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Enhanced non-peptidergic intraepidermal fiber density and an expanded subset of chloroquine-responsive trigeminal neurons in a mouse model of dry skin itch

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Abstract

Chronic pruritic conditions are often associated with dry skin and loss of epidermal barrier integrity. In this study, repeated application of acetone and ether, followed by water (AEW) to the cheek skin of mice produced persistent scratching behavior with no increase in pain-related forelimb wiping, indicating the generation of itch without pain. Cheek skin immunohistochemistry showed a 64.5% increase in total epidermal innervation in AEW-treated mice compared to water-treated controls. This increase was independent of scratching, because mice prevented from scratching by Elizabethan collars showed similar hyperinnervation. To determine the effects of dry skin treatment on specific subsets of peripheral fibers, we examined Ret-positive, CGRP-positive, and GFRα3-positive intraepidermal fiber density. AEW treatment increased Ret-positive fibers, but not CGRP-positive or GFRα3-positive fibers, suggesting that a specific subset of non-peptidergic fibers could contribute to dry skin itch. To test whether trigeminal ganglion neurons innervating the cheek exhibited altered excitability after AEW treatment, primary cultures of retrogradely labeled neurons were examined using whole-cell patch clamp electrophysiology. AEW treatment produced no differences in measures of excitability compared to water-treated controls. In contrast, a significantly higher proportion of trigeminal ganglion neurons were responsive to the non-histaminergic pruritogen chloroquine after AEW treatment. We conclude that non-peptidergic, Ret-positive fibers and chloroquine-sensitive neurons may contribute to dry skin pruritus.

Perspective

This study examines the underlying neurobiological mechanisms of persistent dry skin itch. Our results indicate that non-peptidergic epidermal hyperinnervation and non-histaminergic pruritic receptors are potential targets for chronic pruritus.

Key words: pruritus, Ret, hyperinnervation, xerosis, epidermis, GFRα3

Introduction

Pruritus is a primary complaint associated with xerosis (dry skin) and other dermatoses that compromise skin barrier integrity such as atopic dermatitis and psoriasis.[44, 59] A rodent model of persistent and ongoing dry skin pruritus was previously developed by application of equal parts acetone and ether followed by water (AEW) to the rostral back skin.[33] Adaptation of the AEW model to the hind-limb, where biting and licking behaviors were used to indicate itch, showed an absence of behavioral sensitization to heat and mechanical stimuli, suggesting the dry skin produced itch but not hyperalgesia.[1] Recently, clear differentiation between pain and itch behaviors was achieved by application of algogens or pruritogens to the rodent cheek.[24, 43] When repeated AEW treatments were applied to the cheek, hind-limb scratching behavior was evoked, indicating that dry skin produced ongoing itch.[54]

The mechanisms by which dry skin generates itch are unclear. Intriguingly, human pruritic dermatoses are frequently associated with increased intraepidermal fiber density.[14, 38, 40, 47] Increased intraepidermal fiber density was also reported after a single, acute application of acetone in rodents[23, 49], and has been hypothesized to occur in AEW-induced itch.[57] However, scratching after AEW treatment develops with a latency of 3 days, suggesting that the sprouting fibers observed after a single treatment may be insufficient to induce itch.[1, 33, 37] The identity of the expanded peripheral fibers is not known, nor is it understood whether fiber hyperinnervation directly contributes to pruritus.

In addition to possible changes in epidermal innervation, enhancement of pruritic receptor function and phenotypic switching of sensory neurons into pruriceptors may contribute to the increased itch generated by dry skin. Like many intractable pruritic conditions, AEW-induced itch is thought to be histamine-independent.[33] Novel non-histaminergic neural pathways

and pruritic receptors have recently been identified.[8] Importantly, members of the Mas-related G protein-coupled receptor family (Mrgprs) are activated by the non-histaminergic pruritogen chloroquine, and ablation of MrgprA3 resulted in decreased scratching after AEW treatment.[16, 27] MrgprA3 is functionally coupled to TRPA1, a channel that exhibits sensitivity to a wide range of irritants including mustard oil and formalin.[4, 22, 32, 53] A significant reduction in scratching was observed in TRPA1 knock-out mice exposed to AEW treatment. Furthermore, AEW treatment induced upregulation of MrgprA3 mRNA, suggesting that these receptors may contribute to dry skin-induced itch.[54]

The aim of this study was to determine the effects of dry skin pruritus on peripheral fiber anatomy and the physiological properties of sensory neurons innervating dry skin. Intraepidermal innervation was characterized and quantified with and without scratch-preventing Elizabethan collars to determine whether scratching itself contributes to changes in nerve fiber density in dry skin. We tested the hypothesis that dry skin produces peripheral sensitization by enhancing the excitability of trigeminal neurons. Finally, we monitored calcium responses evoked by chloroquine and mustard oil from AEW-treated and control animals to test for altered expression or function of itch-related receptors.

A preliminary version of this work was presented at the American Pain Society annual meeting in 2014.[50]

Materials and Methods

Animals and AEW treatment

All experiments were conducted in accordance with the National Institutes of Health guidelines and received the approval of the Animal Care and Use Committee of Washington University, School of Medicine. 8-12 week old littermate mice (C57BL/6 (Jackson lab) or Ret-EGFP[15, 18], N=68) were housed on a 12 hour light-dark cycle and allowed ad libitum access to food and

water. Ret-EGFP reporter mice (129/SvJ:C57BL/6) were obtained from Dr. Jain.[18] Cheek skin was shaved with electric clippers one day prior to the start of acetone-ether-water treatments. The acetone-ether group was treated with a 1:1 mixture of acetone and diethyl ether (Sigma, St. Louis, MO.) for 30 seconds by soaking and then applying a gauze-wrapped cotton tip to the cheek, followed by similarly applied water for 30 seconds. Control animals were treated with water only. After 6 days of twice per day treatments (morning and evening), scratching behavior was quantified 6-8 hours after the final AEW treatment. Mice were placed in individual observation chambers and allowed to acclimate for 1 hour prior to observation. Bouts of scratching were then counted for 1 hour with experimenters blinded to treatment. A bout of scratching was defined as any number of individual scratch events separated by a pause. During the pause, behaviors such as licking or biting of the hind-limb, holding the limb motionless, or putting the limb down on the surface, could occur. Wiping behavior was taken to indicate pain and was defined as a rostrally-directed movement of the ipsilateral forelimb across the cheek starting from the ear. [24, 43] Scratching behavior with continued AEW treatment has been reported to persist for at least 2 weeks.[1, 37]

Immunohistochemistry

After six days of AEW treatment, mice were deeply anesthetized (ketamine-xylazine-acepromazine: 38-1.92-0.38 mg/mL; 2.7 mL/kg). The treated skin was dissected and immersion-fixed in Zamboni's fixative for 4 hours, rinsed in PBS, and cryoprotected in 30% sucrose, then sectioned at 30μm and collected on slides. Wild type C57BL/6 mice were used to determine β-III tubulin, CGRP, and GFRα3 fiber innervation, while a separate strain of Ret-EGFP reporter mice[15, 18] were used to determine Ret-positive fiber density. Antibodies and dilutions: rabbit anti-βIII tubulin (1:1000, Covance), goat anti-CGRP (1:1000, Serotec), goat anti-GFRα3 (1:100, R&D Systems), rabbit anti-GFP (1:1000, Invitrogen), Alexa Fluor 488/555 donkey anti-rabbit (1:200-400, Invitrogen), Alexa Fluor 488 donkey anti-goat (1:200-350,

Invitrogen). These concentrations are based on previous demonstrations of intraepidermal fiber staining.[10, 11, 15] Specificity of the GFRα3 antibody was previously shown using a GFRα3 knock-out mouse.[12] All slides were stained with bisbenzamide (1:40,000, Sigma, St. Louis, MO.) and MetaMorph Software (Molecular Devices, Sunnyvale, CA.) was used to measure the length of the dermal-epidermal border. In each examined section labeled fibers crossing the dermal-epidermal border were counted on an upright epifluorescent microscope (Nikon 80i; CoolSnapES camera). Six examined sections separated by >60µm were analyzed for each animal and the mean fiber density was calculated. To determine whether changes in fiber innervation were dependent on scratching, modified Elizabethan collars (Harvard Apparatus, Holliston, MA.) were secured at the start of treatment in both AEW and water-only groups. For hematoxylin and eosin (H&E) staining, tissue was fixed in Zamboni's fixative for 2-4 hours, then embedded in paraffin. Sections 10 µm thick were stained using standard H&E methods.[13] Representative images of fibers stained with the above-described methods were obtained using a Leica SPE Confocal microscope. Images were collected across the z-plane at 1µm width and maximum projection images were generated using ImageJ software.

Culture of trigeminal ganglia

Wild type mice were sacrificed by decapitation after nine days of treatment and the ipsilateral trigeminal ganglia (TG) were removed and cut into several pieces. Ganglia were incubated in 45U papain (Worthington, Lakewood, NJ) in 3 mL Hank's buffered saline solution without Ca^{2+} or Mg^{2+} and with 10 mM HEPES at 37℃, 5% CO_2 for 20 minutes. TG were washed and then incubated in collagenase (1.5 mg/ml) for 20 minutes. TG were triturated with a fire-polished Pasteur pipette, then passed through a 40µm filter, and the dissociated cells were plated on poly-D-lysine and collagen coated glass coverslips. Cells were cultured overnight in Neurobasal A media supplemented with B27, 100U/mL penicillin/streptomycin, 2 mM Glutamax, and 5%

fetal bovine serum (Gibco). No additional growth factors were added to the media. All experiments were performed within 24 hours of plating.

Whole-cell patch clamp electrophysiology

For electrophysiology experiments, FastDiI (Sigma, St. Louis, MO.) was injected intradermally into the cheek of wild type mice on day 2 of AEW or water treatment to label trigeminal neurons innervating the skin at the treatment site. After seven additional days of AEW or water treatment to allow maximum retrograde labeling of trigeminal neurons, including sprouting terminals, trigeminal ganglia were cultured as described above. Retrogradely labeled trigeminal neurons from AEW- or water-treated mice were then identified using an Olympus BX-50 epifluorescence microscope and subsequently examined for differences in membrane excitability. Cells were tested in an external recording solution consisting of (in mM): 145 NaCl, 3 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 7 Glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH and 305 mOsm with sucrose. Borosilicate, filamented glass electrodes with 2-5 MΩ resistance (Warner Instruments, Hamden, CT) contained internal solution (in mM): 130 K-gluconate, 5 KCl, 5 NaCl, 3 Mg-ATP, 0.3 EGTA, 10 HEPES, adjusted to pH 7.3 with KOH and 294 mOsm with sucrose. After acquiring gigaseal and break-in, neurons were given 2 minutes to stabilize and then a series of protocols to determine membrane excitability were performed. Action potentials were evoked in current clamp mode using a series of increasing 1 second ramp current injections. The first action potential of a train was used to determine threshold, defined as the voltage at which the first derivative of the membrane potential increased by 10 V/s. Rheobase was established from the step current pulse at which the first action potential was triggered. Data were collected with a HEKA EPC 10 amplifier, digitized at 20 kHz, and recorded on a PC running Patchmaster software (v2x-71). Series resistance was kept below 10 MΩ in all recordings and only DiI labeled cells with a diameter less than 30 µm were studied.

Calcium imaging

The protocol for calcium imaging was adapted from our previous studies.[5, 19, 51, 58] Cells from wild type mice were incubated for 45 minutes in 3 µg/mL of the cell-permeant ratiometric calcium indicator Fura-2 AM (Molecular Probes) and then incubated in external solution (in mM): 130 NaCl, 5 K, 2 $CaCl_2$, 1 $MgCl_2$, 30 Glucose, 10 HEPES for a 30 minute de-esterification prior to recording. Coverslips were positioned in a recording chamber and perfused with external solution at room temperature. Cells were viewed under an inverted microscope (Olympus Optical, Tokyo, Japan) and images were captured with a Hamamatsu Orca camera. Regions of interest encompassing all Fura-loaded cells were identified a priori and the ratio of fluorescence emission at an alternating excitation wavelength of 357 and 380 nm was recorded with SimplePCI Software. The experimental protocol consisted of a 2 minute baseline followed by 30 second bath application of 100 µM mustard oil (MO, Sigma, St. Louis), 8 minutes of external solution, 30 seconds of 1mM chloroquine (CQ, Sigma, St. Louis), 8 minutes of external solution, and 10 seconds of high KCl (50mM). Peak responses were determined by calculating the absolute increase in Fura-2 signal above baseline immediately prior to each stimulus. A change from resting level of ≥20% was set as the threshold for a response to a bath applied chemical. Cells unresponsive to high K+ were excluded from physiological analysis.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 6.04 (2014). For comparisons between AEW-treated and water-treated scratching behavior, unpaired t-test was used to compare the total scratch bouts or wipes per 60-minute interval. Electrophysiological data comparisons were performed using unpaired t-test. Differences between the proportion of responders in AEW and water groups were determined using a χ^2 test. Peak calcium increase in response to stimuli was analyzed with unpaired t-test. For all statistical analyses, significance was defined as $p<0.05$. Data are presented as mean ± S.E.M.

Results

AEW-induced dry skin elicits scratching but not wiping behavior

AEW treatment induced grossly visible, dry and scaly skin on the treated cheek of mice, whereas skin from water treated mice appeared unchanged (Figure 1A, B). AEW treatment also induced a hyperproliferation of keratinocytes resulting in thickening of the epidermis that was not observed in the water-only controls. The dry skin group was marked by spongiosis and large pieces of dissociating stratum corneum which still contained nucleated keratinocytes (Figure 1C, D). AEW treatment induced a significant increase in the number of site-directed bouts of scratching (H_2O = 4.0 ± 2.3 scratch bouts, AEW = 60.7 ± 17.0 scratch bouts, unpaired t-test, $p \leq 0.01$; Figure 1E). In contrast, very little wiping behavior was observed in general and no difference in the number of wipes was observed between water-only and AEW treatment groups (H_2O = 1.0 ± 0.8 wipes, AEW = 4.4 ± 1.7 wipes; unpaired t-test, Figure 1F). These results indicate that AEW-induced dry skin elicits ongoing itch without pain.

Dry skin induces intraepidermal hyperinnervation independent of scratching

To determine whether dry skin alters intraepidermal nerve fiber density (IENFD), we quantified fiber innervation in the cheek epidermis from AEW- and water-treated mice. IENFD was measured using an antibody against β-III tubulin, which is specific for neurons and labels axons and their terminals (Figure 2).[25] IENFD was significantly increased in the AEW-treated group compared to water-only controls (H_2O = 26.8 ± 4.2 fibers/mm, AEW=44.1 ± 5.4 fibers/mm, n=5 animals per group, unpaired t-test, $p<0.05$) (Figure 2A - C).

Scratching itself is thought to promote itch via a positive-feedback loop known as the "itch-scratch cycle", and could alter fiber innervation. We sought to determine whether AEW-induced dry skin is itself capable of generating epidermal hyperinnervation, or whether

scratching behavior was necessary to observe the increased IENFD. To this end, mice were fitted with Elizabethan collars to prevent scratching of the cheek for the duration of the AEW and water treatments. AEW-treated mice that wore collars also showed an increase in IENFD (H_2O = 45.5 ± 1.8 fibers/mm, AEW = 70.2 ± 1.7 fibers/mm, n=3 animals per group, unpaired t-test, $p<0.001$) (Figure 2D, E). The magnitude of hyperinnervation relative to water controls (Fold Change) was not different between the no collar and collar groups (no collar = 1.5 ± 0.2 fold increase relative to water, n=5 animals; collar = 1.5 ± 0.04 fold increase relative to water, n=3 animals, unpaired t-test, p=0.89) (Figure 2F), indicating that AEW treatment induced epidermal hyperinnervation independent of scratching.

Dry skin selectively induces hyperinnervation by Ret-positive, non-peptidergic fibers

Both peptidergic and non-peptidergic fibers are present in the epidermis and may contribute to pruritus. However, βIII-tubulin is an indiscriminant marker of nerve fibers. Therefore, to determine the subsets of sensory fibers that are increased in AEW-induced dry skin, peptidergic fiber density was assessed with an anti-CGRP antibody. There were no significant changes in CGRP-positive IENFD in the dry skin group when compared to water controls (H_2O = 10.2 ± 1.4 fibers/mm, AEW = 8.9 ± 2.1 fibers/mm, n = 5 animals per group, unpaired t-test, p=0.62) (Figure 3A - C). Ret-EGFP reporter mice were used to identify Ret-positive (non-peptidergic) fibers, which were visualized using an anti-GFP antibody.[15] The density of Ret-positive epidermal fibers was significantly increased in the dry skin group (H_2O = 40.2 ± 1.1 fibers/mm, AEW = 70.1 ± 7.6 fibers/mm, n = 4 animals per group, unpaired t-test, $p<0.01$) (Figure 3D – F). A small subset of fibers that express both peptidergic and non-peptidergic markers can be identified by their expression of the artemin co-receptor GFRα3.[34] Intraepidermal fiber density of these fibers was quantified using an antibody directed against the GFRα3 receptor and no change in innervation after AEW treatment was observed (H_2O = 16.0 ± 1.2 fibers/mm, AEW = 14.9 ± 2.0 fibers/mm, n = 4-5 animals per group, unpaired t-test, p=0.68) (Figure 3G-I). These data

suggest that dry skin induces branching and extension of non-peptidergic, Ret-positive epidermal fibers, which may be important for the development or maintenance of dry skin-induced itch.

We determined the proportion of trigeminal neurons projecting to the skin that were also Ret-positive by intradermal cheek injection of the retrograde tracer DiI into untreated Ret-EGFP mice. Trigeminal ganglia were harvested and cultured and we found that the majority of retrogradely labeled neurons were also Ret-positive (Figure 4A, B). Of all retrogradely labeled neurons, we found that 70.4 ± 4.1% were Ret-positive (Figure 4C). Additionally, when tested for physiological responses to KCl, we found that 92.4 ± 2.7% of the KCl-responsive, DiI-positive cells were also Ret-positive (Figure 4C). This increased proportion of viable Ret-positive skin-projecting neurons suggests a small loss of Ret-negative neurons in culture.

Effects of dry skin on trigeminal neuron physiology

We hypothesized that AEW treatment could alter the membrane excitability of trigeminal neurons innervating the dry skin. We specifically targeted neurons with known peripheral projections by retrogradely labeling trigeminal neurons with DiI injected into the cheek skin of C57BL/6 AEW- and water-treated mice (Figure 5A). Whole-cell patch clamp electrophysiology was then used to assess changes in membrane excitability. Rheobase was assessed using a short step and action potential threshold was determined with the first spike evoked from a ramp current (Figure 5B, C). Resting membrane potential of trigeminal neurons was not significantly different between AEW- and water-treated mice (H_2O = -60.8 ± 2.7 mV, n=14 neurons, AEW = -57.2 ± 2.5 mV, n=15 neurons, unpaired t-test) (Figure 5D), and neither group showed spontaneous activity. There was also no significant difference in the current amplitude required to elicit an action potential between AEW- and water-treated animals (H_2O = 614.3 ± 70.4 pA, n=15; AEW = 453.3 ± 50.0 pA, n=15, unpaired t-test) (Figure 5E). Action potentials evoked by a ramp current injection showed no difference between AEW and water treatment groups in the

threshold for activation (H_2O = -8.7 ± 3.7 mV, n=10; AEW = -3.8 ± 1.9 mV, n=12, unpaired t-test) (Figure 5F). Together, these data show that dry skin treatment did not produce ongoing activity or changes in membrane excitability that could be determined *in vitro*.

We next determined whether AEW-induced dry skin altered the responses of pruritic receptors and pruriceptive neurons. Trigeminal ganglion neurons from AEW- and water-treated wild type C57BL/6 mice were harvested, cultured, and loaded with Fura2-AM (Figure 6A). Wilson et al., (2013) showed that TRPA1 is crucial for dry skin-induced itch and that MrgprA3 mRNA is upregulated in sensory neurons after AEW treatment. To determine whether corresponding functional changes occur in trigeminal neurons, intracellular calcium was monitored to test the responses to the TRPA1 agonist mustard oil (MO) and non-histaminergic pruritogen chloroquine (Figure 6B). We observed no significant difference between AEW- and water-treated groups in the proportion of MO-responsive neurons (H_2O = 89/1042 cells (8.54%), AEW = 107/1199 cells (8.92%), χ^2 test, p=0.81), or in the peak amplitude of the MO response (H_2O = 101.4 ± 7% above baseline, AEW = 112.7 ± 6% above baseline, unpaired *t*-test, p=0.24) (Figure 6C). On the other hand, the proportion of CQ-responsive neurons in AEW-treated animals was significantly increased by 27.2% (H_2O = 127/1042 cells (12.19%), AEW = 186/1199 cells (15.51%), χ^2 test, $p<0.05$) (Figure 6D). Peak calcium responses to CQ were not different between the two groups (H_2O = 73.4±4% above baseline, AEW = 68.7±3% above baseline, unpaired *t*-test, p=0.34).

Most MO-responsive neurons also responded to CQ regardless of treatment (H_2O = 77/89 (86.5%), AEW = 93/107 (86.9%), χ^2 test, p=1). Of the CQ-responsive neurons, most responded to MO in the water-treated group (77/127, 60.6%), while in the AEW group, half of CQ-responsive neurons responded to MO (93/186, 50.0%). Therefore, a large proportion of CQ-responsive neurons did not respond to MO. We tested whether the proportion of CQ-responsive neurons that did not respond to MO was increased in the AEW group, but this did not reach significance (χ^2 test, p=0.066).

Discussion

Dry skin pruritus is a common problem and is often associated with other dermatoses. Here we show that persistent dry skin induced both pruritus and epidermal hyperinnervation in mice. We found that Ret-positive fibers contributed to the increased fiber density, but peptidergic, CGRP-positive and GFRα3-positive fibers did not. Moreover, preventing scratching of the affected area did not prevent dry skin-induced hyperinnervation. To understand whether sensitization or ongoing activity of sensory neurons contributes to persistent dry skin pruritus, we performed *in vitro* recordings from trigeminal neurons that were determined to have innervated the treated skin. No evidence was found supporting the hypothesis that altered membrane excitability was responsible for persistent dry skin itch. On the other hand, AEW treatment produced an increase in the proportion of trigeminal neurons responsive to the histamine-independent pruritogen chloroquine, supporting the concept that the Mrgpr family of receptors is upregulated and functionally contributes to persistent dry skin itch.

In this study, AEW treatment of the cheek skin evoked scratching, but not forelimb wiping, indicating the treatment produced ongoing itch without pain. A common feature in the affected skin of patients with pruritic disease is increased epidermal innervation.[9, 14, 38, 47] Likewise, increased fiber growth in the murine epidermis after AEW treatment has been noted.[23, 49] Here, persistent AEW treatment increased total epidermal fiber density by 65%, as indicated by the pan-neuronal marker βIII-tubulin. We tested the possibility that mechanical stimulation from scratching contributed to the fiber growth. When Elizabethan collars were fitted to prevent scratching, intraepidermal innervation was still greater than in water-treated skin. The relative increase did not differ from the hyperinnervation observed in the AEW-treated animals without collars. These results demonstrate that dry skin itself is sufficient to induce hyperinnervation without the presence of scratching.

In addition to hyperinnervation, histological studies of patients with atopic dermatitis or psoriasis indicate that itch severity correlates positively with nerve growth factor (NGF) in the skin and the NGF-receptor TrkA in nerve fibers.[9, 21, 36, 47, 56] Increased epidermal fibers and expression of NGF have been observed in a mouse model of atopic dermatitis[17, 48], and in mice with acute acetone-induced skin barrier dysfunction.[23, 49] While the specific contribution of hyperinnervation to itch sensation is not clear, these observations suggest the idea that peptidergic, TrkA-positive fibers may be important regulators of atopic and dry skin pruritus. The present study shows that repeated AEW treatment resulted in persistent dry skin itch, but we observed no increase of the CGRP-positive or GFRα3-positive fibers which likely express TrkA.[34] This may be due to differences between the biology underlying human atopic dermatitis and mouse models of acute dry skin. Our data do not rule out the possibility of functional contributions to dry skin itch from the CGRP-positive or GFRα3-positive subset of fibers or other peptidergic fibers, and it should be noted that fiber sprouting is not a prerequisite for sensory neurons to signal itch.

A majority of the fibers innervating the epidermis are non-peptidergic and express the receptor tyrosine kinase for the GDNF family of neurotrophic factor ligands, Ret, rather than TrkA.[15, 60] GDNF release from atopic skin was recently acknowledged to play an important role in sensory neurite outgrowth *in vitro* with implications for pruritus.[41] Artemin, which activates Ret and GFRα3, is increased in human atopic skin and artemin-treated mice displayed increased sprouting of peripheral nerve fibers and itch-like behaviors.[35] Likewise, an increase in GFRα3 immunostained fibers was found in artemin over-expressing mice.[10, 11] However, in the AEW model of dry skin no sprouting of GFRα3-positive fibers was observed. Neurturin is another potentially interesting GDNF family ligand but its role in pruritus is currently unknown. The Mrgpr family of histamine-independent receptors has been shown to be selectively localized to Ret-positive DRG neurons and is expressed in epidermal nerve terminals.[16, 26-28, 60] Our results show that AEW treated skin resulted in a significant increase in Ret-positive fibers penetrating into the

epidermis. This suggests that Ret-positive, non-peptidergic fibers could play a role in itch induced by dry skin.

The functional mechanisms engaged by pruriceptive sensory neurons to produce itch in dry skin conditions remain unresolved, but are thought to be independent of histamine signaling. Anti-histamines are generally not effective for treating chronic itch, including itch from dry skin.[39, 59] Furthermore, mast cell-deficient mice exhibit normal scratching after AEW treatment, indicating that factors released from mast cells are unlikely to generate the itch from dry skin.[33] Recent studies have pointed to the involvement of specific non-histaminergic mechanisms for dry skin itch. For example, ablation of the chloroquine-activated MrgprA3-expressing subset of sensory neurons drastically reduced AEW-induced scratching.[16] Also, in vivo recordings from spinal dorsal horn neurons showed enhanced responses to non-histaminergic pruritogens after AEW treatment, but not to histamine.[2] Taken together, these studies support the idea that dry skin pruritus signals through a non-histaminergic pathway.

Several signaling pathways for histamine-independent itch have now been identified. Scratching in mice deficient for TRPA1 was greatly reduced after chloroquine or AEW treatment, suggesting an important role for TRPA1 in non-histaminergic itch.[53, 54] Moreover, AEW treatment increased the message for MrgprA3 in both skin and sensory neurons.[54] MrgprA3, which functionally couples to TRPA1, is present on Ret-positive neurons, suggesting that dry skin itch involves non-peptidergic fibers.[16] Although TRPA1 was initially shown to be expressed in peptidergic sensory neurons,[45] recent data have demonstrated robust modulation of TRPA1 function in Ret-positive neurons and TRPA1 expression in non-peptidergic IB4-positive fibers that innervate the epidermis.[3, 6, 10, 11, 30, 31] While the hyperinnervation of Ret-positive neurons is consistent with a potential role in dry skin itch, functional studies are necessary to determine whether changes in neural sensitization or receptor expression occur after AEW treatment.

To gain insight into the functional changes of potential pruriceptive neurons exposed to dry skin, we hypothesized that locally released inflammatory mediators or neurotrophic factors act directly on pruriceptive sensory neurons to induce sensitization. Peripheral sensitization could explain the hyperknesis (heightened itch) and alloknesis (itch produced by a non-itchy stimulus) commonly associated with pruritic diseases. To target neurons that directly innervated the treated skin, we performed whole-cell recordings from trigeminal ganglion neurons retrogradely labeled from the cheek skin of animals exposed to AEW or water treatment. With this strategy, we found no evidence for sensitization of trigeminal neurons in the AEW versus water-treated groups. It is possible that culture conditions reset differences that may have existed *in vivo* and future studies to examine the excitability of sensory fibers may yield different results. It is also possible that dry skin is associated with changes in excitability that are more pronounced at the fiber terminals in the skin and these did not translate into our *in vitro* studies of sensory neuron cell bodies. On the other hand, previous studies have shown that manipulations *in vivo* have produced altered sensory neuron physiology *in vitro*.[7, 52, 55]

To test whether changes occur in the expression of functional pruritic receptors, we examined receptor-mediated responses to mustard oil and chloroquine in cultured trigeminal neurons from AEW- and water-treated animals. Interestingly, we found that AEW treatment expanded the population of sensory neurons with functional responses to chloroquine. In contrast, we found that responses to mustard oil were no different between AEW- and water-treated animals. These results mirror data showing an increase in mRNA for MrgprA3, but not TRPA1, in AEW-treated animals.[54] Overall, we found that ~10 percent of trigeminal neurons responded to mustard oil, which is consistent with the expression of TRPA1 in sensory ganglion in several reports.[20, 29, 42] While other studies have shown as many as 25% of sensory neurons are TRPA1-positive, TRPA1 expression can vary by innervation target, exposure to growth factors, and changes in other experimental methods.[22, 30, 45] Interestingly, many of the neurons that responded to chloroquine after AEW showed no mustard oil responses at all, suggesting

that the MrgprA3 receptor may couple to other channels in addition to TRPA1. In favor of this idea, MrgprA3 was recently found to modulate TRPM8, TRPC, and TRPV1.[46] Additionally, TRPV3-deficient mice exhibited greatly reduced scratching after AEW treatment compared to water-treated controls, indicating that other mechanisms in addition to TRPA1 may account for AEW-induced scratching.[57]

In summary, our results show that dry skin-induced pruritus is associated with non-peptidergic fiber growth into the epidermis and an expanded population of sensory neurons responsive to the non-histaminergic pruritogen chloroquine. Although sprouting of peptidergic, TrkA-positive fibers is observed in the skin of patients with atopic dermatitis and other dermatoses, our results suggest that non-peptidergic fibers may also play a role in chronic itch related to xerosis and compromised barrier integrity. Dry skin-induced itch, which particularly affects the older population and often co-exists with other dermatoses, may be improved by topical treatment directed at prevention of Ret-positive neural sprouting in the epidermis.

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Disclosures

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Figure Legends

Figure 1. Dry skin treatment induces itch without pain. A, B) Photographs of shaved mouse cheeks after treatment with water or acetone/ether and water. AEW treatment induced scaly, dry skin. **C, D)** H&E staining shows epidermal hyperplasia and hyperkeratosis in the AEW treated group but not in the water treated group. Stratum corneum still containing nucleated

keratinocytes can be seen in the process of dissociating from the epidermis. Scale bar = 50µm. **E, F)** Quantification of the mean number of scratch bouts and wipes during 1 hour of observation after 6 days of AEW treatment. AEW treatment greatly enhanced scratching ($p < 0.01$, unpaired t-test, water-treated n=9; AEW-treated n=11) but little wiping occurred and was not significantly different between water and AEW groups (n=5 each group, unpaired t-test).

Figure 2. Dry skin induces intraepidermal hyperinnervation independent of scratching. A, B) Mice treated with acetone/ether and then water showed increased fiber innervation and epidermal hyperplasia compared to the water treated animals. Scale bar = 20µm. **C)** Intraepidermal fiber innervation was significantly increased by AEW treatment. **D, E)** Mice with Elizabethan collars placed at the start of treatment also showed increased fiber density and thickening of the epidermis. Scale bar = 20µm. **F)** No change in the magnitude of AEW-induced hyperinnervation relative to water-only controls was observed between collared and non-collared mice after AEW treatment.

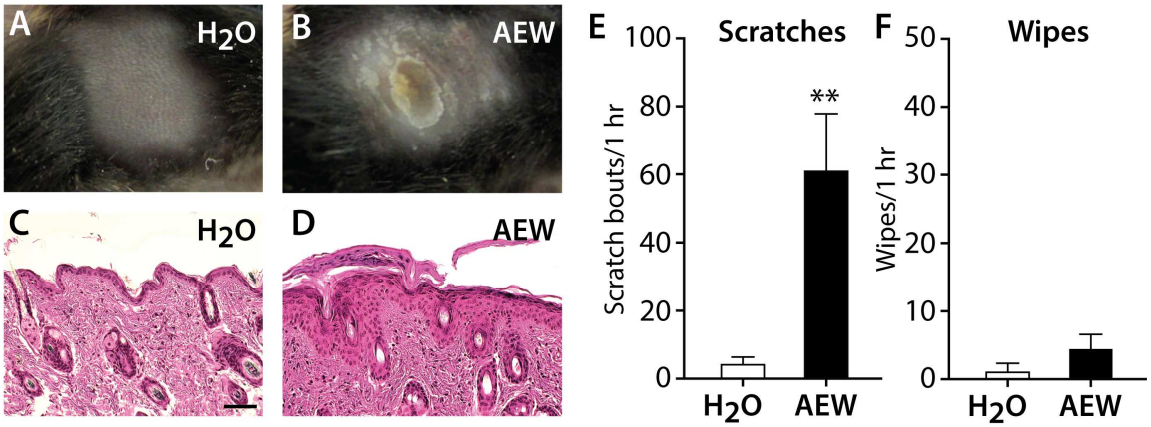
Figure 3. Hyperinnervation of Ret+, but not CGRP+ or GFRα3+ fibers after AEW treatment. A, B) Intraepidermal CGRP+ fibers appeared no different in the AEW and water groups. Scale bar = 20µm. Arrowheads indicate large pieces of dissociating stratum corneum which still contained nucleated keratinocytes. **C)** Quantification of CGRP+ density. **D, E)** Photomicrographs from a strain of mice in which eGFP is expressed from the Ret locus. eGFP immunostained fibers in the cheek epidermis show increased fiber density after AEW-treatment. Scale bar = 20µm. **F)** Quantification of Ret+ fiber density. **G, H)** IENFD of GFRα3+ fibers was not different between AEW and water groups. **I)** Quantification of GFRα3+ density.

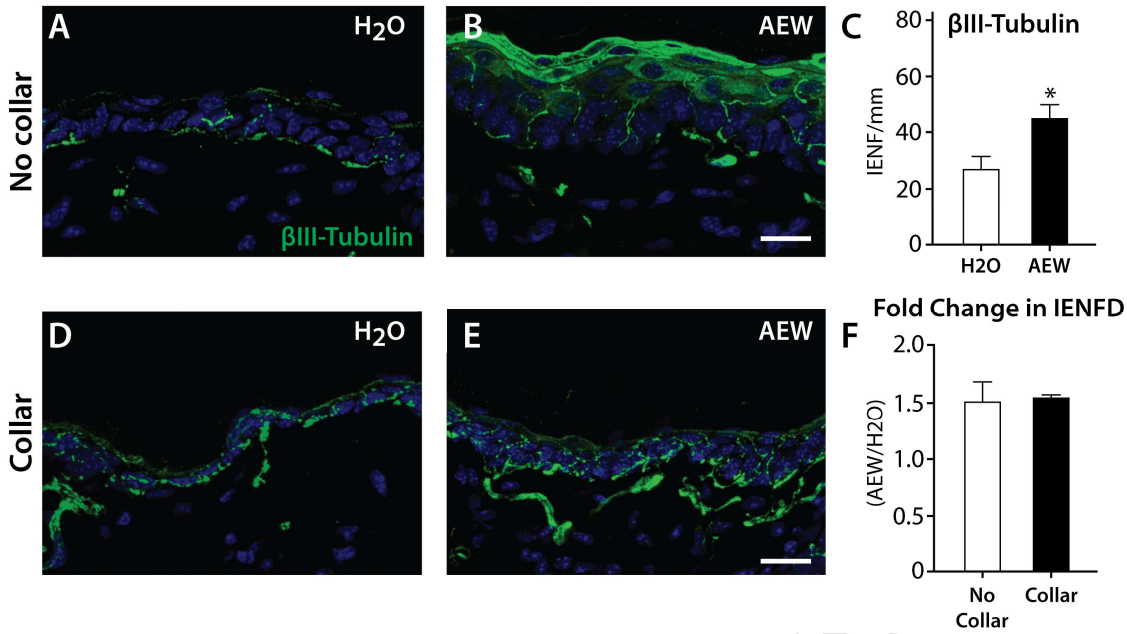
Figure 4. Retrograde labeling of trigeminal afferent fibers innervating the cheek. A, B) Two injections of DiI (10 uL each) into the cheek of an untreated Ret-EGFP mouse retrogradely labeled trigeminal ganglion neurons that were later cultured. Arrows show double-labeled

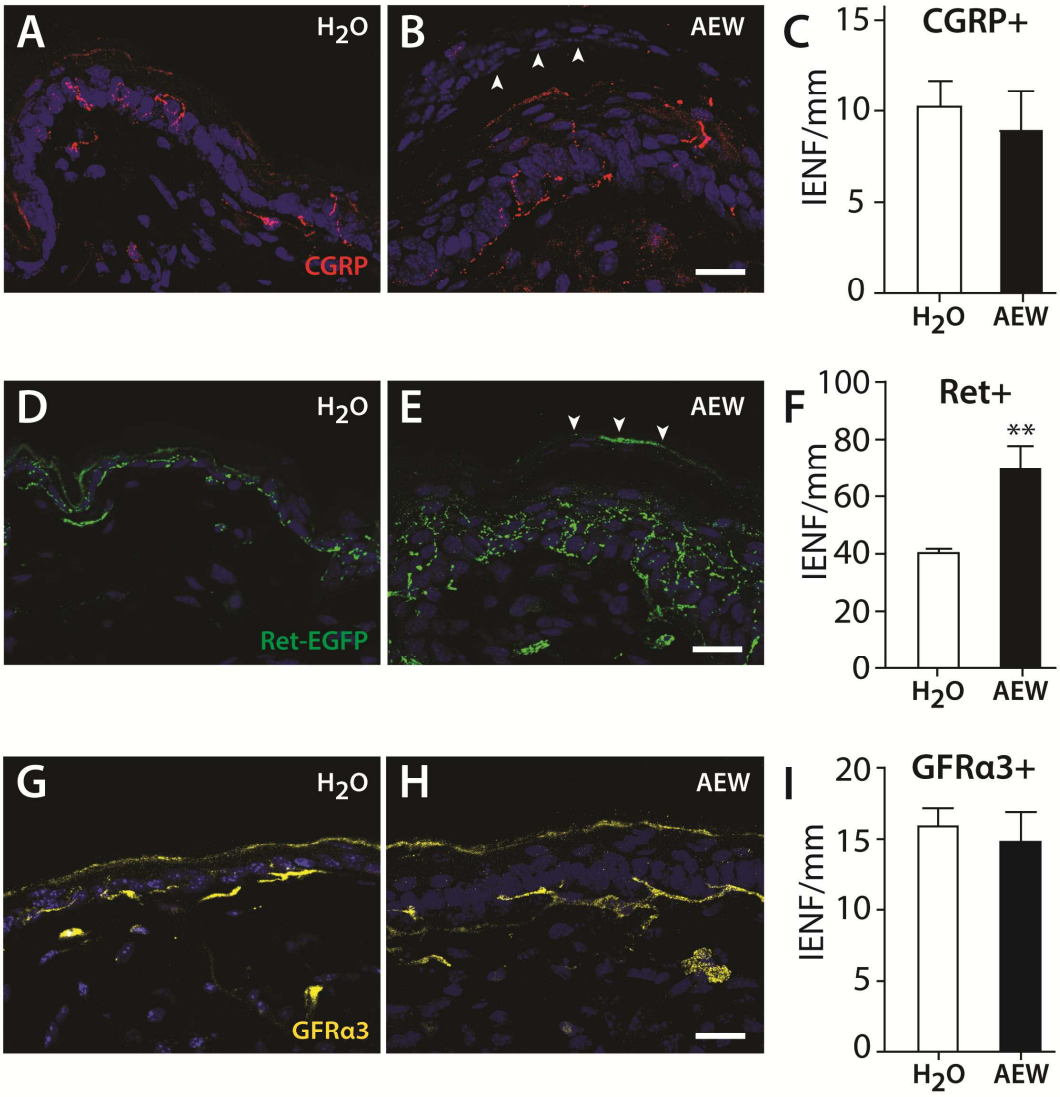
neurons. **C)** 70.4 ± 4.1% of all DiI+ neurons were also Ret + (n=10 coverslips, each circle represents % per coverslip); 92.4 ± 2.7% KCl-responsive, DiI+ neurons were also Ret+ (n=5 coverslips). Total of 3 Ret-eGFP mice used.

Figure 5. Electrophysiology of AEW- and water-treated trigeminal neurons. A) Photomicrograph of a patched wild type trigeminal neuron retrogradely labeled from the cheek with DiI. (Scale bar = 30μm) **B)** Example trace of a short step protocol and evoked action potential to determine rheobase. **C)** Example traces of the ramp current and evoked action potentials from AEW- and water-treated trigeminal neurons. **D)** Resting membrane potential, **E)** Rheobase, and **F)** Action potential threshold were not different between AEW- and water-treated groups.

Figure 6. Increased calcium responses to chloroquine in AEW-treated trigeminal neurons in vitro. A) Image of Fura-2AM loaded trigeminal neurons with responses shown in B. (Scale bar = 50μm) **B)** Example traces of mustard oil (MO, 100 μM) and chloroquine (CQ, 1 mM) responders, and a chloroquine responder without mustard oil sensitivity. **C)** Quantification of the proportion of neurons responsive and magnitude of the response to mustard oil from AEW- and water-treated animals. **D)** AEW increased the proportion of neurons responsive to chloroquine but did not alter the magnitude of the response.



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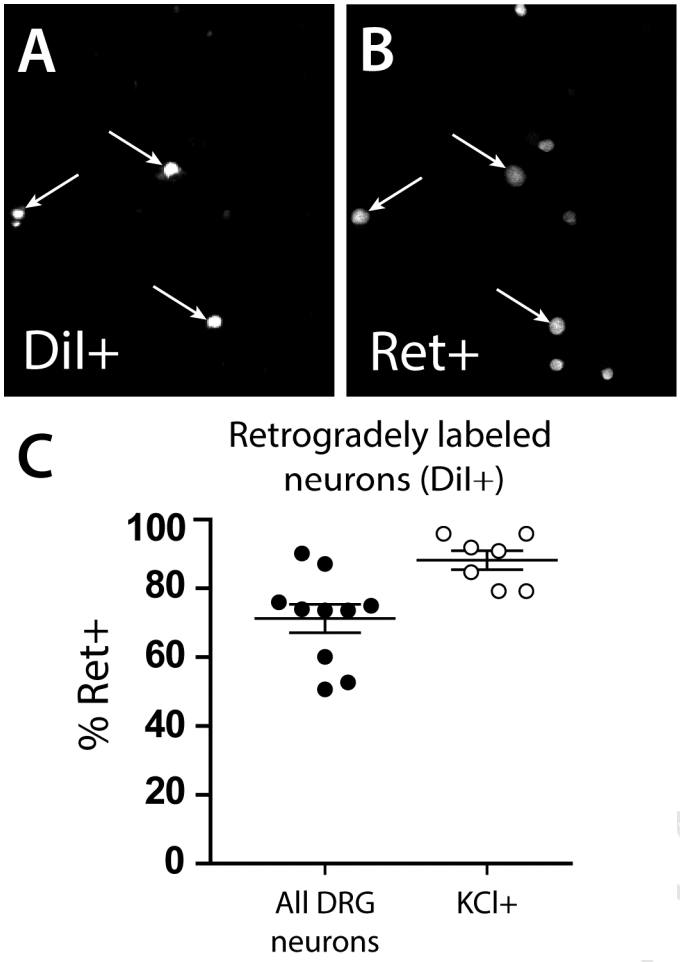

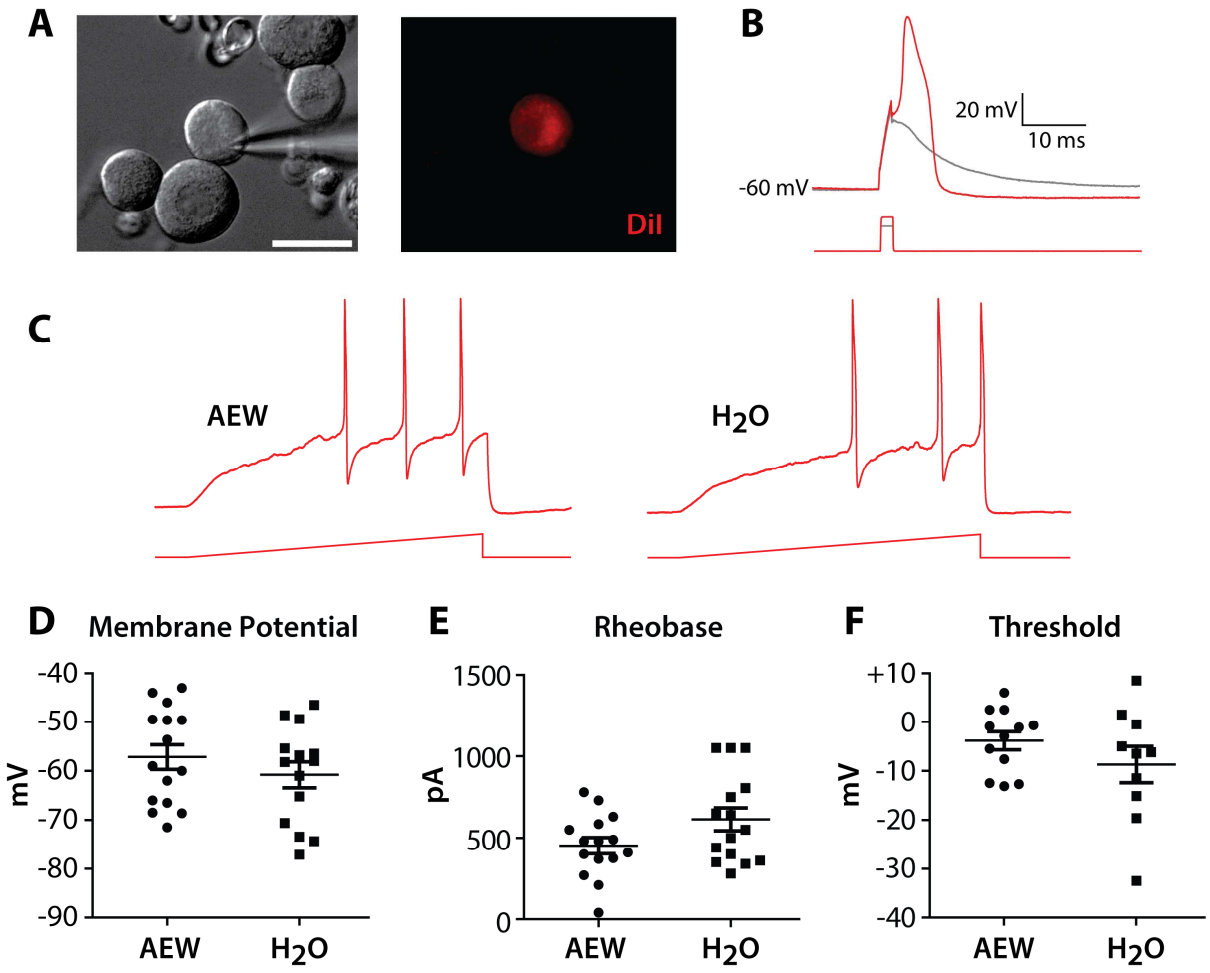
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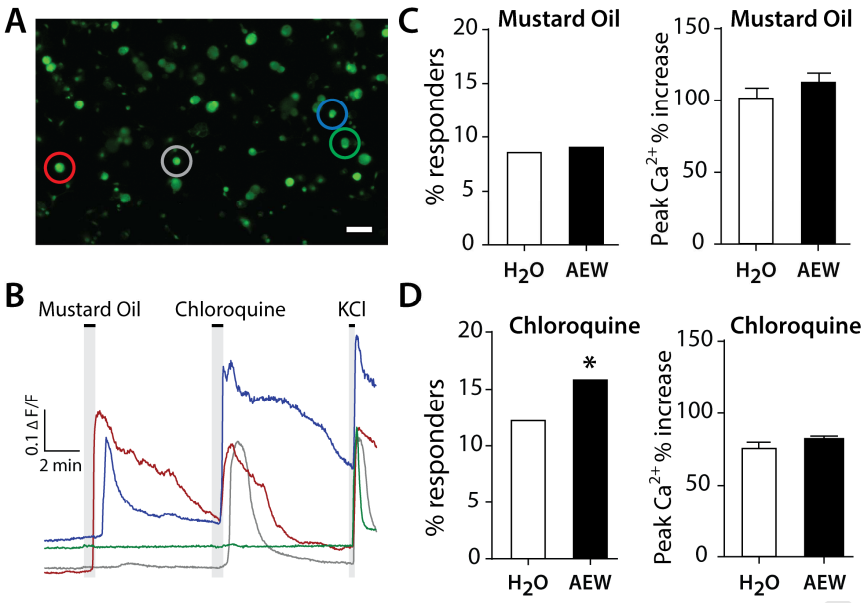

Highlights

- Dry skin produced persistent itch without pain in a mouse model
- Non-peptidergic fibers hyperinnervated the epidermis in dry skin
- The proportion of sensory neurons responsive to chloroquine was increased
- Non-peptidergic and histamine-independent mechanisms are likely important for dry skin itch