chronic itch.

A variety of cell types contribute to chronic itch pathologies and sensations. In the skin, tissue-resident cells such as keratinocytes and cells that infiltrate during inflammation, such as lymphocytes, mast cells, and eosinophils, release pruritogens that activate primary afferent neurons. These pruritogens are detected by the free nerve endings of primary afferent C-fibers located within the epidermis. Itch is mediated by both
histamine-sensitive and histamine-insensitive C-fibers, although most forms of chronic itch are insensitive to antihistamine treatment and are mediated by histamine-insensitive neurons\textsuperscript{125,126}.

The ion channel TRPA1 is required for acute histamine-independent itch\textsuperscript{54}. However, whether TRPA1 ion channels are required for chronic itch has not been examined. Using a mouse cheek model of chronic itch adapted from the model used by Miyamoto et al., we show that scratching evoked by a dry skin model is abolished in TRPA1-deficient animals. This model recapitulates many of the pathophysiological hallmarks of chronic itch that are observed in prevalent human diseases such as psoriasis, including robust scratching, extensive epidermal hyperplasia, and changes in gene expression in the skin\textsuperscript{121}. We provide clear evidence that TRPA1-expressing sensory neurons drive all of these dry-skin-evoked phenotypes. In addition, we show for the first time that a model of chronic itch leads to significant expressional changes in the sensory neurons that innervate the affected skin. Finally, we demonstrate that dry, itchy skin triggers TRPA1-dependent hyperplasia in both the presence and absence of scratching. Remarkably, TRPA1 is required not only for transduction of chronic itch signals to the CNS, but also for the changes in gene expression that we observed in both neurons and skin.

RESULTS

Cheek model of dry skin pruritus induces itch behavior and epidermal thickening

To determine whether TRPA1 plays a role in chronic itch, we developed and validated a cheek assay for analyzing the molecular and cellular mechanisms underlying dry-skin-evoked chronic itch. Previous studies have shown that topical application of AEW to the rostral back/neck area of mice for 3–7 d induces dry skin and increases scratching significantly\textsuperscript{127,128}. However, injection of both algogens and pruritogens into the rostral back triggers scratching, making it difficult to distinguish between itch and pain behaviors\textsuperscript{109}. This ambiguity confounds studies of pruritus because chronic itch lesions, which are described to be both painful and itchy, develop in chronic itch models\textsuperscript{129,130}. This uncertainty is particularly relevant when studying genes such as Trpa1, which are implicated in both itch and pain\textsuperscript{51,54}. In the cheek model of acute itch, injection of algogens triggers wiping with the forelimbs, whereas injection of pruritogens leads to scratching with the hindlimbs\textsuperscript{109}. We combined these approaches to develop a cheek model of AEW-evoked dry skin in which the acetone/ether mixture and water are applied to the mouse cheek for 5 d (Fig. 1A,B).

We observed that, like AEW treatment in the rostral back/neck, cheek AEW treatment significantly increased scratching starting on the third day of treatment that doubled by day five (DAY 3 VEH = 0 ± 0 s, DAY 3 AEW = 52.43 ± 12.39 s, DAY 5 VEH = 4.00 ± 5.23 s, DAY 5 AEW = 114.33 ± 22.76 s; Fig. 1C). Consistent with previous studies in untreated mice, topical application of mustard oil, a TRPA1 agonist and algogen, to the cheek evoked robust nocifensive behaviors (cheek wiping, 20 s in 2 min), which were never observed during AEW treatment\textsuperscript{131}. These data show that the AEW-cheek model triggers robust itch behaviors in mice.
Figure 1. Cheek model of dry skin pruritus induces scratching and epidermal thickening.

A, Time course for the dry skin assay in the mouse cheek. First day: the right cheek of each mouse was shaved. Days one to five: the shaved cheek was treated twice daily with a 1:1 mixture of acetone and ether, followed by water. Scratching behaviors were recorded for 20 min on days three and five after the second AEW treatment. Mouse cheeks were removed for histological analysis following recording on either day three or day five. B, Photo displaying the area of treatment in the cheek model of itch. C, Scratching behaviors were observed on day three and day five of cheek treatment. The total time spent scratching was quantified for 20 min. Application of vehicle (VEH, water) failed to elicit scratching or wiping. All error bars represent SEM (n ≥ 12 mice/group, **p < 0.01, one-way ANOVA). D, H&E-stained cheek skin sections from mice treated for 3 d (top) or 5 d (bottom) with AEW on ipsilateral and VEH on contralateral cheeks (left). E indicates epidermis; D, dermis. E, Thickness of nucleated epidermal layers was quantified from AEW-treated and VEH-treated skin. Means ± SEM are shown (n = 16–24 sections from two mice per group, ***p < 0.002, Student’s two-tail t test).
AEW treatment was shown previously to induce epidermal thickening in skin isolated from the nape of the neck, a hallmark of psoriatic chronic itch in human\textsuperscript{127}. We performed quantitative histological analysis to investigate whether AEW-evoked itching is accompanied by keratinocyte hyperplasia in the cheek skin. To control for variability between mice, we modified our AEW protocol and treated one cheek with AEW and the contralateral cheek with water (vehicle). Cheek skin treated with AEW for 3 d showed a significant increase in the thickness of nucleated epidermal layers compared with vehicle-treated contralateral cheeks (VEH = 7.4 ± 0.2 µm, AEW = 11.1 ± 0.8 µm; Fig. 1D,E). After 5 d of AEW treatment, the treated epidermis was ~4-fold thicker than the contralateral control skin (VEH = 8.8 ± 0.4 µm, AEW = 35.6 ± 4.1 µm; Fig. 1E).

Epidermal hyperplasia may occur from dry skin treatment alone and/or from dry-skin-evoked scratching\textsuperscript{132-138}. To examine the contribution of scratching to AEW-evoked hyperplasia, animals must be prevented from scratching the AEW-treated skin. We were unsuccessful in preventing AEW mice from scratching the cheek using all commercially available restraint methods. Unlike water-treated mice, AEW-treated mice were highly motivated to remove such restraints and succeeded in removing apparatuses within 24 h. Therefore, we instead returned to the back model of dry skin to address this question, specifically focusing on a caudal area of the back, just rostral to the tail, which mice cannot directly access to scratch, bite, or wipe (Fig. 2A). Both the rostral and caudal back have been extensively used in rodents to study acute and chronic itch\textsuperscript{2,25,89,139-148}. For example, pruritogen injection into the caudal back was shown to cause a 3.4-fold increase in itch behavior adjacent to the injection site (off-site scratching) and 5.4-fold increase in the activity of cutaneous nerves innervating the caudal back\textsuperscript{4}. Likewise, an animal model of allergic chronic itch in the mouse back triggered itch behaviors, as well as epidermal hyperplasia and immune cell infiltration in the back skin of treated mice\textsuperscript{148}. Indeed, a previous study in rodents showed that off-site scratching (within 5–17 mm of a pruritogen injection site) was antipruritic\textsuperscript{149}.

Several additional lines of data show that AEW on the caudal back is an effective model and that mice treated with AEW on the caudal back display itch behaviors. First, these mice spend a significant amount of time scratching areas of their body that can be reached (Fig. 2B). As observed on-site in the cheek and rostral back, mice treated with AEW on the caudal back showed off-site hair loss and skin lesions consistent with repetitive scratching. We observed similar off-site scratching after acute injection of the histamine-independent pruritogen, chloroquine (Fig. 2B). In addition, mice with AEW treatment on the caudal back spend a significant amount of time attempting to press their backs against the behavior chamber wall (Fig. 2C). Moreover, these mice appeared hyperactive, spending considerably more time moving around, with repetitive failed attempts to contact the site of treatment to the wall (Fig. 2D). Such behaviors were never observed in control mice. AEW also induces skin thickening on the caudal back, as it does on the cheek (Fig. 2E,F) and rostral back\textsuperscript{127}. Interestingly, hyperplasias developed to a much lesser extent than that observed in the presence of scratching (Fig. 2F). Based on these results, we conclude that the AEW model induces itch behaviors, as well as scratch-dependent and -independent epidermal hyperplasia, and
Figure 2. AEW caudal back model of dry skin pruritus induces off-site scratching, epidermal thickening, and increased locomotor activity.

A, Photo displaying the area of treatment in the caudal back model of itch. This assay produces dry skin, but the inaccessible location prevents scratching, wiping, or biting of the treated area. B, Image displaying location of off-site scratching after treatment to the caudal back. Off-site scratching was observed in response to both acute subcutaneous injection of chloroquine (CQ) and chronic (5 d) AEW treatment to the caudal back. AEW-evoked scratching behaviors were recorded on day five of treatment. After both treatments, the total time spent scratching or biting was quantified for 20 min. Application of vehicle (VEH, water) failed to elicit scratching or biting toward any site. C, Image displaying AEW site-to-wall contact behavior. Site-to-wall behaviors were observed on day five of treatment. The total time spent in a back-to-wall configuration was quantified for 20 min. Application of VEH failed to elicit back-to-wall contact. D, AEW-treated mice display a significant increase in locomotor activity in which they move and frequently attempt to contact the caudal back to the chamber wall. The percentage of time spent moving was quantified for 20 min. Application of VEH failed to elicit back-to-wall contact. E, H&E-stained caudal back skin sections from mice treated for 5 d with AEW or VEH on the caudal back (VEH; water; left). E indicates epidermis; D, dermis. F, Thickness of nucleated epidermal layers was quantified from AEW-treated and VEH-treated skin. All error bars represent SEM (n ≥ 6 mice/group, ***p < 0.001, one-way ANOVA).
thus represents a valid model for studying the cellular and molecular mechanisms underlying chronic itch conditions.

**A subset of TRPV1-expressing sensory neurons is required for dry-skin-evoked itch behaviors**

Primary afferent sensory neurons that express the ion channels TRPV1 and TRPA1 are required for acute itch. TRPV1 is required for transducing histamine-dependent itch and TRPA1 (expressed in a subset of TRPV1 fibers) is required for signaling in response to the histamine-independent pruritogens chloroquine and BAM8-22\textsuperscript{36,54,750,151}. We thus assessed the role of TRPV1-positive fibers in the generation of dry-skin-evoked chronic itch behaviors. Previous studies have shown that ablation of TRPV1- and TRPV1/TRPA1-positive sensory neurons with capsaicin or the capsaicin analog RTX results in axonopathy and, consequently, suppression of acute pain and itch\textsuperscript{1,36,150,152}. Consistent with previous studies, RTX treatment of the cheek rendered mice insensitive to topical application of mustard oil, whereas control mice displayed robust wiping of the cheek\textsuperscript{153} (Fig. 3A). No significant differences in skin thickness or integrity were observed between RTX- and water-treated mice (data not shown). Interestingly, RTX-treated mice displayed a significant decrease in AEW-evoked scratching behaviors compared with control mice on both day three and day five of AEW treatment (Fig. 3B). These data show that the TRPV1- and TRPV1/TRPA1-positive sensory neurons are required for chronic AEW-evoked scratch behaviors and implicate TRPV1 and TRPA1 ion channels as candidate chronic itch transducers.

To gain insight into the molecules responsible for transducing chronic itch signals in TRPV1- and TRPV1/TRPA1-positive sensory neurons, we investigated whether dry skin alters gene expression in trigeminal neurons that innervate the mouse cheek. RNA from the TG of AEW- and vehicle-treated mice was hybridized to Affymetrix Mouse Genome 430 2.0 microarrays. Of ∼34,000 genes (45,102 probe sets), 1843 genes (2423 probe sets) were significantly differentially expressed. We focused on genes that belong to functional classes relevant to sensory neuron transduction, itch, and inflammation, as well as uncharacterized receptors and ion channels (202 genes; Fig. 3C). These genes included the itch receptors Mas-related G-protein coupled receptor A3 (MrgprA3) and Protease Activated Receptor-2 (PAR2) and the inflammatory bradykinin receptor\textsuperscript{51,53,108,125,154-157} (Bdkr2) all of which were more highly expressed in the TG of AEW-treated mice compared with VEH-treated mice (Fig. 3C, green stars). We and others have shown that activation of MrgprA3, PAR2, and a Bdkr family member (Bdkr1) lead to opening of the ion channel TRPA1 and that TRPA1 activation is required for MrgprA3-evoked itch and Bdkr1-evoked pain behaviors in mice\textsuperscript{51,54,158}. These data, combined with the role of TRPA1 in acute histamine-independent itch, strongly implicate a role for TRPA1 in chronic itch.
Figure 3. Sensory neurons are required for dry-skin-evoked itch behaviors.

A, Mustard oil (MO, 10% in PBS)-evoked wiping was measured in wild-type, vehicle (CONTROL, 0.05% ascorbic acid, and 7% Tween 80, black), or RTX-injected (RTX ABLATED; 1 µg/ml RTX in 0.05% ascorbic acid, and 7% Tween 80, red) mice 3 d after injection. Nocifensive behavior was quantified for 2 min. All error bars represent SEM (n ≥ 4 mice/genotype, *p < 0.05). B, Dry-skin-evoked scratching was measured in wild-type vehicle (CONTROL, 0.05% ascorbic acid, and 7% Tween 80, black) or RTX-injected (RTX ABLATED; 1 µg/ml RTX in 0.05% ascorbic acid, and 7% Tween 80, red) mice on days three and five of treatment. The total time spent scratching was quantified for 20 min. Error bars represent SEM (n ≥ 9 mice/genotype; *p < 0.05) C, Gene expression was measured in whole TG isolated from VEH- and AEW-treated mice normalized to VEH-treated mice. Green indicates AEW-evoked increase in expression; magenta, AEW-evoked decrease in expression. Differentially expressed genes are clustered based on cellular function. Among differentially expressed receptors, the itch receptors MrgprA3 and Bdkr2 (*) were highly expressed in the TG of AEW-treated mice relative to VEH-treated mice.
TRPA1 is required for dry-skin-evoked itch behaviors and morphological changes in the skin

We next investigated the role of TRPA1 in mediating chronic itch behaviors in mice. Trpa1−/− and wild-type littermates were treated using the AEW cheek assay. AEW treatment triggered prolonged periods of scratching in wild-type mice (DAY 3 VEH = 0 ± 0 s, DAY 3 AEW = 52.43 ± 12.39 s, DAY 5 VEH = 4.00 ± 5.23 s, DAY 5 AEW = 114.33 ± 22.76 s; Fig. 4A, black); this scratching behavior was significantly reduced in TRPA1-deficient mice (A1−/− DAY 3 = 7.5 ± 2.5 s, A1−/− DAY 5 = 36.7 ± 10.39 s; Fig. 4A, red). In contrast, no significant differences in itch behaviors were observed between Trpv1+/+ mice and Trpv1−/− (gray) littermates (V1−/− DAY 3 = 45.12 ± 11.23 s, V1−/− DAY 5 = 98.76 ± 15.71 s; Fig. 4A) or between TRPV1+/+ and TRPA1+/+ animals. These experiments demonstrate that TRPA1 is selectively required for dry-skin-evoked chronic itch behaviors.

We next investigated whether acute inhibition of TRPA1 attenuates AEW-evoked scratching. The TRPA1 inhibitor HC-030031 was injected subcutaneously into the cheek immediately before recording AEW-evoked itch behaviors on treatment days three and five. Like genetic ablation of TRPA1, HC-030031 significantly reduced AEW-evoked itch behaviors (Fig. 4B). These data suggest that TRPA1 is required for the transduction of itch sensation in chronic conditions. We also found that TRPA1 promoted epidermal hyperplasia in this chronic itch model. In wild-type mice, 5 d treatment with AEW triggered a 4-fold increase in the thickness of nucleated epidermal layers compared with vehicle-treated contralateral skin samples (VEH = 8.7 ± 0.6 µm, AEW = 32.5 ± 6.4 µm; Fig. 4C,D, gray). TRPA1−/− mice showed comparatively modest epidermal hyperplasia in response to AEW (VEH = 9.7 ± 2.1 µm, AEW = 20.4 ± 1.9 µm) and the magnitude was significantly less than in wild-type littermates (WT = 375 ± 50%, A1−/− = 210 ± 20%; Fig. 4D, red). These data show that TRPA1 promotes disturbances in epidermal homeostasis in chronic itch and supports a role for TRPA1, not only in the transduction of, but also in the development of dry-skin-evoked itch.

Although dry skin treatment alone caused epidermal thickening, the itch-scratch cycle further promoted hyperplasia (Fig. 1F). Therefore, a lack of scratching behaviors may contribute to the decreased epidermal thickening in TRPA1-deficient mice. Indeed, dry-skin evoked-epidermal hyperplasia was not significantly different between the "no-scratch" skin versus TRPA1-deficient skin (Fig. 4D, gray). However, this does not rule out the possibility that TRPA1 may also directly influence hyperplasia via signaling between sensory neurons and keratinocytes. We next examined dry-skin-evoked gene expression changes in the skin to determine the relative contribution of scratching and TRPA1 to the phenotypic changes observed in the skin.

TRPA1 is required for dry-skin-evoked expressional changes in the skin

Significant changes in epidermal gene expression are observed in the skin of patients with chronic itch121,159,160. Studies have shown that the itch-scratch cycle contributes to the chronic itch disease-associated changes observed in patient skin132,138 133-138. Does
Figure 4. TRPA1 is required for dry-skin-evoked itch behaviors and morphological changes in the skin.

A. Dry-skin-evoked scratching was measured in wild-type (WT AEW; black), TRPV1−/− (V1 AEW; gray), and TRPA1−/− (A1 AEW; red) mice after 3 and 5 d of AEW treatment to the cheek. The total time spent scratching was quantified for 20 min. Treatment with vehicle on wild-type mice (VEH, water) failed to elicit scratching or wiping (VEH; white). All error bars represent SEM (n ≥ mice/genotype, **p < 0.01). B, Dry-skin-evoked scratching was measured in wild-type mice on AEW treatment on days three and five immediately after (≤2 min) subcutaneous injection with control PBS (CON) or the TRPA1 inhibitor HC-030031 (HC). The total time spent scratching was quantified for 20 min postinjection and normalized to CON-treated mice. All error bars represent SEM (n ≥ 9 mice/genotype or treatment, **p < 0.01). C, H&E-stained skin sections from wild-type mice (WT; top) and TRPA1−/− littermates (A1−/−; bottom) treated ipsilaterally with AEW (left) and contralaterally with VEH. E indicates epidermis; D, dermis. D, The increase in epidermal thickness induced by AEW was quantified from four to five mice per condition. NS indicates the “no scratching” condition in the caudal back model. Means ± SEM are shown (*p = 0.01, Student's two-tail t test).
the AEW model of chronic itch induce expressional changes in the skin? If so, are these changes mediated by dry skin alone, by the dry-skin-evoked itch-scratch cycle, or both? And to what extent does TRPA1 contribute to AEW-evoked expression changes? To answer these questions, we used microarray analysis to examine AEW-evoked gene expression in skin.

We compared changes in expression between AEW- and vehicle-treated skin in three sets of animals: wild-type mice that were free to scratch the patch of dry skin on their cheek, wild-type animals that could not scratch the patch of dry skin on their back, and TRPA1-deficient animals that were free to scratch the patch of dry skin on their cheek (however, as shown in Figure 4, these mice display significantly reduced itch behaviors). Vehicle-treated skin isolated from the cheek and caudal back displayed few differences in gene expression (<2%). However, to compare gene expression among all groups, expression was normalized to vehicle treatments within the same tissue (e.g., AEW-treated back skin normalized to VEH-treated back skin; Fig. 5A). Overall, AEW treatment of wild-type mice triggered a significant change in expression in 9340 (14,884 probe sets) genes (Fig. 5A). We categorized these AEW genes into three groups: (1) TRPA1-dependent genes (those that do not display altered expression in the absence of TRPA1), (2) scratch-dependent genes (those that do not display altered expression in the absence of scratching), and (3) scratch- and TRPA1-dependent genes (those that do not display altered expression in the absence of TRPA1 and scratching). Quantitative PCR validated expresional changes of several genes that were previously linked to chronic itch. For example, the scratch-independent gene Fillagrin (FLG) is upregulated by AEW treatment in a TRPA1-independent manner. The scratch-independent genes Aquaporin 3 (AQP3) and interleukin 33 (IL33) were also differentially expressed in AEW-treated versus VEH-treated mouse skin and were TRPA1 dependent. Likewise, the scratch-dependent gene IL31 receptor A (IL31RA) was upregulated in AEW-treated versus VEH-treated mouse skin, but not in TRPA1-deficient skin. Antibody staining was also used to validate the enriched expression of the scratch-independent gene KRT6 (Fig. 5C), a hair follicle keratin upregulated in the interfollicular epidermis upon injury. Microarray analysis showed that AEW increased the KRT6 signal intensity 9-fold in wild-type skin, but only 4-fold in TRPA1−/− skin. Consistent with these data, immunohistochemistry demonstrated an upregulation of KRT6 protein in the interfollicular epidermis, but not in the hair follicles of AEW-treated skin (Fig. 5C). Although upregulation was observed in both genotypes, KRT6 expression was more abundant in the WT than in TRPA1−/− interfollicular epidermis (Fig. 5C).

Of the upregulated genes, most were scratch-dependent (76%), suggesting that the itch-scratch cycle contributes greatly to AEW-evoked changes in the skin (Fig. 6A). The majority of the upregulated genes (77%) were also TRPA1-dependent (Fig. 6A). TRPA1 also contributed to scratch-independent expressional changes; of the 24% of AEW genes that were not affected by scratching, 45% were TRPA1 dependent. We next analyzed a subset of the genes affected by itch that were previously implicated in processes related to chronic itch (195 genes); this subset includes genes altered by
Figure 5. TRPA1 is required for dry-skin-evoked expressional changes in the skin.

A. Gene expression was measured in cheek skin biopsies isolated from VEH- or AEW-treated TRPA1+/+ mice that were free to scratch (WT SCR), and TRPA1−/− mice. Gene expression was also measured in caudal back skin biopsies in which scratching was prevented (WT NSCR). The AEW gene expression data are normalized to VEH for each genotype and treatment area. Green indicates the AEW-evoked increase in expression; magenta, the AEW-evoked decrease in expression. All genes significantly altered in WT mice are shown (p < 0.05). B. Changes in gene expression with AEW treatment for FLG, IL31RA, AQP3, and IL33 in WT (black) and A1−/− (white) mice as measured by quantitative PCR. Expression is reported as ΔΔCt. C. Micrographs of WT and A1−/− skin are stained with KRT6. In VEH-treated skin, KRT6 is localized to hair follicles (arrows), whereas AEW-treated skin shows induction of KRT6 in the interfollicular epidermis (brackets). Nuclei are stained in blue. Scale bar, 50 µm.
pathological pruritic diseases, genes controlling neuronal growth, and genes involved in itch signal transduction (Fig. 6B). Within this subset of itch genes, 42% were dependent solely on TRPA1, compared with only 9% percent observed for the larger dataset inclusive of all genes; only 57% of the itch subset were scratch dependent, compared with 76% of the overall dataset of genes, suggesting that independently of the role of TRPA1 in scratching, TRPA1 may play a more direct role in the regulation of genes related to chronic itch disease.

To gain insight into the biological functions affected by dry, itchy skin and the role of scratching and TRPA1 in promoting these changes, we performed Gene Ontology (GO) analysis to identify classes of genes that are significantly affected by AEW treatment. Most GO terms were both scratch- and TRPA1-dependent and were no longer enriched in the absence of TRPA1 or scratching. These include many categories, such as regulation of epithelial cell proliferation, regulation of axon extension, and activation of immune response. However, GO terms for water transport were only dependent on TRPA1, which was surprising because increased water loss, which has been linked to the water channel AQP3, is a hallmark of chronic itch skin and disruption of skin barrier function.4,122,159,163,165

DISCUSSION
Our findings demonstrate a new role for TRPA1 in multiple facets of chronic itch. Here we used a dry skin mouse model of itch to probe the mechanisms of chronic itch in skin and sensory neurons. We adapted the dry skin model to the 132 cheek, which allowed for the distinction between itch and pain behaviors.127 Then we adapted the model to the caudal back, an area that the mouse cannot access for scratching, biting, or wiping, to examine mechanisms of chronic itch in the absence of scratching. The cheek model displays many of the phenotypes observed in human chronic itch, including itch behaviors, epidermal hyperplasia, and expression changes in the skin.121 This model triggers increased expression of many genes implicated in human pruritic dry skin conditions: AQP3, Ccl27, FLG, and Tnc for atopic dermatitis; IL33, Ccl20, Cxcl2, Cxcr2, lipocalin, and Slc9a3r1 for psoriasis; and S100a9, involucrin, and Ccr5, which have been implicated in both psoriasis and atopic dermatitis.121,159,160 Because the cheek AEW model promotes dry itchy skin and the itch-scratch cycle, in which repetitive scratching worsens chronic itch symptoms, we sought to look at these phenotypes in isolation.132-138

We performed, for the first time, a comparison of hyperplasia and gene expression changes in itchy, yet unscratched skin. The ideal experiment to determine the role of scratching in AEW-evoked hyperplasia and gene expression would be to compare AEW-treated cheek skin isolated from mice that were permitted to or prevented from scratching. However, we were unsuccessful in preventing AEW mice from scratching the cheek using all commercially available restraint methods. We thus instead turned to the back model of dry skin to address this question, specifically focusing on a caudal area of the back just rostral to the tail, which is inaccessible. In the caudal back model,
Figure 6. TRPA1 promotes scratch-dependent and scratch-independent expressional changes in the skin.

A, Venn diagram displaying relative numbers of scratch-dependent, TRPA1-dependent, and scratch-and TRPA1-dependent genes significantly modulated in WT AEW mouse cheek skin. B, Analysis of a subset of genes that were previously implicated in processes related to chronic itch to determine dependence on scratching and/or TRPA1 (Fig. 5A). Green indicates AEW-evoked increase in expression; magenta, AEW-evoked decrease in expression. C, Venn diagram displaying relative numbers of scratch-dependent, TRPA1-dependent, and scratch- and TRPA1-dependent itch-related genes significantly modulated in WT AEW mouse cheek skin.
mice display off-site scratching, but the location of the AEW-treated skin is such that the mice cannot directly scratch, bite, or lick the affected skin.

The caudal back model induced a 230% increase in epidermal thickness, whereas the cheek model induced a 350% increase. Like the hyperplasia, dry-skin-evoked gene-expression changes in the skin were not entirely dependent on the itch-scratch cycle, because 24% of the genes upregulated in the cheek model were also upregulated in the caudal back model. These findings suggest that the itch-scratch cycle contributes greatly to dry-skin-evoked changes in the skin, but many changes also occur in the absence of scratching. One caveat with this interpretation is the assumption that cheek skin responds identically to AEW treatment as caudal back skin. We see no significant differences in baseline epidermal thickness and few differences in baseline gene expression between these different sites. However, it is possible that differences in the dermis or epidermis, the prevalence and/or distribution of itch-sensitive nerve fibers, or the resident immune cells that contribute to inflammation may vary between the cheek and the back. Regardless of any differences between the cheek and back, the genes identified in the caudal back AEW model represent the first candidate genes that are altered by dry, itchy skin independently of scratching.

Chronic itch patients often experience increased sensitivity to itch stimuli, as well as alloknesis, the perception of itch in response to a previously non-itchy stimulus (e.g., a light brush of the skin that is normally perceived as innocuous touch induces an intense desire to scratch)\(^4,166\). The changes in sensitivity are hypothesized to occur as a result of increased innervation of itch-sensitive primary afferent fibers and expressional changes that drive hyperexcitability in sensory neurons\(^3,4,167-169\). Whereas extensive transcriptome profiling of pruritic skin has been performed, much less is known about the changes within the sensory neurons that innervate this skin. We show here for the first time that AEW-evoked drying of the cheek skin leads to significant expressional changes in the sensory neurons of the TG. Although we cannot distinguish whether these changes are scratch dependent or independent, they are nevertheless a consequence of the normal itch-scratch cycle associated with chronic itch. Therefore, these genes represent novel candidate transducers of chronic itch. One such candidate is the bradykinin receptor 2. Injection of bradykinin evokes weak itch and pain sensations in healthy subjects, whereas bradykinin injection in patients with chronic itch triggers severe itch that is not relieved by antihistamine treatment\(^170\). Therefore, increased expression of the bradykinin receptor in sensory neurons during chronic itch may result in alloknesis. In general, this analysis now provides a list of novel genes that represent candidate neuron-specific factors that might play a role in the pathophysiology of itch conditions. Future studies will be required to test the roles of such candidates in chronic itch models.

The ion channel TRPA1 was previously shown to mediate acute histamine-independent itch: TRPA1 is required for sensory neuron activation and itch behavior downstream of two histamine-independent pruritogens, chloroquine and BAM8-22, which activate MrgprA3 and MrgprC11, respectively\(^53,54\). Likewise, a MrgprA3-positive subset of
neurons that express TRPA1 were recently shown to be required for acute and chronic itch. Our data now demonstrate a new role for TRPA1 ion channels in multiple facets of chronic itch. We found that functional TRPA1 is required for all of the dry-skin-evoked phenotypes documented here, including AEW-evoked scratching, hyperplasia, and expressional changes in the skin. One striking result is that TRPA1 promotes dramatic changes in gene expression in the skin. Seventy-nine percent of the itch-related genes upregulated in the cheek model are TRPA1-dependent, 11% of which are independent of scratching. Among the human disease genes modulated in the cheek model, TRPA1 regulates both scratch-dependent and scratch-independent changes: AQP3, IL-33, Cxcr2, lipocalin, Slc9a3r1, and S100a9 require TRPA1 and are independent of the itch-scratch cycle, whereas Ccl27 and Tnc are scratch and TRPA1 dependent. These genes play diverse roles in the initiation and maintenance of chronic itch. For example, AQP3, the predominant aquaporin in human skin, is upregulated in atopic dermatitis and mice lacking AQP3 display reduced hyperplasia in mouse models of atopic dermatitis. Our findings that AQP3 expression is TRPA1 dependent in the absence of scratching support a model for sensory neurons signaling to keratinocytes under chronic itch conditions. Another TRPA1-dependent gene, IL-33, displays enhanced expression in psoriatic skin and may act in concert with substance P to increase VEGF expression and release in psoriatic skin. This also supports an active role for neuronal signaling in the periphery in the development of chronic itch.

It is quite striking that TRPA1 channels mediate expressional changes of so many chronic itch genes. Key next steps in characterizing the role of TRPA1 will include identifying the endogenous upstream factors that activate TRPA1 during chronic itch and determining whether hyperplasia is caused by a TRPA1-dependent release of inflammatory agents. Peptide release promotes vasodilation, vascular leakage, recruitment of immune cells, and, notably, epidermal hyperplasia in chronic itch through modulation of keratinocyte growth. Indeed, a role for neurogenic inflammation has been suggested in PAR2-mediated itch behavior in mice. Consistent with this model, we show here that genes involved in the regulation of leukocyte activation and chemotaxis are upregulated in AEW-treated mice in a TRPA1-dependent fashion. It is also possible that TRPA1 expression in keratinocytes contributes to the AEW phenotype, although non-neuronal expression of TRPA1 is controversial. Future studies using tissue-specific TRPA1-deficient animals are required to resolve whether TRPA1 in neurons or non-neuronal cells is necessary for AEW-evoked dry skin. Nonetheless, we show here for the first time a role for TRPA1 in epidermal homeostasis under a disease state, provide new insight into the role of TRPA1 in chronic itch, and highlight the potential benefit of TRPA1 antagonists as therapies for pathological itch conditions.

**EXPERIMENTAL PROCEDURES**

*Mice and behavior.*

All experiments were performed on male mice, 8–14 weeks of age. Female mice were not used due to effects of the estrus cycle on itch behaviors. Mice (20–35 g) were housed with a 12 h light-dark cycle at 21°C. For assessing dry-skin-evoked itch
behaviors in the mouse cheek, each cheek was shaved (Fig. 1B) and treated twice daily
with a cotton swab immersed in either a 1:1 mixture of acetone and ether or water for 15
s, followed by water for 30 s for a duration of either 3 or 5 d. For assessing dry-skin-
evoked itch behaviors in the mouse caudal back, the mouse back was shaved (Fig. 1E)
and treated twice daily with a cotton swab immersed in either a 1:1 mixture of acetone
and ether or water for 15 s, followed by water for 30 s for a duration of either 3 or 5 d.
For resiniferatoxin (RTX) treatments, RTX (100 µg/ml, 10 µl in 0.05% ascorbic acid, and
7% Tween 80 vehicle) was injected subcutaneously into the cheek 3 d before the first
acetone, ether, and water (AEW) treatment. All mice were videotaped for 25 min to
assess scratching. The amount of time each mouse spent scratching was quantified
over a 20 min period. One bout of scratching was defined as an episode in which a
mouse lifted its paw and scratched continuously for any length of time until the paw was
returned to the floor. Mustard oil (10% in PBS, 377430; Sigma) was applied topically
with a cotton swab to shaved mouse cheeks. Nocifensive behavior was quantified for 2
min after application. Behavioral scoring was performed while the observer was blind to
the experimental condition. All experiments were performed under the policies and
recommendations of the International Association for the Study of Pain and were
approved by the University of California, Berkeley, Animal Care and Use Committee.

Histology.
Skin specimens were dissected immediately after animals were killed and were then
fixed in 4% paraformaldehyde overnight at 4°C, dehydrated in 70% ethanol, and
embedded in paraffin. Sections (8 µm) were stained with H&E or with rabbit anti-keratin-
6 (KRT6) polyclonal antibodies (Covance), followed by immunoperoxidase labeling.
Slides were imaged with a bright-field microscope outfitted with 10×, 0.3 numerical
aperture (NA) and 20×, 0.4 NA lenses and an AxioCam color CCD camera
(AxioObserver.Z1; Zeiss). All specimens were blinded with respect to genotype and
treatment before imaging. The thickness of nucleated epidermal layers was measured
from 20 × bright-field images taken at 2–3 random fields per section using ImageJ
software.

Real-time quantitative PCR.
Total RNA from skin was extracted using TRIzol reagent (Invitrogen) according to the
manufacturer’s specifications; 500 ng of total RNA was used to generate cDNA using
SuperScript III Reverse Transcriptase (Invitrogen). Real-time PCR was performed using
SYBR GreenER qPCR SuperMix for ABI PRISM (Invitrogen) on a StepOnePlus ABI
machine. To determine gene expression in keratinocytes, threshold cycles for each
transcript \( \Delta\Delta C_t \) were normalized to GAPDH \( \Delta C_t \). Calibrations and normalizations were
performed using the \( 2^{-\Delta\Delta C_t} \) method in which GAPDH was used as the reference gene,
samples from vehicle-treated mice were used as the calibrator, and \( \Delta C_t = [(C_t (target \text{ gene}) - C_t (reference \text{ gene})) - [C_t (calibrator) - C_t (reference \text{ gene})]] \). Real-time PCR
measurements were performed in triplicate.

Microarray.
Trigeminal ganglia (TG) were removed from either wild-type C57BL6 or Trpa1\(^{-/}\)
C57BL6 6- to 8-week-old mice treated with either AEW or vehicle for 5 d and RNA was
isolated with TRIzol (Invitrogen) according to the manufacturer’s protocol. RNA integrity was confirmed with an Agilent 2100 BioAnalyzer. RNA (100 ng) was used to make cRNA and samples were analyzed for gene expression with Affymetrix Mouse Genome 430.2 GeneChip arrays, which cover transcripts and variants from 34,000 well characterized mouse genes using standard Affymetrix reagents and protocols. Probe sets on this array are derived from sequences from GenBank and dbEST. Samples from three independent mice from each group were analyzed using one microarray per biological sample. Microarray data were normalized using the GCRMA algorithm; ratios of normalized probe set intensity values were calculated for each sample pair, in which \( M = \log_2[\text{ICAM1}(+)/\text{ICAM1}(−)] \), and then averaged among the three replicate pairs. Fold change was calculated to compare AEW- and VEH-treated mice within the same group (fold change = average AEW M value/average VEH M value, e.g., WT AEW/WT VEH).

**Data analysis.**
Data are shown as means ± SEM. Statistical significance was evaluated using a one-way ANOVA followed by a Tukey–Kramer post hoc test, unpaired two-tailed Student's t test for comparing difference between two populations, or paired two-tailed Student's t tests for datasets containing in-animal controls.
CHAPTER III: The Epithelial Cell-Derived Atopic Dermatitis Cytokine TSLP Activates Neurons to Induce Itch.

This chapter is a reproduction of the paper by the same name published in Cell in October 2013. My contributions were to Figures 1c-f, 2b-c, 3, 4, 5, 6, 7a, 7d-e, 7f-g, and S1b-d. For this paper I performed cellular imaging, siRNA, pharmacology, PCR, ELISA, and mouse behavior experiments and made figures and wrote the manuscript with L.T. and D.M.B.

SUMMARY
Atopic dermatitis (AD) is a chronic itch and inflammatory disorder of the skin that affects one in ten people. Patients suffering from severe AD eventually progress to develop asthma and allergic rhinitis, in a process known as the “atopic march.” Signaling between epithelial cells and innate immune cells via the cytokine Thymic Stromal Lymphopoietin (TSLP) is thought to drive AD and the atopic march. Here we report that epithelial cells directly communicate to cutaneous sensory neurons via TSLP to promote itch. We identify the ORAI1/NFAT calcium signaling pathway as an essential regulator of TSLP release from keratinocytes, the primary epithelial cells of the skin. TSLP then acts directly on a subset of TRPA1-positive sensory neurons to trigger robust itch behaviors. Our results support a new model whereby calcium-dependent TSLP release by keratinocytes activates both primary afferent neurons and immune cells to promote inflammatory responses in the skin and airways.

INTRODUCTION
Atopic dermatitis (AD) is a chronic itch and inflammatory disorder of the skin that affects one in ten people. AD is primarily characterized by intolerable and incurable itch. Up to 70% of AD patients go on to develop asthma in a process known as the “atopic march.” Numerous studies suggest that the cytokine Thymic Stromal Lymphopoietin (TSLP) acts as a master switch that triggers both the initiation and maintenance of AD and the atopic march (Moniaga et al., 2013; Ziegler et al., 2013). TSLP is highly expressed in human cutaneous epithelial cells in AD, and bronchial epithelial cells in asthma. Over-expression of TSLP in keratinocytes, the most prevalent cell type in the skin, triggers robust itch-evoked scratching, the development of an AD-like skin phenotype and ultimately asthma-like lung inflammation in mice. However, the mechanisms by which TSLP triggers itch and AD remain enigmatic.

Itch is mediated by primary afferent somatosensory neurons that have cell bodies in the dorsal root ganglia (DRG) that innervate the skin and are activated by endogenous pruritogens to drive itch behaviors. Hallmarks of AD skin include robust itch sensations, increased neuronal activity and hyper-innervation. While many studies have shown that epithelial cell-derived TSLP activates T cells, dendritic cells and mast cells, the role of sensory neurons in this pathway has not been studied. How does TSLP lead to sensory neuron activation to promote itch?
In vitro studies suggest that keratinocytes may directly communicate with sensory neurons via neuromodulators. Indeed, many of the factors that keratinocytes secrete act on both immune cells and primary afferent sensory neurons. Thus, TSLP may evoke itch behaviors directly, by activating sensory neurons, indirectly, by activating immune cells that secrete inflammatory mediators that target sensory neurons, or both. While TSLP's action on immune cells is well characterized, its effects on sensory neurons, and the contribution of sensory neurons to TSLP-evoked atopic disease, have not been studied. Furthermore, the mechanisms regulating TSLP release by keratinocytes are unknown.

The GPCR Protease-Activated Receptor 2 (PAR2) plays a key role in keratinocyte TSLP production. Studies have shown a correlation between PAR2 activity and TSLP expression in the skin of AD patients and in mouse models of atopic disease. In addition, PAR2 activation triggers robust TSLP expression in keratinocytes. While there is a strong correlation between PAR2 activity and TSLP levels in the skin, virtually nothing is known about the molecular mechanisms by which PAR2 leads to TSLP expression.

Here we sought to elucidate the mechanisms that regulate TSLP secretion and that promote TSLP-evoked itch. Our findings show that keratinocyte-derived TSLP activates sensory neurons directly to evoke itch behaviors. We define a new subset of sensory neurons that require both functional TSLP receptors and the ion channel, TRPA1, to promote TSLP-evoked itch behaviors, and we identify the ORAI1/NFAT signaling pathway as a key regulator of PAR2-mediated TSLP secretion by epithelial cells.

RESULTS
TSLP evokes robust itch behaviors in mice
To identify proteins that mediate itch transduction in somatosensory neurons, we looked for biomarkers of AD in the mouse DRG transcriptome. We were surprised to find expression of the TSLP Receptor (TSLPR) in mouse sensory ganglia. While studies have shown that TSLP acts on various immune cells, TSLP signaling in the nervous system has not been reported. TSLPR is a heterodimer, composed of the IL7 receptor alpha (IL7Ra) chain and a TSLP-specific receptor chain (TSLPR; also Crlf2). Consistent with the presence of TSLPRs in sensory neurons, we detected both TSLPR and IL7Ra transcripts in mouse and human DRG using RT-PCR.

Somatosensory neurons mediate itch, touch and pain. Thus, we asked if TSLP injection triggers itch and/or pain behaviors by using a mouse cheek model of itch, which permits easy distinction between these behaviors. Injection of TSLP into the cheek of wild type mice evoked robust scratching that was not observed following vehicle injection. Wiping was never observed, indicating that TSLP triggers itch, rather than pain. Intradermal injection of TSLP has been previously shown to evoke inflammation of the skin and lung over the course of hours or days. However, we observed robust itch behaviors within 5 minutes of TSLP injection (latency to scratch = 4.1 ± 0.3 min).
Figure 1. TSLP triggers robust itch behaviors in mice by activating sensory neurons

(A) PCR analysis of TSLPR and IL7Ra in mouse (left) and human (right) dorsal root ganglia (DRG). No product was amplified from the “no RT” control. (B) Image of itch-evoked scratching following intradermal injection of TSLP (2.5 µg/20 µl) into the cheek. (C) Quantification of scratching following TSLP injection in the cheek. TSLP (black) induced robust scratching compared to vehicle (white). n≥18 per group. (D) Itch behavior in RAG1+/+, RAG1−/−, NOD, and NOD/SCID mice following vehicle (PBS) or TSLP cheek injection. n≥8 per group. (E) Itch behavior in cKIT+/+ and cKIT−/− mice following vehicle (PBS) or TSLP injection. n≥8 per group. (F) TSLP-evoked scratching following neuronal ablation by RTX (red) versus control (black). n≥6 per group. *P<0.05; **P<0.01; ***P<0.001. Error bars represent s.e.m.
While immune cells play a key role in long-term TSLP-evoked inflammation, whether immune cells are required for acute TSLP-triggered itch behaviors is unknown. The current model posits that TSLP acts on various immune cells to promote TH2 cell differentiation and inflammation. We thus compared TSLP-evoked itch behaviors of wild type mice to mouse strains lacking either T and B cells (RAG1−/−, NOD SCID) or mast cells (Kit(W-sh), Fig. 1D-E). TSLP triggered robust itch behaviors in all strains, with no significant differences between transgenic and congenic wild type littermates. Together, these data indicate that acute TSLP-evoked itch does not specifically require lymphocytes or mast cells, nor does it require the cytokines or other products produced when these cells are activated, and suggest that TSLP may act directly on sensory neurons.

Previous studies have shown that intradermal injection of the TRPV1 agonist, resiniferatoxin (RTX), results in ablation of primary afferent sensory neurons that express TRPV1, or TRPV1 and TRPA1, and consequently eliminates pain and itch behaviors. TSLP-evoked scratching was significantly decreased in RTX-treated mice as compared to control mice (Fig. 1F). These findings show for the first time that the AD cytokine, TSLP, induces itch via sensory neurons.

**TSLP directly activates an uncharacterized subset of sensory neurons**

We next asked whether TSLPRs are expressed in sensory neurons. DRG neurons are a heterogeneous population of cells, including a subset of small-diameter, peripherin-positive neurons that transmit itch and pain signals to the CNS, and release inflammatory mediators in the skin and other target organs. We thus examined the prevalence of TSLPR-positive neurons and co-localization with known neuronal markers. In situ hybridization revealed that TSLPR and IL7Ra were expressed in a subset of small diameter DRG neurons (Fig. 2A). Using antibodies against TSLPR, we observed TSLPR protein expression in 5.9% of cells in DRG sections (Fig. 2B). Co-staining of TSLPR and peripherin, a marker of small-diameter DRG neurons, demonstrated that all TSLPR-positive neurons are also peripherin-positive, with an average diameter of 18.1±0.6µm (Fig. 2B). Overall, the characteristics of TSLPR-positive neurons match those of sensory neurons that mediate itch and/or pain.

If TSLPRs mediate somatosensory transduction, they should localize to primary afferent nerve terminals in the skin. We thus performed immunohistochemistry with antibodies against TSLPR and the pan-neuronal fiber marker PGP9.5 on mouse skin (Fig. 2C). We observed TSLPR staining in 9% of PGP9.5-positive free nerve endings in the skin (Fig. 2C). These data show that TSLPRs are localized to sensory neuronal endings that innervate the skin in close apposition to keratinocytes in the epidermis. Taken together, these data demonstrate that the TSLPR subunits are expressed in a subset of sensory neurons that innervate the skin and mediate itch and/or pain transduction.

To test whether TSLPR is functional in sensory neurons, we used ratiometric Ca^{2+} imaging (Fig. 3A-B). We found that 4.1 ± 0.6% of DRG neurons showed robust increases in intracellular Ca^{2+} following TSLP application (Fig. 3E); this is similar to the
Figure 2. TSLP receptor components are expressed in sensory neurons
(A) DIC overlay images of in situ hybridization with cDNA probes detecting TSLPR, IL7Ra and TRPV1 in mouse DRG. Scale bar = 400µm. (B) Immunostaining of DRG sections with antibodies against peripherin and TSLPR in DRG sections. White arrows (right) mark peripherin- and TSLPR-positive neurons. Scale bar = 400µm. n≥4 mice/condition. (C) Immunostaining of PGP 9.5 and TSLPR in glabrous hind paw skin. The white arrows (right) mark PGP 9.5- and TSLPR-positive neurons. Scale bar = 200µm. n≥3 mice per condition.
percentage of neurons that respond to other endogenous pruritogens, like BAM8-22. Previous studies have shown that small diameter sensory neurons transduce itch and/or pain signals via the ion channels TRPA1 and TRPV1. Indeed, subsequent exposure to the TRPA1 agonist, allyl isothiocyanate (AITC), or the TRPV1 agonist, capsaicin (CAP), further increased Ca^{2+} levels in all TSLP-positive cells (Fig. 3A-B). Similarly, TSLP triggered action potential firing in a subset of CAP-sensitive neurons (Fig. 3C). These data suggest that TSLP activates a subset of TRPV1- and TRPA1-positive sensory neurons. The itch compounds histamine, chloroquine (CQ) and BAM8-22 have been shown to activate 5-20% of sensory neurons that express TRPA1 and/or TRPV1. TSLP appears to activate an undescribed subset of itch neurons, as most TSLP-positive neurons were insensitive to other itch compounds (Fig. 3A, B, D).

**TSLPR and TRPA1 mediate TSLP-evoked neuronal activation**

To ask whether TSLPRs mediate TSLP-evoked neuronal activation, we examined TSLP-evoked Ca^{2+} signals in neurons isolated from IL7Ra-deficient mice. TSLP-, but not AITC- or CAP-evoked Ca^{2+} signaling, was abolished in IL7a-deficient neurons (Fig. 3E). These results are consistent with previous studies in immune cells showing that functional IL7Ra is required for TSLP signaling. Here we show that functional TSLPRs are required for TSLP-evoked neuronal activation.

TRPV1 and TRPA1 channels are required for acute itch signaling and behavior. TRPV1 and TRPA1 inhibition by the nonselective inhibitor, ruthenium red, significantly decreased neuronal sensitivity to TSLP (Fig. 3E). We also compared neurons isolated from TRPA1- and TRPV1-deficient mice to those from wild type littermates. TSLP-evoked Ca^{2+} signals were significantly attenuated in TRPA1-deficient neurons, but not TRPV1-deficient neurons (Fig. 3E). Our results show that TRPA1 channels mediate TSLP-evoked neuronal excitability.

We next examined the mechanisms by which TSLPR activation promotes TRPA1 activity. Two signaling pathways have linked itch receptors to TRPA1 activation: Phospholipase C (PLC) signaling couples MrgrprC11 to TRPA1; and, Gbg signaling couples MrgrprA3 to TRPA1. Treatment of cells with the PLC inhibitor, U73122, significantly reduced the prevalence of TSLP-sensitive neurons (Fig. 3F). In contrast, gallein, a Gbg inhibitor, had no effect on TSLP-evoked Ca^{2+} signals (Fig. 3F). Consistent with TSLP activation of the PLC pathway, TSLP triggers both release of Ca^{2+} from intracellular stores, and subsequent Ca^{2+} influx in sensory neurons (Fig. 3G). Overall, these experiments suggest that TSLPR and TRPA1 communicate via PLC signaling.

**TSLPR and TRPA1 mediate TSLP-evoked itch**

To test whether TSLP and TRPA1 receptors are required for TSLP-evoked itch behaviors, we used the cheek model of itch. TSLP-evoked scratching was significantly attenuated in IL7Ra-deficient mice, supporting a role for TSLPRs in TSLP itch signaling (Fig 4A). These mice were not generally deficient in itch behaviors, as CQ-evoked
Figure 3. TSLP directly activates a subset of sensory neurons

(A) Representative images of Fura-2 loaded DRG neurons treated with vehicle, TSLP (2 ng/mL), histamine (HIS, 1mM), AITC (200µM) and capsaicin (CAP, 1µM). (B) Representative trace shows a neuron that responds to TSLP, AITC and CAP, but not HIS. (C) Current-clamp recording showing TSLP- and CAP-evoked action potential firing in a DRG neuron. n≥60 cells. (D) A small percentage of the TSLP-sensitive population overlaps with the population of histamine-sensitive (HIS, 6%) or chloroquine-sensitive neurons (CQ, 6%), but not the BAM8-22 population (BAM, 0%). (E) Left: Prevalence of TSLP sensitivity in wild-type neurons (black), IL7Ra-deficient (grey) neurons, neurons treated with 20µM ruthenium red (RR; red), TRPA1-deficient neurons (blue) and TRPV1-deficient neurons (white). Right: prevalence of AITC and CAP sensitivity in wild-type (black) and IL7Ra-deficient (grey) neurons n≥1000 cells. (F) Prevalence of TSLP sensitivity in neurons pre-treated with vehicle (black), a PLC blocker, U73122 (red) and the Gbg blocker, gallein (grey) n≥600 cells. (G) Representative response to TSLP in the absence (0mM Ca²⁺) and presence (2mM Ca²⁺) of extracellular Ca²⁺ n≥200 cells. *P<0.05; **P<0.01; ***P<0.001. Error bars represent s.e.m.
scratching, which occurs via MrgrpA3, was normal (Fig. 4B). These data demonstrate that TSLP targets TSLPRs to trigger itch behaviors in vivo.

We next asked whether TSLP-evoked itch behaviors require TRP channels. TSLP-evoked scratching was abolished in TRPA1-deficient mice, but normal in TRPV1-deficient mice (Fig. 4D). These experiments show that both functional TSLPRs and TRPA1 channels are required for TSLP-evoked itch. PLC signaling is also required for the functional coupling between TSLPR and TRPA1 in vivo, as TSLP-evoked scratching was significantly attenuated by intradermal injection of U73122. Such treatment selectively silenced TSLP-evoked behaviors, as these mice displayed normal CQ-evoked scratching, which is PLC-independent. Overall, these data demonstrate a new role for TSLP as a pruritogen and a robust activator of sensory neurons, and suggest that these neurons may contribute to the initiation of TSLP-evoked inflammatory responses in the skin in AD, and airways in asthma.

Keratinocyte release of TSLP is Ca\(^{2+}\)-dependent

Our data establish a new cellular target for TSLP, supporting a model whereby both immune cells and sensory neurons are activated by keratinocyte-derived TSLP to drive itch and AD. What are the upstream mechanisms that govern the expression and release of TSLP by keratinocytes? Protease signaling via PAR2 plays a key role in TSLP production and AD. PAR2 activity, and levels of the endogenous PAR2 agonist, tryptase, are increased in the skin of AD patients. Consistent with a previous study, injection of tryptase induced robust itch behaviors in mice (Fig. 5A). Tryptase-evoked itch was significantly attenuated in both PAR2- and IL7Ra-deficient mice (Fig. 5A), consistent with a pathway where PAR2 signaling promotes the release of TSLP from keratinocytes, which then acts on TSLPR-positive neurons to drive itch behaviors. We next sought to determine the signaling pathways that control PAR2-induced TSLP expression in keratinocytes.

Studies on keratinocytes have shown that the endogenous PAR2 agonist, tryptase, and the widely used PAR2 ligand mimetic, Ser-Leu-Ile-Gly-Arg-Leu (SLIGRL), elicits Ca\(^{2+}\) influx and triggers the Ca\(^{2+}\)-dependent release of inflammatory mediators. For example, SLIGRL triggers a rise in intracellular Ca\(^{2+}\) in keratinocytes and also promotes TSLP expression. We thus asked if PAR2-evoked TSLP expression is Ca\(^{2+}\)-dependent. ELISA measurements revealed that treatment of keratinocytes with tryptase or SLIGRL, but not vehicle, triggered the robust secretion of TSLP (Fig. 5B). These data show that PAR2 stimulation of keratinocytes triggers TSLP release.

TSLP secretion was highly dependent on Ca\(^{2+}\). First, TSLP secretion was not observed in keratinocytes treated with tryptase or SLIGRL in the absence of external Ca\(^{2+}\) (Fig. 5B). In addition, treatment with the drug thapsigargin (TG), which promotes depletion of intracellular Ca\(^{2+}\) stores and subsequent Ca\(^{2+}\) influx, caused a significant increase in TSLP secretion (Fig. 5B). These data demonstrate that Ca\(^{2+}\) is required and sufficient to drive TSLP secretion.
Figure 4. TSLP induces robust TSLPR- and TRPA1-dependent itch behaviors
(A) Itch behaviors following intradermal cheek injection of vehicle (10µL PBS, white) or TSLP (2.5µg/10 µL) into wild type (WT; black) or IL7Ra-deficient (red) mice. (B) Scratching in WT (black) and IL7Ra-deficient (red) mice following chloroquine (CQ) injection in the cheek. (C) Scratching in WT (black), TRPA1-deficient (red) and TRPV1-deficient (white) mice following TSLP injection (2.5 µg/10 µL). (D) Attenuation of TSLP-evoked scratching by 30 min preinjection with the PLC blocker, U73122 (U7) compared to vehicle (VEH). (E) CQ-evoked scratching in mice preinjected with U73122 or vehicle. The time spent scratching was quantified for 20 min after injection. n≥7 mice/condition. **P<0.01; ***P<0.001. Error bars represent s.e.m.
Figure 5. PAR2 activation promotes itch behaviors and Ca^{2+}-dependent release of TSLP

(A) Itch-evoked scratching following injection of tryptase into the cheek (100 pg/20 µL) of wild type (WT; black), PAR2-deficient (blue) or IL7Ra-deficient mice (red), or PBS (white, 20µL) injection into WT mice, n≥8 mice per condition. The time spent scratching was quantified for 1 h after injection. (B) TSLP secretion evoked by 24 h treatment with vehicle (VEH), tryptase (TRY, 100nM), tryptase in the absence of extracellular Ca^{2+} (TRY 0Ca), SLIGRL (100µM), SLIGRL in the absence of extracellular Ca^{2+} (SLIGRL 0Ca), or TG (1µM). n≥4 replicates/condition *P<0.05; **P<0.01; ***P<0.001. Error bars represent s.e.m.
A recent study has shown that some PAR2 agonists, including SLIGRL, also activate the sensory neuron-specific itch receptor, MrgrpC11 (MrgrpX1 in human, \(^{210}\)). However, this result does not impact our *in vitro* studies for several reasons. First, keratinocytes do not express MrgrpX1 (supplementary Fig. 1A). Second, keratinocytes are insensitive to the MrgrpX1-specific ligand, BAM8-22 (supplementary Fig. 1B). Third, tryptase-evoked itch is dependent on PAR2 (Figure 5A). Finally, tryptase does not activate MrgrpC11 in mice (supplementary Fig. 1C-D). Overall, our findings support a model where tryptase- and SLIGRL treatment of keratinocytes promotes PAR2-evoked Ca\(^{2+}\) signaling and subsequent secretion of TSLP.

**ORAI1 and STIM1 are required for PAR2-evoked Ca\(^{2+}\) influx**

We next used ratiometric Ca\(^{2+}\) imaging to dissect the mechanisms underlying PAR2-evoked Ca\(^{2+}\) signals. Consistent with previous studies, tryptase and SLIGRL evoked a rise in intracellular Ca\(^{2+}\) in keratinocytes (Fig. 6A-C; \(^{207}\)). In some cells, PAR2 signals via PLC\(^{158}\), and PLC activation leads to Ca\(^{2+}\)-release from IP\(_3\)-dependent stores and influx via the store-operated Ca\(^{2+}\) entry (SOCE) pathway. Indeed, PAR2 activation in keratinocytes induced both Ca\(^{2+}\) release from intracellular stores and Ca\(^{2+}\) influx, consistent with activation of SOCE (Fig. 6A-B).

What are the molecules mediating PAR2-evoked SOCE in keratinocytes? Both ORAI and TRPC channels have been implicated in SOCE \(^{211,212}\). We next asked whether PAR2 activates SOCE via ORAI or TRPC channels, which can be distinguished by their distinct pharmacological profiles \(^{213-215}\). The drugs 2-Aminoethoxydiphenyl borate (2-APB) and lanthanum (La\(^{3+}\)) inhibit ORAI1 and ORAI2 channels, but not ORAI3 or TRPC channels \(^{213-215}\). Tryptase and SLIGRL-evoked Ca\(^{2+}\) influx was significantly attenuated by treatment with 2-APB or La\(^{3+}\). These data show that tryptase and SLIGRL activate the same SOCE pathway and support a role for ORAI channels in PAR2-evoked SOCE (Fig. 6C).

ORAI1 and TRPC channels can also be distinguished by their distinct biophysical characteristics: ORAI1 and ORAI2 are Ca\(^{2+}\)-selective channels that are inwardly-rectifying, while TRPC channels are outwardly-rectifying, non-selective channels \(^{211,216,217}\). Thus, we measured SLIGRL-evoked currents using perforated-patch, voltage-clamp recordings on keratinocytes. Treatment with SLIGRL triggered an ORAI1/2-like current; the currents were dependent on extracellular Ca\(^{2+}\), displayed an inwardly-rectifying current-voltage relationship, and displayed no measurable reversal potentials below +80 mV (Fig. 6D). These results implicate ORAI1 and/or ORAI2 in PAR2-evoked SOCE.

qPCR demonstrated that keratinocytes express ORAI1, ORAI2 and the ORAI regulator, Stromal Interaction Molecule 1 (STIM1). We thus examined the role of ORAI1, ORAI2, and STIM1 in SOCE using siRNA-mediated knockdown. Depletion of ORAI1 transcripts by 71% or STIM1 transcripts by 84% significantly diminished Ca\(^{2+}\) entry in response to SLIGRL as compared to scrambled control siRNA (Fig. 6E-G). ORAI1 and STIM1 knockdown also significantly attenuated tryptase-evoked Ca\(^{2+}\) signals (not shown). In
Supplementary Figure 1. Human keratinocytes do not express human MRGPRX11 and the PAR2 agonist, tryptase, does not activate mouse MrgprC11, Related to Figure 6

(A) PCR analysis of the human BAM8-22 (BAM) receptor, MrgprX1, in human dorsal root ganglia (DRG) and human keratinocytes (KRT). MrgprX1 was amplified from DRG, but not keratinocytes. MrgprX1 and GAPDH were amplified from RT-treated tissue but not from “no RT” controls. (B) Representative response to BAM8-22 (BAM, 2 µM) in human keratinocytes. (C) Representative response to tryptase (TRY, 3 µM) and BAM8-22 (BAM, 2 µM) in the presence or absence of the mouse BAM8-22 receptor, MrgprC11. (D) Left: representative traces showing a neuron that is sensitive to BAM8-22 (BAM) but not tryptase (TRY, blue), and a neuron that is sensitive to tryptase but not BAM8-22 (black). Right: quantification of the prevalence of tryptase-responsive (TRY, black), BAM8-22-responsive (blue, BAM), and tryptase- and BAM8-22-responsive neurons in mouse dorsal root ganglia. n ≥ 500 cells. Data are represented as mean +/- SEM.
Figure 6. ORAI1 and STIM1 are required for PAR2- and TG-evoked Ca\(^{2+}\) influx

(A) Representative response to SLIGRL (100µM) following pretreatment with vehicle (black) or 2-aminoethoxydiphenyl borate (50 µM 2-APB; red). (B) Representative response to tryptase (100nM) following pretreatment with vehicle (black) or 2-APB (red). (C) Average steady state Ca\(^{2+}\) level following SLIGRL- or tryptase (TRY)-evoked Ca\(^{2+}\) influx (2 mM Ca\(^{2+}\)), in the presence of 2-APB (red), lanthanum (50nM La\(^{3+}\), blue), or vehicle (CTRL, black). n≥1000 cells. (D) Representative current-voltage trace in the presence of SLIGRL (100µM) in perforated-patch, whole-cell voltage-clamp recordings. Representative baseline subtracted currents before (red) and during application of SLIGRL (black). n≥3 cells/condition. (E) siRNA-induced silencing of STIM1 (red), ORAI1 (blue), and ORAI2 (grey) mRNA in keratinocytes. Expression was normalized to scrambled-siRNA control (black). n≥1000 cells. (F) Representative traces of SLIGRL-evoked (100µM) Ca\(^{2+}\) signals following treatment with siRNA targeting STIM1 (red) or scrambled control (CTRL, black). (G) Average steady state Ca\(^{2+}\) concentration after treatment with SLIGRL (100µM) or TG (1µM) in cells treated with scrambled siRNA (black), STIM1 (red), ORAI1 (blue), or ORAI2 (grey) siRNA. n≥500 cells. *P<0.05; **P<0.01, ***P<0.001. Error bars represent s.e.m.
contrast, depletion of ORAI2 transcripts by 86% had no effect on SLIGRL-evoked SOCE (Fig. 6E, 6G). These data demonstrate that ORAI1 and STIM1 are required for PAR2-evoked SOCE in human keratinocytes. ORAI1 and STIM1 knockdown also attenuated TG-evoked SOCE (Fig. 6G), suggesting that ORAI1 is the primary store-operated Ca\(^{2+}\) pathway in keratinocytes.

**PAR2-activation induces Ca\(^{2+}\)-dependent NFAT translocation and TSLP secretion**

In immune cells, ORAI1 signaling activates NFAT, which triggers cytokine expression and secretion \(^{218,219}\). The ORAI1/NFAT pathway may play a similar role in keratinocytes, promoting the expression and secretion of TSLP. Consistent with a regulatory role for NFAT in TSLP expression, two NFAT binding motifs (GGAAATN) \(^{207,220}\) are present in the 5'-upstream regulatory region of the human TSLP gene. These findings imply that PAR2 may trigger NFAT-dependent expression and release of TSLP; however, the evidence is merely correlative. To directly test this hypothesis, we measured PAR2-dependent NFAT translocation and TSLP expression and release in keratinocytes.

Following a rise in Ca\(^{2+}\), NFAT is dephosphorylated by the Ca\(^{2+}\)-dependent phosphatase calcineurin and translocates from the cytosol to the nucleus to promote transcription of target genes \(^{220}\). Immunostaining demonstrated that treatment of keratinocytes with SLIGRL for 30 minutes induced robust NFAT translocation to the nucleus (Fig. 7A). This translocation was attenuated by blocking ORAI channels with 2-APB, or by inhibiting NFAT activity with cyclosporine A (CsA), an inhibitor of calcineurin (Fig. 7A); similar results were observed using live cell imaging of a human keratinocyte cell line, HaCat, that expressed NFAT-GFP (Fig. 7B). These results show that PAR2 activation induces Ca\(^{2+}\)-dependent NFAT translocation, which may lead to NFAT-dependent changes in gene expression. In support of this model, PAR2-evoked SOCE robustly increased expression of TSLP transcripts in keratinocytes (Fig. 7C).

We next addressed whether ORAI1/NFAT signaling mediates PAR2-evoked TSLP release. We found that siRNA-mediated knockdown of ORAI1 or STIM1 significantly attenuated SLIGRL-evoked TSLP release by keratinocytes, suggesting that ORAI1 is required for PAR2-evoked TSLP secretion (Fig. 7D). Likewise, inhibition of NFAT-mediated transcription with CsA also attenuated TSLP release (Fig. 7E), but had no effect on SOCE-evoked Ca\(^{2+}\) signals (not shown). In addition to cutaneous epithelial cells, airway epithelial cells of patients with allergic rhinitis, AD and asthma also display high TSLP expression \(^{183}\). Previous studies have shown that TG induces ORAI1-dependent Ca\(^{2+}\) signals in human airway epithelial cells \(^{221}\). Interestingly, we found that, like keratinocytes, SOCE triggers robust TSLP expression in human airway epithelial cells, which can be blocked by CsA (not shown). These data identify ORAI1-dependent NFAT activation as a regulator of TSLP expression and release in both cutaneous and airway epithelial cells.

We next tested the hypothesis that NFAT promotes TSLP expression in vivo. Mice were treated with SLIGRL, SLIGRL and CSA, or vehicle via intradermal injection into the back. TSLP protein levels in treated skin were measured three hours after injection. Co-
Figure 7. PAR2 activation promotes Ca$^{2+}$-dependent NFAT translocation and TSLP secretion

(A) Representative images displaying cytosolic and nuclear localization of NFAT (green) and DAPI (red) in keratinocytes after a 30 min incubation with vehicle (VEH), SLIGRL (100µM), SLIGRL + 2APB (50µM) or SLIGRL + CsA (1µM). Pretreatment with 2APB or CsA prevented SLIGRL-induced NFAT nuclear translocation. n≥300 cells. (B) Fraction of HaCaT keratinocytes displaying nuclear localization of NFAT-GFP following treatment with SLIGRL (100µM; black), SLIGRL and 2APB (50 µM; red), SLIGRL + CsA (1µM; blue) or vehicle (VEH; white). n≥1000 cells. (C) TSLP expression in human keratinocytes following a 3h treatment with vehicle (VEH, black) or SLIGRL (100µM, red). n≥3. (D) SLIGRL-evoked TSLP release in cells treated with scrambled (black), STIM1 (red) or ORAI1 siRNA (blue). Secretion was normalized to vehicle-treated cells (white). n≥3. (E) TSLP release in response to treatment with vehicle (VEH, black), SLIGRL (100 µM, red) or SLIGRL + CsA (1µM, blue). (F) Western blot of skin lysates from mice following intradermal injection with vehicle (VEH), SLIGRL, or SLIGRL+CsA. Samples were probed with antibodies against TSLP and calnexin (loading control). n≥3 mice. (G) Western blot of skin lysates isolated from mice following intradermal injection with vehicle (VEH), treptase (TRY; 100pg/20µL), or treptase+CsA (TRY + CsA). Samples were probed with antibodies against TSLP, and actin (loading control). n≥3 mice. *P<0.05; **P<0.01, ***P<0.001. Error bars represent s.e.m. (H) Schematic diagram depicting the ORAI1 signaling pathway in keratinocytes that links PAR2 to TSLP secretion and activation of itch neurons. Activation of PAR2 triggers release of Ca$^{2+}$ from the ER and activation of STIM1, which opens ORAI1 channels to promote Ca$^{2+}$ influx. Ca$^{2+}$ activates the phosphatase calcineurin, which dephosphorylates NFAT and causes nuclear translocation, thus inducing transcription of TSLP. Secreted TSLP depolarizes a subset of C-fibers to evoke itch, in a TSLPR- and TRPA1-dependent manner. Activation of TRPA1-expressing sensory neurons can then lead to release of neuropeptides in the skin in a process known as neurogenic inflammation.
injection of CsA significantly attenuated SLIGRL-evoked TSLP protein expression in skin (Fig. 7F). Similar results were also observed with the endogenous PAR2 agonist, tryptase (Fig. 7G), demonstrating that PAR2 triggers TSLP expression via the Ca$^{2+}$-calmodulin/NFAT pathway in vivo.

**DISCUSSION**

TSLP plays a key role in the triad of atopic diseases: asthma, allergic rhinitis and atopic dermatitis. Recent studies have also implicated TSLP in a number of disorders, including cancer, gastrointestinal diseases, and autoimmunity. As such, there is much interest in understanding the mechanisms of TSLP expression and downstream effects of TSLP secretion. Here we present molecular, cellular and behavioral data showing that ORAI1/NFAT signaling regulates TSLP release by keratinocytes, and that TRPA1 is required for TSLP-evoked activation of sensory neurons and subsequent itch behaviors. Our data support a new model whereby TSLP released from keratinocytes acts directly on sensory neurons to trigger robust itch-evoked scratching (Fig. 7H).

**Sensory neurons mediate TSLP-evoked itch**

Studies on the role of TSLP in promoting atopic disease have focused solely on its effects on immune cells. A variety of immune cells are activated by TSLP, including dendritic cells, T cells, B cells, natural killer cells, mast cells, basophils and eosinophils, which together promote allergic inflammation. The inflammatory cytokines produced by these immune cells can activate sensory neurons. TSLP expression in keratinocytes leads to robust scratching in mice, which was previously assumed to occur solely downstream of immune cell cytokine release.

The current model is that sensory neurons are activated downstream of TSLP-activated immune cells to induce itch. Our data support the direct activation of sensory neurons by TSLP. First, we show that mast cell release of histamine, or other pruritogens, is not required for TSLP-evoked itch behaviors. In addition, histamine-dependent itch requires TRPV1, and our data show that TRPV1-deficient mice display normal TSLP-evoked itch behaviors. Finally, we show that acute TSLP-evoked itch does not require lymphocytes. These results were surprising given the well-known role of immune cells in TSLP-evoked atopic disease. However, until now, studies have focused on the long-term, rather than the acute effects of TSLP. These data suggest that the acute versus chronic phases of TSLP-evoked inflammation may be mediated by distinct mechanisms. In addition, because activation of primary afferent neurons triggers the release of inflammatory agents that promote immune cell chemoattraction and activation, neuron-to-immune cell communication may also play a key role in the development of AD. Thus far, all published studies have focused on global knockouts of TSLPR. Future studies using tissue specific TSLPR knockout mice are required to determine the relative contributions of sensory neurons and immune cells to both the acute and chronic phases of AD.
TRPA1 is required for TSLP-evoked itch
TSLP activates a subset of sensory neurons that express TSLPRs and the irritant receptor TRPA1. TRPA1-positive sensory neurons are required for the transmission of itch and pain stimuli to the CNS. Recent studies have shown that TRPA1 is also required for dry skin- and allergen-evoked chronic itch, but the endogenous signaling molecules that promote TRPA1 activation in these itch models are unknown. We now show that the endogenous AD cytokine, TSLP, leads to TRPA1 activation, downstream of TSLPR. Inhibition of PLC significantly attenuates such coupling both in vitro and in vivo. Despite the extensive literature on TSLP in immune cells, little is known about the signaling pathways activated downstream of TSLPR. The JAK/STAT pathway has been implicated in TSLP signaling but thus far, neither TSLPR nor IL7R have been linked to PLC.

TRPA1 afferents are not restricted to the skin, but also densely innervate the airways and gastrointestinal tract. Indeed, TRPA1 activation promotes lung inflammation in mouse models of airway inflammation and asthma and triggers inflammation in mouse models of inflammatory bowel disease. Interestingly, we found that like keratinocytes, Ca\(^{2+}\) signaling through ORAI1 triggers robust TSLP expression in human airway epithelial cells (data not shown). Thus, crosstalk between sensory neurons and epithelial cells via TSLP and TRPA1 may not be restricted to the skin, but may also occur in the airways and gut. The “atopic march” has been largely attributed to the actions of epithelial and immune cells (Holgate, 2007). Future studies using tissue specific TSLPR-deficient animals are required to resolve the contributions of neuronal and immune cell TSLP signaling to atopic disease. Nonetheless, our findings highlight a potential new role for TRPA1 and sensory neurons in promoting the atopic march.

ORAI1/NFAT regulates TSLP release in keratinocytes
ORAI1 was first identified as the channel that mediates store-operated Ca\(^{2+}\) influx required for NFAT-dependent cytokine expression during immune cell activation; loss of function mutations in ORAI1 and STIM1 lead to severe combined immunodeficiencies in patients. Our work shows that in addition to lymphocytes, epithelial cells also utilize ORAI1-mediated Ca\(^{2+}\) influx to regulate cytokine expression and release, suggesting that ORAI1 plays a more general role in the pathogenesis of inflammatory disease. Thus, ORAI1 may represent a new therapeutic target for atopic disease.

A role for the ORAI1/NFAT pathway in AD is consistent with a number of disparate clinical findings. First, SNPs in the ORAI1 gene have been linked to susceptibility to atopic disease in humans but the role of ORAI1 in AD had not been studied. Second, NFAT displays an abnormally high degree of nuclear localization in the keratinocytes of chronic itch patients, but the consequences of NFAT activation on AD was unknown. Finally, CsA, an inhibitor of NFAT-mediated transcription, is a potent immunosuppressant drug and is often prescribed for itchy inflammatory skin diseases, such as psoriasis and AD. While its effects have been mainly attributed to immune cell inhibition, our work suggests that the effectiveness of CsA in treating chronic itch may, in part, be due to its effects on keratinocyte-mediated TSLP release.
EXPERIMENTAL PROCEDURES

Cell culture.
Primary human epidermal keratinocytes (PromoCell) and HaCaT cells were cultured in PromoCell Keratinocyte Medium 2 and DMEM, respectively. siRNA directed against ORAI1, ORAI2, and STIM1 (Qiagen; 100ng) were transfected using HiPerFect (Qiagen). HaCaT cells were transiently transfected with Lipofectamine 2000 (Invitrogen) using 1µg HA-NFAT1(1-460)-GFP plasmid (Addgene 11107). DRG neurons were isolated from P18-30 mice and cultured as previously described. All media and cell culture supplements were purchased from the UCSF Cell Culture Facility.

Ca^{2+} imaging.
Ca^{2+} imaging was carried out as previously described. Physiological Ringer solution: 140mM NaCl, 5mM KCl, 10mM HEPES, 2mM CaCl_{2}, 2mM MgCl_{2}, 10mM D-(+)-glucose, pH 7.4 with NaOH. Images were collected and analyzed using MetaFluor (Molecular Devices). \([Ca^{2+}]_i\) was determined from background-corrected \(F_{340}/F_{380}\) ratio images using the relation \([Ca^{2+}]_i = K^*(R-R_{min})/(R_{max}-R)\) (Almers 1985), with the following parameters measured in keratinocytes: \(R_{min}=0.3; R_{max}=2.2;\) and \(K^*=3\mu M\). Cells were classified as responders if \([Ca^{2+}]_i\) increased 15% above baseline.

Electrophysiology.
Recordings were collected at 5 kHz and filtered at 2 kHz using an Axopatch 200B and PClamp software. Electrode resistances were 2-6 MΩ. Perforated patch internal solution: 140mM CsCl, 5mM EGTA, 10mM HEPES, pH 7.4 with CsOH, 0.24 mg ml^{-1} Amphotericin B. Stimulation protocol: 10ms step to -80 mV, 150ms ramp from -80mV to +80mV. Current clamp internal solution: 140mM KCl, 5mM EGTA and 10mM HEPES (pH 7.4 with KOH). Series resistance of all cells were <30 MΩ and liquid junction potentials were < 5mV (no correction).

RT-PCR.
RNA was extracted using RNeasy (Qiagen) and reverse transcription was performed using Superscript III. RT-PCR was carried out using SYBR Green (Invitrogen) on a StepOnePlus ABI machine. Threshold cycles for each transcript (Bogiatzi et al., 2012) were normalized to GAPDH (ΔCΔ). Calibrations and normalizations used the \(2^{-\Delta\Delta C_i}\) method where \(\Delta\Delta C_i = [(C_i\ (target\ gene) - C_i\ (reference\ gene))] - [C_i\ (calibrator) - C_i\ (reference\ gene)];\) GAPDH=reference gene; scrambled siRNA=calibrator. Experiments were performed in triplicate.

Histology.
Histology was carried out as previously described. Antibodies: rabbit anti-PGP9.5 and rabbit anti-peripherin (Millipore) 1:1000; goat anti-TSLPR and mouse anti-NFATc1 (Santa Cruz Biotechnology) 1:100. IL7Ra and TSLPR probes (Panomics) were used for in situ hybridization following the QuantiGene protocol (Panomics).
**Protein detection.**
TSLP protein levels were measured using the DuoSet ELISA kit (R&D Systems) on media collected 24h after stimulation. TSLP release was normalized to vehicle. For western blots, 50µg of cleared tissue lysate was resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with Anti-TSLP (1:250, Genetex), Anti-Calnexin (1:2,000, Abcam) and Anti-Actin (1:2,000).

**Mice and Behavior.**
Mice (20-35g) were housed in 12h light-dark cycle at 21°C. Behavioral measurements were performed as previously described. Compounds injected: 2.5µg TSLP, 200µg CQ, 100pg tryptase dissolved in PBS, or RTX 1µg/mL in 0.05% ascorbic acid and 7% Tween 80 (two days prior to pruritogen injection). For AITC behavior, 5µL 10% AITC in mineral oil was applied to the right hind paw. Behavioral scoring was performed while blind to treatment and genotype. All experiments were performed under the policies and recommendations of the International Association for the Study of Pain and approved by the University of California, Berkeley Animal Care and Use Committee.

**Data analysis.**
Data are shown as mean ± s.e.m. Statistical significance was evaluated using a one-way ANOVA followed by a Tukey-Kramer post hoc test or unpaired two-tailed Student’s t-test for comparing difference between two samples. *p < 0.05, **p < 0.01, ***p < 0.00
SUMMARY

There remains a great deal to learn about the molecules, cell types, and circuitry mediating acute and chronic itch. The work presented here represents a step forward in describing some of the pathways underlying itch transduction. Our work suggests that TRPA1 plays a role in mediating multiple forms of acute and chronic itch and that, as a convergence point for multiple itch related signaling pathways, it is a promising target for pharmacological intervention in clinically relevant itch. Indeed, recent studies have shown that TRPA1 is required for other models of chronic itch. Further studies will elucidate whether TRPA1 inhibitors can alleviate itch in humans.

The relative role of peripheral TRPA1 in sensory neurons, keratinocytes, and immune cells remains elusive. TRPA1 expression and function has been confirmed by many groups in primary somatosensory afferents, although non-neuronal expression of TRPA1 is controversial. While keratinocyte TRPA1 expression has been most commonly explored, a recent study suggests that functional TRPA1 is expressed in mast cells and mediates IL-13-mediated itch. The experiments performed in this body of work cannot rule out a role for nonneuronal TRPA1 in acute or chronic itch behavior. Consequently, use of tissue specific TRPA1 mutant mice will be required to fully dissect the role of neuronal and nonneuronal TRPA1 in itch behavior, as well as pain behaviors, which have been subject to similar constraints. These studies may also provide evidence lending toward the relative role of keratinocytes, sensory neuron afferent endings, immune cells, and endothelial cells to the complex process of itch stimulus transduction in the skin.
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