Published in final edited form as:
Pain. 2010 November ; 151(2): 378–383. doi:10.1016/j.pain.2010.07.024.

Enhanced scratching evoked by PAR-2 agonist and 5-HT but not histamine in a mouse model of chronic dry skin itch

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Abstract

Chronic itch is a symptom of many skin conditions and systemic disease, and it has been hypothesized that the chronic itch may result from sensitization of itch-signaling pathways. We induced experimental chronic dry skin on the rostral back of mice, and observed a significant increase in spontaneous hindlimb scratches directed to the dry skin. Spontaneous scratching was significantly attenuated by a PAR-2 antibody and 5-HT2A receptor antagonist, indicating activation of these receptors by endogeneous mediators released under dry skin conditions. We also observed a significant increase in the number of scratch bouts evoked by acute intradermal injections of a protease-activated receptor (PAR)-2 agonist and serotonin (5-HT), but not histamine. We additionally investigated if pruritogen-evoked activity of dorsal root ganglion (DRG) neurons is enhanced in this model. DRG cells from dry skin mice exhibited significantly larger responses to the PAR-2 agonist and 5-HT, but not histamine. Spontaneous scratching may reflect ongoing itch, and enhanced pruritogen-evoked scratching may represent hyperknesis (enhanced itch), both potentially due to sensitization of itch-signaling neurons. The correspondence between enhanced behavioral scratching and DRG cell responses suggests that peripheral pruriceptors that respond to proteases and 5-HT, but not histamine, may be sensitized in dry skin itch.

INTRODUCTION

Chronic itch associated with dermatitis, liver or kidney disease, HIV and many other conditions represents a large and poorly-treated medical condition worldwide [5]. Our understanding of the neural basis for normal itch transmission is at a beginning stage, and virtually nothing is known about pathophysiological changes associated with chronic itch. Sensitization of itch-signaling pathways has been suggested as a mechanism underlying chronic itch of atopic dermatitis patients, since histamine elicits greater itch, and noxious stimuli elicit itch instead of pain in lesional skin [15,16]. Sensitization may be triggered by spontaneous firing of pruriceptors from lesional skin [25], and the switch from pain to itch may reflect a pathological reduction in the normal inhibitory effect of pain on itch transmission.

To date, there have been few if any experimental studies of itch sensitization associated with dermatitis. We addressed this issue by investigating if scratching, a behavioral manifestation of itch, is enhanced in an animal model of chronic dry skin itch [20,22]. In this model, chronic

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The authors have no conflict of interest.

dry skin is induced by twice-daily skin treatments with acetone:ether (50:50) followed by water (AEW) over a 5–7 day period. These results in a significant increase in the number of spontaneous hindlimb scratch bouts directed to the dry skin treatment area on the rostral back, accompanied by increased epidermal thickness, decreased stratum corneum hydration and increased transepidermal water loss of the treated skin area [20]. We reasoned that chronic dry skin itch would sensitize itch-signaling pathways, such that mice would exhibit increased spontaneous scratching as a manifestation of chronic ongoing itch and increased scratching to acute challenge with intradermally injected pruritogens. One potential mechanism contributing to enhanced scratching is peripheral sensitization of pruritogen-responsive primary afferent fibers. To test this possibility, we investigated dorsal root ganglion (DRG) cells from cervical segments innervating skin on the rostral back. We tested if DRG cells from mice receiving AEW treatment on the rostral back exhibited a higher incidence of responsiveness to pruritogens, and if their pruritogen-evokes responses were larger, compared to DRG cells taken from control mice. We presently tested histamine, serotonin (5-HT) and an agonist of the protease-activated receptor type 2 (PAR-2), all of which elicit dose-dependent scratching behavior in mice [2,7,26,31]. Histamine is a prototypical itch mediator in humans, serotonin elicits mild itch [9], and PAR-2 has recently been implicated in chronic itch of atopic dermatitis [27].

METHODS

Experiments were conducted using ICR mice (29–34 g, 6–7 wk; Harlan, Oxnard CA) under a protocol approved by the UC Davis Animal Care and Use Committee.

Behavior

To induce chronic dry skin on the hindpaw, we followed a previouslyreported procedure [20, 22]. Briefly, a mixture of acetone and diethylether (1:1) was applied to a circumscribed area at the nape of the neck for 15 s, followed immediately by distilled water for 30 sec, twice-daily for 5 days. Control mice were treated in the same manner with application of water only for 45 sec. The animals' toenails were clipped so that the mice could direct hindlimb scratch movements to the treatment area such that the skin was rubbed by the toes but not scratched. After the 5^{th} treatment day, animals were placed in an arena and videotaped for 30 min to assess spontaneous scratching. Following the recording, animals were tested with id injection of 10 μl of one of the following: vehicle (isotonic saline), histamine (Sigma-Aldrich, St. Louis MO, 35 μg in saline), the PAR-2 agonist SLIGRL-NH2 (Quality Controlled Biochemicals, Hopkinton, MA, and GenScript, Piscataway, NJ; 35 μg in saline), or 5-HT (Sigma; 4.7 nmol in saline). Id microinjections were made as described in our recent study [2]. Immediately following the id microinjection, mice were placed in the arena and videotaped for 30 min from above. Scratching elicited by each pruritogen subsided by the end of the 30-min recording period. Investigators left the room during videotaping.

In separate studies, we tested the effect of antagonists of suspected itch mediators in AEW-treated mice. In one experiment, after 5 days of AEW treatment, mice received an id injection of PAR-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 16 μg/80 μL, id) within the AEW treatment area. The fairly large id injection was intended to saturate as much of the dry skin treatment area as possible. Five min later, they were placed in the recording arena and videotaped for 80 min. In the second experiment, AEW-treated mice received ketanserin (Sigma-Aldrich, 3 mg/kg, ip). After 30 min they were placed in the recording arena and videotaped for 80 min. IgG antibody (Santa Cruz Biotechnology) and 5% tween 80 in saline served as respective vehicle controls.

In all experiments, videotapes were played back and the number of scratch bouts counted at 5-min intervals over a 30-min (for acute pruritogen-evoked scratching) or 80-min recording

period (for antagonist studies) by an investigator blinded as to treatment. For comparison, we also include data from naïve animals that had been collected and analyzed in an identical manner in previous studies [2]. Scratch bouts were defined as previously reported [2,7] and scratch movements directed away from the treatment area (e.g., ears) were not included. Between-group comparisons of counts of scratch bouts were made by unpaired t-test and by repeated measures analysis of variance (ANOVA) followed by post-hoc Least Significant Difference (LSD) tests with time as with-subject variable and group as between-subject factor, using SPSS 9.0 software. A $p<0.05$ was considered to be significant.

Calcium Imaging

Upper- to mid-cervical DRGs removed from mice in each treatment group were enzymatically digested at 37°C for 10 min in Hanks's balanced salt solution (HBSS; Invitrogen, Carlsbad, CA) containing 20 units/ml papain (Worthington Biochemical, Lakewood, NJ) and 6.7 mg/ml L-cysteine (Sigma), followed by 10 min at 37°C in HBSS containing 3 mg/ml collagenase (Worthington Biochemical). The ganglia were then mechanically triturated using fire-polished glass pipettes. Cells were pelleted, suspended in MEM Eagle's with Earle's BSS (Gibco) containing 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 1×vitamin (Gibco) and 10% horse serum (Quad Five, Ryegate, MT), plated on poly-D-lysine-coated glass coverslips, and cultured for 16–24 hr.

Cells were incubated in Ringers solution (pH7.4; 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 4.54 mM NaOH) with 10 μM of Fura-2 AM and 0.05% of Pluronic F-127 (Invitrogen). Coverslips were mounted on a custom-made aluminum perfusion block and viewed through an inverted fluorescence microscope (Nikon Eclipse TS100, Melville, NY). Fluorescence was excited by UV light at 340 nm and 380 nm alternately and emitted light was collected via a CoolSnap camera attached to a Lambda LS lamp and a Lambda optical filter changer (Sutter Instrument Company, Novato, CA). Ratiometric measurements were made using computer software Simple PCI (Compix Inc, Cranberry Township, PA) every 3 sec. Approximately 40 cells were observeded per dish and subjected to the identical stimulus sequence. Solutions were delivered by a solenoid-controlled 8-channel perfusion system (ValveLink, AutoM8) at a flow rate at 6 ml/min. Histamine (100 μM), the PAR-2 agonist SLIGRL-NH2 (100 μM) and 5-HT (100 μM) were delivered in random order. After applications of pruritogens, 1 μ M capsaicin and 144 mM potassium were applied in this order. Stimulus duration was 30 sec (10 sec for capsaicin). Ratios were normalized to prestimulus baseline. Cells were judged to be responsive if the ratio value increased by more than 10% of the resting level following chemical application.

The incidence of DRG cell responses to each pruritogen (histamine, PAR-2 agonist, 5-HT) was compared between W and AEW treatment groups using Fisher's exact test. Between-group comparisons were made of the magnitude of response to each pruritogen using unpaired t-tests. For this comparison, we only used data for the initially-tested chemical so as to avoid possible order effects. A $p<0.05$ was considered to be significant.

True blue labeling

To determine the percentage of upper- to mid-cervical DRG cells innervating skin on the rostral back, we injected the fluorescent tracer True Blue (Sigma) intradermally. Using a 30 G hypodermic needle, a total of 5, 10 μl injections of 4% True Blue was made within within the region of AEW treatment on the rostral back in mice anesthetized with isoflurane. After a 1-wk survival period, the mice were euthanized under deep sodium pentobarbital anesthesia and DRG at mid-cervical levels were removed, prepared and cultured as described above. The number of True Blue-labeled cells was counted using a fluorescence microscope (Nikon

Eclipse Ti; Technical Instruments, San Francisco CA) equipped with a UV filter (excitation wavelength: 350 nm, emission wavelength: 420 nm).

RESULTS

Behavior

After 5 days of AEW treatment on the rostral back, ICR mice exhibited a significant elevation in the number of spontaneous hindlimb scratch bouts directed to the dry skin area (Fig. 1A, black bar to left) as compared to control mice treated twice-daily with water only (Fig. 1A, open bar to left) or naïve mice (Fig. 1A, gray bar to left). Intradermal injection of vehicle (saline) resulted in significantly more scratching in AEW-treated mice compared to water-treated or naïve controls but did not differ significantly from spontaneous scratching in the AEW group (Fig. 1A).

Acute id injection of each pruritogen elicited scratching that began during the initial 5-min observation period and persisted for about 20–25 min with histamine and SLIGRL-NH2 and for about 15 min with 5-HT. Fig. 1A shows that histamine, the PAR-2 agonist SLIGRL-NH2, and 5-HT, each elicited significantly more scratch bouts in AEW-compared to water-treated and naïve groups. In water-treated and naïve control animals, injection of each pruritogen elicited a significantly greater number of scratch bouts compared to saline. In Fig. 1B, scratching associated with vehicle injections was subtracted from pruritogen-evoked scratching for both AEW and water groups. Both the PAR-2 agonist and 5-HT still elicited significantly more scratching in the AEW compared to water groups, while scratching elicited by histamine was not significantly greater in the AEW vs. water group. Thus, even after spontaneous and vehicle-associated scratching behavior was subtracted, the PAR-2 agonist and 5-HT elicited significantly more scratching in the AEW groups, indicating a true enhancement under this treatment condition.

Since PAR-2 agonist- and 5-HT-evoked scratching was enhanced in dry skin, we wished to determine if PAR-2 and 5-HT2A receptors might be involved in spontaneous scratching in AEW-treated mice. Local pretreatment of the dry skin area with a PAR-2 antibody significantly reduced spontaneous scratching in AEW-treated mice as compared to vehicle controls (Fig. 2A). Systemic administration of the 5-HT2A antagonist, ketanserin also significantly reduced spontaneous scratching (Fig. 2B).

Calcium Imaging

The Venn diagrams in Fig. 3 show the relative proportions of DRG cells responsive to histamine, the PAR-2 agonist SLIGRL-NH2, 5-HT, and various combinations of these, for the W and AEW treatment groups. In the W group, the incidence of histamine, PAR-2 agonist and 5-HT responsive cells, respectively, were 15.1%, 2.9% and 6.4% (Fig. 3A). Eighty one percent, 72% and 76% of histamine-, PAR-2 agonist- and 5-HT-responsive cells, respectively, were also activated by capsaicin. In thr AEW-treated group, the incidence of histamine, PAR-2 agonist and 5-HT responsive cells, respectively, were 19.7%, 10.3% and 11.2% (Fig. 3B). Sixty six percent, 58% and 85% of histamine-, PAR-2 agonist- and 5-HT-responsive cells, respectively, were also activated by capsaicin. Proportions of histamine-, PAR-2 agonist-, and 5-HT-sensitive cells in AEW group were significantly greater compared to W group ($p<0.05$ for each, Fisher's exact test). The proportion of cells responsive to two or more pruritogens was 1.3% or less in the W and 2.4% or less in the AEW groups.

Fig. 4 shows individual examples of pruritogen-evoked responses of DRG cells. Each pruritogen elicited a response within the first 60 sec. The data are summarized in Fig. 5, which shows averaged responses vs. time of DRG cells in each treatment group to application of

histamine (Fig. 5A), the PAR-2 agonist (Fig. 5C) and 5-HT (Fig. 5E). Mean peak responses to the PAR-2 agonist (Fig. 5D) and 5-HT (Fig. 5F), but not histamine (Fig. 5B) were significantly larger in the AEW- vs. W-treated group.

Following id injections of True Blue in rostral back skin, 26.25% (42/160) DRG cells were labeled. This compares with a value of 29.7% of lumbar DRG cells labeled by injection of another tracer, DiI, into the hindpaw of rats [19].

DISCUSSION

The increased spontaneous scratching behavior observed in the AEW-treated group is consistent with the idea that dry skin produces ongoing itch that is relieved by scratching directed to the treatment area. This confirms the original report showing that spontaneous hindlimb scratching behavior progressively increases over a 5-day period of AEW treatment of the rostral back, becoming significant at day 3 and peaking at day 5 [20]. This model appears to mimic the itch and other skin conditions associated with senile xerosis [11,18,29,35]. A novel finding is that acute challenge of the dry skin region with injected pruritogens elicited significantly more scratching compared to challenge of control skin areas. When spontaneous and vehicle-associated scratching was accounted for, a significant enhancement of scratching in AEW-treated mice was observed with the PAR-2 agonist SLIGRL-NH2, and 5-HT, but not histamine. This implies that acute stimulus-evoked itch elicited by certain, but not all pruritogens, is enhanced in chronic itchy skin. This reflects hyperknesis (enhanced itch elicited by a normally itchy stimulus), which is consistent with sensitization of itch-signaling pathways.

Another novel finding was that spontaneous scratching behavior in dry skin-treated animals was significantly attenuated by a PAR-2 antibody and a 5-HT2A receptor antagonist (ketanserin), implicating a role for endogenous agonists of these receptors in chronic itch. Proteases and 5-HT are important mediators in the skin [21,23]. Increased serine protease activity has been observed in atopic skin [32]. Elevated levels of 5-HT have also been reported in psoriasis [13] and chronic eczema [14]. These findings are consistent with our hypothesis that endogenous proteases and 5-HT may contribute to enhanced scratching and chronic itch in dry skin.

DRG cells from AEW-treated mice exhibited significantly larger responses to the PAR-2 agonist and 5-HT, but not to histamine. The correspondence between enhancement of behavioral scratching and DRG cell responses suggests that dry skin itch partly involves peripheral sensitization of pruriceptors that respond to protease and 5-HT but not histamine. Such sensitization may partly involve increased spontaneous firing in pruriceptive primary afferent fibers [25]. It may also involve central sensitization of a subpopulation of itch-signaling spinal cord neurons. In the mouse, populations of superficial dorsal horn neurons identified using a pruritogen were responsive to intradermal injections of SLIGRL-NH2, histamine or 5-HT [3], and a large majority of neurons were excited by both the PAR-2 agonist and histamine [1]. Given this central convergence of pruriceptive inputs, peripheral sensitization of protease- and 5-HT-responsive pruriceptive fibers might better account for the enhanced scratching elicited by these mediators. Presumably, histamine-responsive pruriceptors are not sensitized by the dry skin treatment which did not result in an enhancement of histamine-evoked scratching behavior.

Using the same dry skin model applied to the mouse hindpaw, we observed a significant increase in numbers of spontaneously active neurons (assessed by c-fos) in the superficial dorsal horn of the lumbar spinal cord ipsilateral to AEW as compared to water-treated controls [22] consistent with central sensitization and enhanced neuronal excitability.

In the present study, histamine and 5-HT activated 15.9% and 11% of DRG cells, respectively, from naïve mice, consisting with previous reports [12,24]. In naïve mice, 4.8% of DRG cells responded to PAR-2 agonist. This value is relatively low compared to a previous study [28]. A possible explanation is a difference in culture conditions (with or without NGF) or species (mice or rats).

Responses to PAR-2 agonist were larger in DRG cells from AEW mice. PAR-2 is coexpressed with TRPV1, TRPV4 and TRPA1 in DRG cells [4,8,10]. Genetic knockout of TRPV1 diminished scratching behavior mediated by PAR-2 agonists [6]. Since TRPV1 is directly activated by diacylglycerol produced by G_q protein activation [33], there is the possibility that TRPV1 can function downstream of a G_q protein-coupled H1 histamine receptor as well as the PAR-2 receptor [17]. Enhanced responses of DRG cells to the PAR-2 agonist may result from phosphorylation of TRPV1.

DRG cells from AEW mice exhibited larger response to 5-HT. A high percentage of 5-HT-responsive DRG cells also responded to capsaicin. This result is consistent with the inhibition of scratching following depletion of capsaicin-sensitive peripheral fibers [17]. However, 5-HT-evoked scratching is observed in TRPV1 knockout mice to the same extent as in wild type mice [17], suggesting that TRPV1-expressing neurons, but not TRPV1 per se, play a major role in 5-HT-evoked scratching. Scratching evoked by 5-HT is inhibited by 5-HT2 receptor antagonists [34]. Enhanced responses to 5-HT of DRG cells from AEW-treated mice may reflect upregulation of the 5-HT2 receptor under this dry skin condition.

In dry skin-treated mice, many nerve fibers are observed in the thickened epidermis [20]. Dry skin is a feature in patients with atopic dermatitis. Many gastrin-releasing peptide-positive fibers are observed in the epidermis of lesional skin from NC/Nga mice, an atopic dermatitis model; these fibers co-express PAR-2, TRPV1, μ-opioid receptors and tropomyosin-receptor-kinase A [30]. Extension of this type of fiber into the epidermis may contribute to spontaneous itch and sensitization of itch signaling pathways.

The present data provide the first experimental evidence for behavioral enhancement of itch-related scratching in a dry skin model, potentially mediated via sensitization of peripheral pruritogen-sensitive afferent fibers. We believe that this model will prove useful for future studies of mechanisms of itch sensitization and potential treatment of chronic itch.

Acknowledgments

Supported by National Institutes of Health Grants DE013685 and AR057194.

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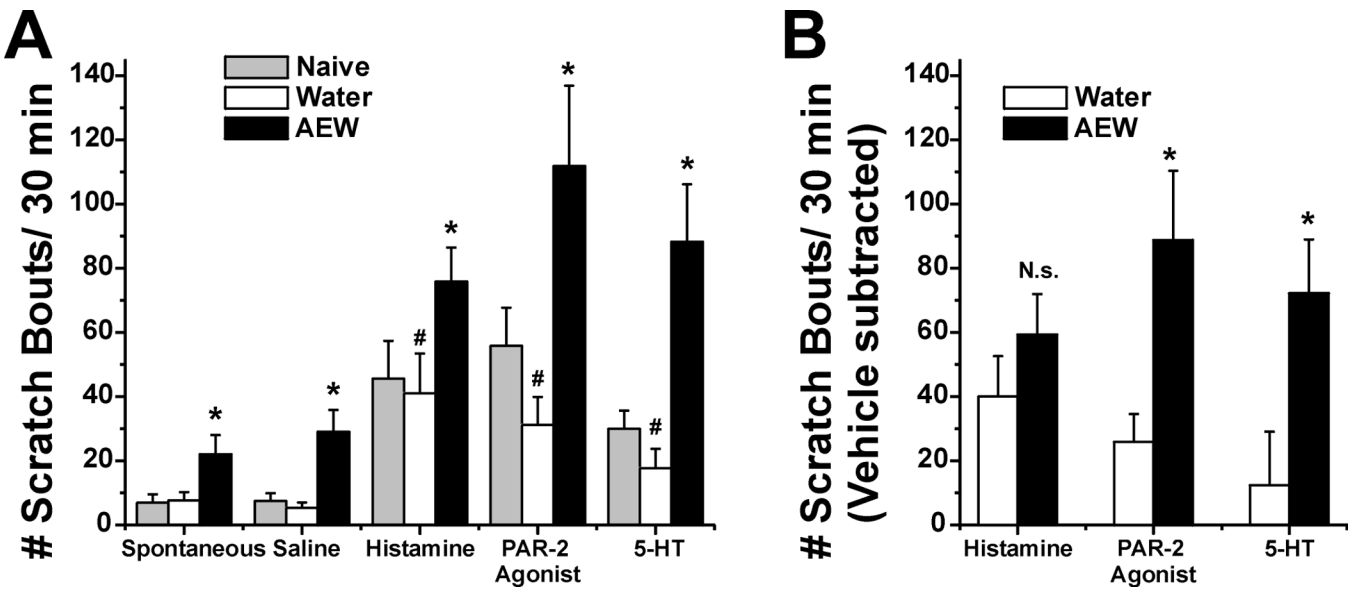

Fig. 1.

Spontaneous and pruritogen-evoked scratching behavior in AEW- and control mice. A: Bar graph plots mean number of scratch bouts recorded over a 30-min period without id injection (spontaneous) or following id injection of saline, histamine, the PAR-2 agonist SLIGRL-NH2, and 5-HT. Black bars: AEW dry skin treatment group; open bars: control water treatment; gray bars: naïve (untreated) group. Error bars: SEM. *: AEW group significantly different compared water and naïve groups for each stimulus condition ($p<0.05$ for all comparisons; repeated-measures ANOVA). #: significantly different compared to saline injection in water-treated group ($p<0.05$, unpaired t-test). B: Graph as in A, with vehicle-associated scratching subtracted. *: AEW group significantly different compared water group ($p<0.05$ for both; repeated-measures ANOVA). N.s.: after subtracting vehicle-associated scratching, there was no difference between AEW and W groups for histamine-evoked scratching behavior. (*n* =6–9/group).

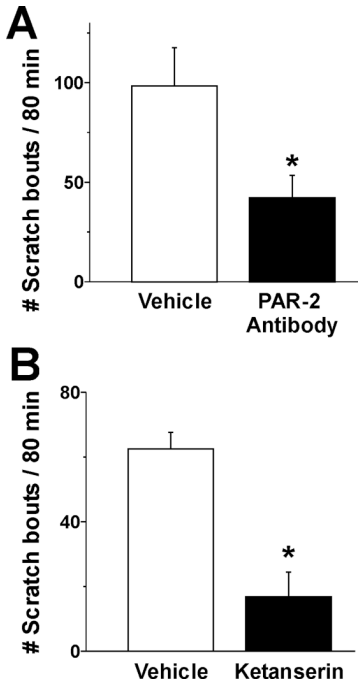

Fig. 2.

Reduced spontaneous scratching following treatment with PAR-2 antibody or 5-HT2A antagonist ketanserin. A: PAR-2 antibody. AEW-treated mice received an id injection of PAR-2 antibody, or vehicle (IgG antibody) within the AEW treatment area. Graph plots mean number of scratch bouts recorded over 80 min observation period, beginning 5 min following antibody pretreatment. *: significantly different from vehicle; $p<0.05$, paired t-test, n=6/group). B: Ketanserin. AEW-treated mice received systemic ketanserin (3 mg/kg, ip) or vehicle (5% tween 80 in saline). The graph plots the mean number of scratch bouts recorded over an 80 min period, beginning 30 min after ketanserin or vehicle pretreatment. *: significantly different from vehicle; $p<0.05$, paired t-test, n=6/group).

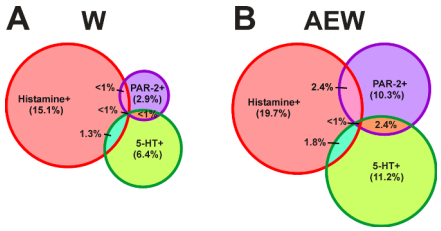

Fig. 3.

Venn diagrams showing relative proportions of DRG cells responsive to histamine, PAR-2 agonist SLIGRL-NH2, and 5-HT, and various combinations, for each treatment group. A: Water-treated (W) controls (n=1,391 cells). 81%, 72% and 76% of histamine-, PAR-2 agonist- and 5-HTresponsive cells, respectively, were also activated by capsaicin. B: AEW (n=726). Proportions of histamine-, PAR-2 agonist-, and 5-HT-sensitive cells in AEW group were significantly greater compared to W group ($p<0.05$ for each, Fisher's exact test). 66%, 58% and 85% of histamine-, PAR-2 agonist- and 5-HT-responsive cells, respectively, were also activated by capsaicin.

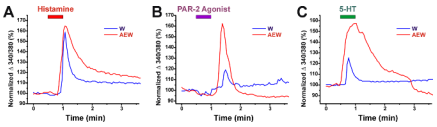

Fig. 4.

Individual examples of pruritogen-evoked responses of DRG cells. A: Histamine. Graph plots normalized 340/380 nm ratio vs. time for DRG cells from AEW-and W-treated mice. Histamine was applied at time 0:30 (red bar). B: PAR-2 agonist. C: 5-HT. Note larger responses of cells from AEW group to PAR-2 agonist and 5-HT.

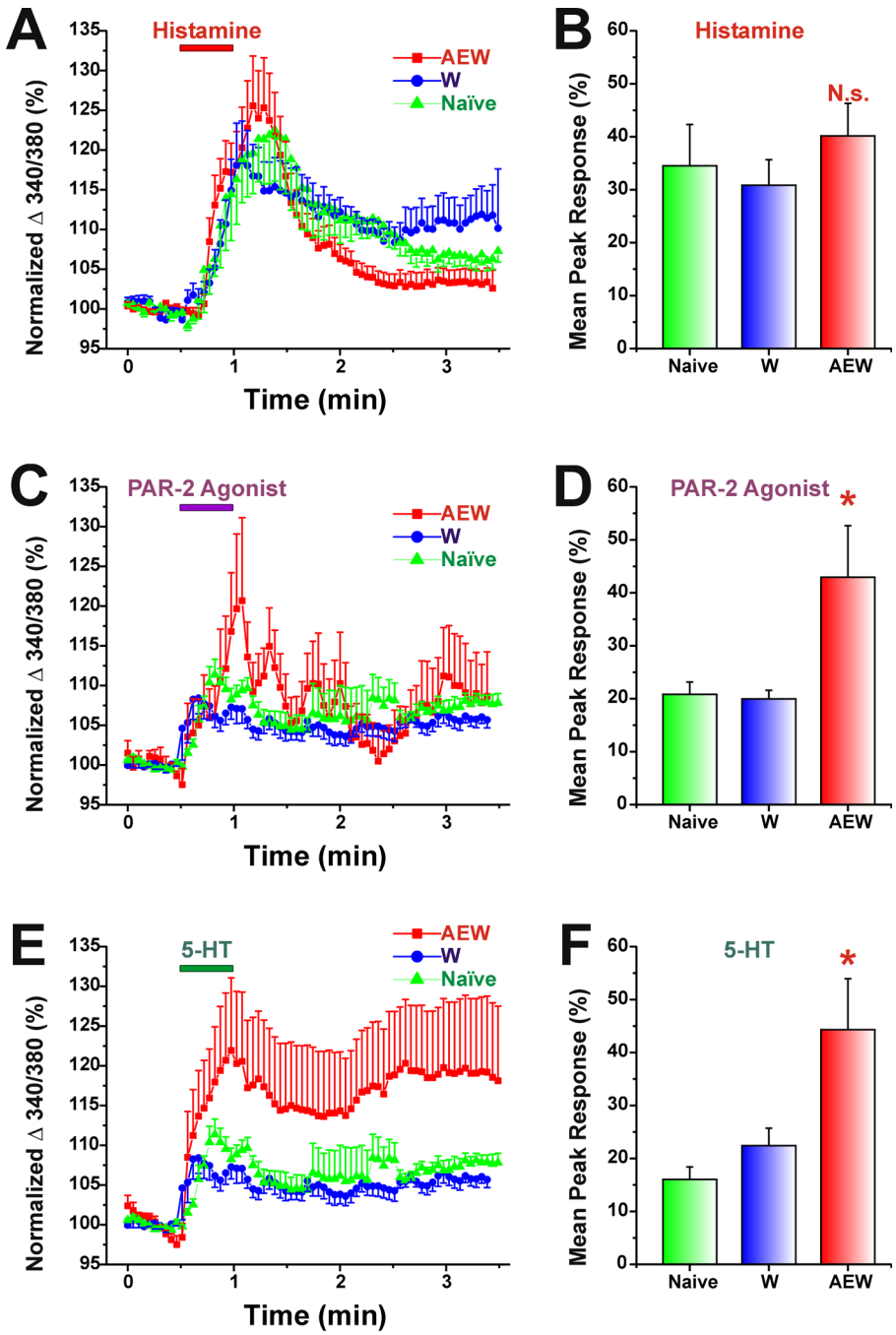

Fig. 5.

DRG cells from AEW-treated mice show enhanced responses to PAR-2 agonist and 5-HT but not histamine. A: Histamine. Mean normalized ratiometric responses of DRG cells from each treatment group vs. time relative to histamine perfusion (red bar). Error bars: SEM. B: Mean peak response (% of baseline) of DRG cells to histamine for each treatment group. N.s.: no significant difference compared to W. C, E: as in A for PAR-2 agonist and 5-HT, respectively. D, F: as in B for PAR-2 agonist and 5-HT, respectively. *: significantly different compared to W ($p<0.05$, unpaired t-test). (n =14–23/group). Naïve data from cervical DRG cells (n=719) obtained from untreated mice.

Dry skin treatment enhanced pruritogen-evoked scratching behavior in mice and responses of dorsal root ganglion cells, indicating peripheral sensitization of itch-signaling afferent fibers.