INVESTIGATIVE REPORT

Differential Itch- and Pain-related Behavioral Responses and *μ*-opioid Modulation in Mice

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Intradermal microinjection of the pruritogen histamine, or the algogen capsaicin, in the mouse cheek differentially elicits mainly hindlimb scratching or ipsilateral forelimb wiping, respectively. We investigated the dose-dependency of these responses elicited by various pruritogens and algogens, and μ-opioid modulation. Histamine, 5-hydroxytryptamine (5-HT) and agonists of protease-activated receptors PAR-2 and PAR-4, all elicited dose-related hindlimb scratching bouts with little forelimb wiping. In contrast, capsaicin, allyl isothiocyanate and bradykinin elicited dose-related forelimb wiping with little scratching. Morphine reduced capsaicin-evoked wiping but not pruritogen-evoked scratching. The μ-antagonist naltrexone decreased pruritogen-evoked scratching but not capsaicin-evoked wiping. A cowhage spicule inserted intradermal elicited equivalent scratching and wiping, while inactivated cowhage spicules loaded with histamine or capsaicin elicited significantly more scratching or wiping, respectively. The mouse cheek injection model appears to be a useful behavioral test that distinguishes between itch and pain. *Key words: itch; scratch; pain; wipe; mouse; algogen; pruritogen.*

Chronic itch frequently accompanies dermatologic conditions as well as systemic diseases and is poorly controlled by antihistamines or other treatments (1, 2). There is a pressing need for improved treatments of itch, which can be informed by investigations of the neural mechanism of normal acute itch using animal models. Rodents are commonly used in animal studies of itch. Because itch is almost always accompanied by a desire to scratch, scratching behavior is used as a behavioral readout of itch in animal studies. This usually involves counting numbers of hindlimb scratch bouts directed to the site of intradermal (i.d.) microinjection of a pruritogen in the nape of the neck (3–5). Biomechanical limitations make this essentially the only motor act capable of accessing the injection site. It was recently reported that stimuli delivered to the cheek can elicit hindlimb scratching or ipsilateral forelimb wiping, and that these responses may discriminate between itch and pain (6). To extend this important finding, we investigated the extent to which i.d. cheek injections of a variety of pruritogens and algogens elicit scratching and/or wiping behaviors in mice.

μ-opioid antagonists reduce experimental itch in humans (7), chronic itch in patients with atopic dermatitis (8), and pruritogen-evoked scratching in rodents (3, 9–11) as well as spontaneous scratching in murine models of chronic itch (12). μ-opioid agonists including morphine frequently elicit itch and scratching (13) and do not attenuate pruritogen-evoked scratching behavior or spinal c-fos expression in rodents (3, 10), while morphine significantly attenuated capsaicin-evoked c-fos expression (10). Based on these results, we additionally investigated μ-opioid modulation of scratching and wiping behaviors, reasoning that itch-related scratching would be reduced by μ-opioid antagonists but not morphine, whereas nocifensive wiping would be reduced by morphine but not μ-opioid antagonists.

Intra-epidermal insertion of spicules from pods of the bean plant cowhage (*Mucuna pruriens*) elicits histamine-independent itch with no accompanying flare (14). Cowhage-evoked itch is typically accompanied by nociceptive sensations of pricking/stinging and burning (15). These sensations are mimicked by heat-inactivated cowhage spicules treated with capsaicin or histamine (16). These latter findings prompted us to test if insertion of a single native cowhage spicule, or inactivated spicules loaded with capsaicin or histamine, elicited scratching and/or wiping behaviors in mice commensurate with human sensations.

METHODS

Experiments were conducted using 7–15-week-old male ICR mice (Harlan, Oxnard CA, USA; 31–44 g) under a protocol approved by the University of California, Davis Institutional Animal Care and Use Committee.

Chemicals

Drugs used were: Protease-activated receptor (PAR-2) agonist SLIGRL-NH2 (10, 50 μg/10 μl; QCB, Hopkinton MA, USA), PAR-4 agonist AYPGKF-NH2 (30–100 μg/10 μl; GenScript, Piscataway NJ, USA), 5-hydroxytryptamine hydrochloride (0.03, 0.1%/10 μl; Sigma-Aldrich, St Louis MO, USA), histamine (10, 50 μg/10 μl; Sigma), capsaicin (10, 30 μg/10 μl;

Sigma), allyl isothiocyanate (AITC = mustard oil; 0.01–0.1%/10 μl; Sigma), bradykinin (0.1–3 μg/10 μl; Sigma), formalin (5%/10 μl; Sigma); morphine sulfate (0.1–1 mg/kg; Sigma), naltrexone (1 mg/kg; DuPont, Garden NY, USA). All chemicals were dissolved in sterile isotonic saline, except capsaicin and AITC, which were dissolved in 7% Tween-80. Cowhage was inactivated by autoclave. Inactivated cowhage spicules were soaked in histamine (10 mg/ml in distilled water) or capsaicin (200 mg/ml in 80% ethanol).

Behavioral studies

The fur on the cheek was shaved and mice were habituated to the Plexiglas recording arena with a clear glass floor one week prior to testing. Microinjections were made i.d. in the cheek using a 30-g needle attached to a Hamilton microsyringe by PE-50 tubing. For studies of cowhage, a single cowhage spicule was held with forceps and inserted into the cheek for 5 s, and then removed. We wished to maintain the stimulus duration constant for spicule insertions, since otherwise animals could scratch out the spicule at variable times after insertion. Immediately after the i.d. injection or spicule insertion the animal was placed back into the arena and videotaped from below for 40 min. Generally 3–4 mice were injected and videotaped simultaneously. Immediately after commencing videotaping all investigators left the room. For studies of opioid modulation, either naltrexone (s.c.), morphine (i.p.) or vehicle were administered 10 min prior to i.d. injection. Videotapes were reviewed and numbers of scratch bouts and forelimb wipes counted and tabulated in 5-min intervals over the 40 min recording session by investigators blinded as to treatment. A scratch bout was defined as one or more rapid back-and-forth motion of the ipsilateral hindpaw directed toward the injected cheek, and ending with licking or biting of the toes and/or placement of the hindpaw on the floor. A wipe was defined as a singular motion of the ipsilateral, but not bilateral, forelimb beginning at the caudal extent of the injected cheek, and proceeding in a rostral direction. The inner aspect of the ankle and/or forelimb typically contacted the cheek with the paw closed. The numbers of scratch bouts and forelimb wipes were compared within and across treatments by analysis of variance (ANOVA) followed by post-hoc least significant difference (LSD) tests, and in some cases by either paired or unpaired t-tests (two-tailed) as appropriate, with a p-value of <0.05 considered to be significant. The durations of ten individual scratch bouts and wipes per animal were measured, as were within-bout scratch frequencies in slow-motion video playback.

RESULTS

Pruritogens

Fig. 1A shows the time-course of hindlimb scratch bouts and ipsilateral forelimb wipes elicited by i.d. injection of histamine in the cheek. Histamine elicited bouts of hindlimb scratching that began within the initial 5–10 min post-injection and persisted over a 20–30 min period with very few accompanying facial wipes. Vehicle (saline) elicited few hindlimb scratch bouts and somewhat more wipes (Fig. 1B). The number of vehicle-evoked scratch bouts was significantly less than the number of wipes ($p<0.05$), and was significantly lower ($p<0.05$) than the number of histamine-evoked scratch bouts. The number of histamine-evoked scratch bouts increased in a dose-related manner (Fig. 2A).

The PAR-2 agonist SLIGRL-NH2 (50 μg) elicited significantly more scratches than wipes over a time-course similar to that of histamine (Fig. 1C). The PAR-4 agonist AYPGKF-NH2, and 5-HT, both elicited significantly more scratches than wipes during the initial 10–20 min post-injection (Fig. 1D, E). Interestingly, i.d. facial injection of formalin, considered to be an algogen, elicited significantly more scratches than wipes in a biphasic temporal distribution (Fig. 1F). The number of PAR-2 agonist-evoked scratch bouts increased in a dose-related manner (Fig. 2B).

Individual scratch bouts had a mean (±SEM) duration of 0.4 ± 0.03 s (range 0.13–0.73 s) with a within-bout frequency of 12.6 ± 0.22 HZ, consistent with previous reports (17, 18). Individual wipes had a shorter mean duration of 0.19 ± 0.002 s that was quite consistent across animals (range 0.16–0.23 s).

μ-opioid modulation of histamine and PAR-2 agonist-evoked behaviors

The μ-opioid antagonist naltrexone (1 mg/kg, sc) almost completely abolished histamine-evoked scratch bouts (Fig. 2A, #) with no effect on wipes. In contrast, neither dosage of the μ-agonist morphine (0.3, 1 mg/kg, ip) had any significant effect on numbers of scratch bouts or wipes (Fig. 2A).

Similarly, naltrexone significantly reduced the number of scratch bouts but not wipes elicited by i.d. injection of the PAR-2 agonist SLIGRL-NH2 (Fig. 2B, #). Morphine did not significantly affect PAR-2 agonist-evoked scratch bouts, but at the highest dose (1 mg/kg) significantly reduced the number of wipes (Fig. 2B, ♦).

Algogens

Fig. 3A shows that capsaicin elicited significantly more ipsilateral forelimb wipes compared with hindlimb scratches ($p<0.05$). Maximal wiping occurred within the initial 5–10 min following the injection, with a second peak at 15–25 min, after which wiping ceased (Fig. 3A). Injection of vehicle (7% Tween 80) elicited very few scratch bouts or wipes, the latter being less ($p<0.05$) compared with capsaicin-evoked wipes (Fig. 3B). Facial injection of the algogens AITC (Fig. 3C) and bradykinin (Fig. 3D) also elicited a significantly greater number of wipes than scratch bouts. The higher capsaicin dose elicited numerically more wipes than the low dose, although the difference was not statistically significant (Fig. 3E).

μ-opioid modulation of capsaicin-evoked behaviors

The μ-opioid drugs had the opposite effect on capsaicin, as compared with pruritogen-evoked behavioral responses. Naltrexone (1 mg/kg, sc) had no effect on capsaicin (30 μg/10 μl)-evoked wipes, although it further reduced
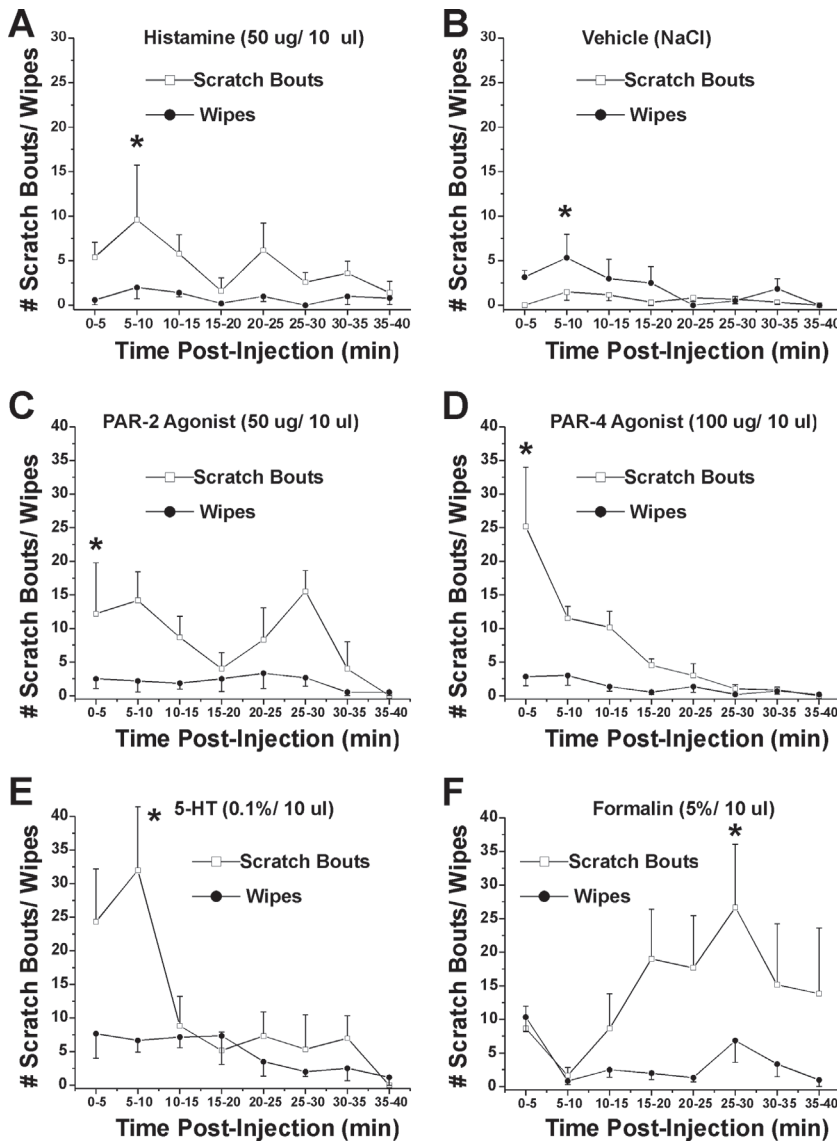
Fig. 1. Histamine and other pruritogens elicit more facial scratching than wiping. (A) Hindlimb scratch bouts and forelimb wipes vs. time after facial intradermal (id) injection of histamine. *Significant difference between number of scratch bouts vs. wipes ($p < 0.05$, paired *t*-test). Error bars: standard error of mean (SEM) ($n = 6$/group). (B) As in A for vehicle (saline) facial injection. (C) As in A for PAR-2 agonist SLIGRL-NH2. (D) As in A for PAR-4 agonist (AYPGKF-NH2). (E) As in A for 5-hydroxy-tryptamine (5-HT) facial injection. (F) As in A for formalin facial injection.

at 10–15 min post-insertion and then decreased, while a relatively constant number of wipes occurred throughout the 30 min period (Fig. 4A). There was no significant difference between the number of cowhage-evoked scratch bouts vs. wipes, although both were significantly greater compared with inactivated cowhage (Fig. 4A, B). The inactivated spicule loaded with histamine elicited significantly more scratch bouts and wipes compared with the inactivated cowhage spicule (Fig. 4C). The histamine-loaded spicule elicited significantly more scratch bouts than wipes over a time-course similar to that of the active cowhage spicule (Fig. 4A, C). The capsaicin-loaded cowhage spicule also evoked significantly more scratch bouts and wipes than the inactivated cowhage spicule (Fig. 4D). The capsaicin-loaded spicule elicited a significantly greater number of wipes than scratch bouts (Fig. 4D).

Summary of pruritogen- and algogen-evoked behaviors

Fig. 5 plots the mean numbers of scratch bouts and wipes elicited by different absolute doses (nmol) of injected pruritogens and algogens to allow direct comparisons. The pruritogens histamine, 5-HT, and agonists of PAR-2 and PAR-4 each evoked dose-related scratches (Fig. 5A) and few wipes (Fig. 5B), with the exception of 5-HT which elicited some wiping that, however, was significantly less compared with scratches ($p < 0.05$). In contrast, the algogens capsaicin, AITC and bradykinin each induced dose-related wiping (Fig. 5D) with much lower numbers of hindlimb scratch bouts (Fig. 5C).

DISCUSSION

Scratching and wiping reflect itch and pain, respectively

We confirm a previous report (6) that histamine elicited dose-dependent hindlimb scratches directed to the cheek, but few ipsilateral forelimb wipes, whereas capsaicin elicited dose-related wipes but very few scratches. We extend this finding by showing that other pruritogens, including PAR-2 and PAR-4 agonists and

the already-low number of capsaicin-evoked scratch bouts significantly (Fig. 3E, #). Morphine significantly attenuated the number of capsaicin-evoked wipes in a dose-dependent manner (Fig. 3E, ♦) with no significant effect on the number of scratch bouts.

Cowhage-evoked scratching and wiping behaviors

Cheek insertion of a single spicule of native cowhage elicited clear-cut scratch bouts and/or wipes in each mouse tested. In three cases cowhage elicited more scratch bouts than wipes, in one case an equal number, and in the remaining two cases more wipes than scratch bouts. Cowhage-evoked scratch bouts peaked
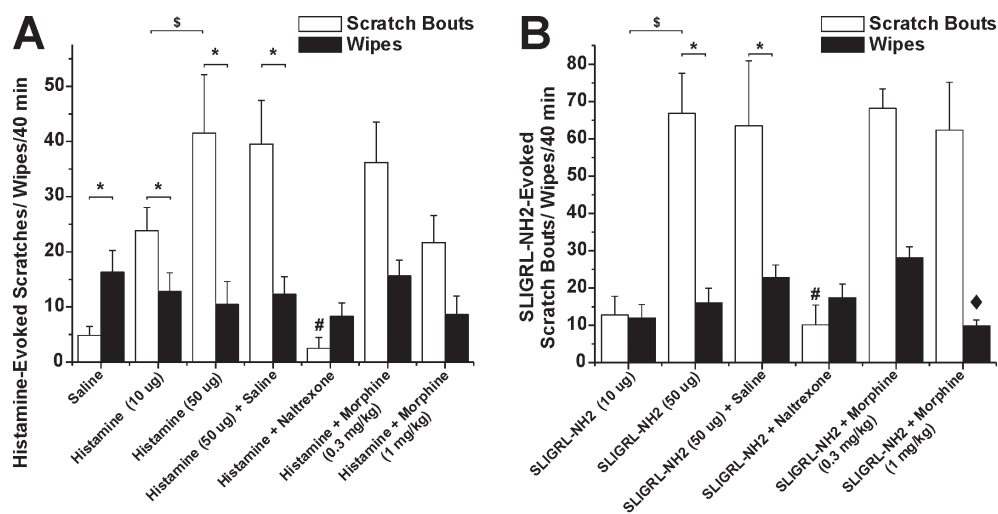
Fig. 2. Naltrexone inhibits pruritogen-evoked scratching, while morphine has no effect. (A) Histamine. Mean number of scratch bouts (□) and ipsilateral forelimb wipes (■). Saline elicited more wipes than scratches; histamine elicited more scratches than wipes (*$p < 0.05$ for all). $^\$$Significant difference in scratch bouts between 10 and 50 μg histamine ($p < 0.05$). #Naltrexone significantly ($p < 0.05$) reduced scratch bouts. Morphine had no significant effect. Error bars: standard error of mean (SEM) ($n = 6$/group). (B) SLIGRL-NH2 (format as in A). *Significantly more scratch bouts than wipes ($p < 0.05$). $^\$$Significant difference between 10 and 50 μg SLIGRL-NH2 ($p < 0.05$). #Naltrexone significantly reduced scratch bouts ($p < 0.05$ vs. SLIGRL 50 μg + saline) but not wipes. ♦Morphine significantly reduced wipes ($p < 0.05$ vs. saline) but not scratches. Error bars: SEM ($n = 6$/group).

5-HT, also elicited dose-dependent scratches and little wiping, consistent with previous studies showing that i.d. injection of these mediators in the nape of the neck elicited dose-related hindlimb scratching directed to the injection site (3, 19, 20). In contrast, the algogens AITC and bradykinin evoked dose-dependent wiping but little scratching, consistent with previous studies showing that these compounds elicit dose-related nocifensive behaviors (21, 22). It was interesting that capsaicin elicited a biphasic time-course of wiping (Fig. 3A), reminiscent of formalin-evoked nocifensive hindpaw behaviors. Curiously, however, cheek injection of formalin elicited significantly more scratches than wipes (Fig. 1F). Formalin injected in the nape of the neck was previously reported to elicit significant hindlimb scratching in rats (23) and mice (24), and formalin as well as pruritogens elicited enhanced scratching in mice lacking a population of inhibitory spinal interneurons (25), suggesting that formalin-evoked nocifensive behavior may reflect itch rather than, or in addition to, pain. In summary, the present results suggest that facial scratching and wiping behaviors differentially reflect itch and pain, respectively.

Opioid antagonists reduced experimentally-evoked itch in humans (7), and μ-opioid agonists are commonly known to induce or enhance itch while reducing pain. This was confirmed by the present data, in which the μ-opioid antagonist naltrexone inhibited histamine- and PAR-2 agonist-evoked scratching that presumably reflects itch, but not wiping that reflects pain (Fig. 2). Morphine did not significantly affect scratching elicited by histamine or the PAR-2 agonist. These findings confirm our previous reports that hindlimb scratching elicited by SLIGRL-NH2 or 5-HT injected in the nape

of the neck was significantly attenuated by naltrexone but was not significantly affected by morphine (3, 10). In contrast, morphine dose-dependently attenuated capsaicin-evoked wiping with no effect on scratching, whereas naloxone did not affect wiping but further reduced the already-low level of capsaicin-evoked scratching significantly (Fig. 3E). This suggests that capsaicin-evoked scratching reflects itch that accompanies a dominant, morphine-sensitive nociceptive sensation. This finding is consistent with human psychophysical observations that topical cutaneous application of capsaicin initially elicited itch in > 50% of subjects (26), and focal application of capsaicin elicited concomitant itch and nociceptive sensory qualities (15) as discussed below.

Presumably, hindlimb scratches directed to the face allow the claws effectively to remove an irritant stimulus on or just beneath the epidermal surface. It is not clear why the forepaws, which are used in facial grooming, are not used to scratch at a pruritic facial stimulus. Speculatively, such forepaw scratch motions may not generate sufficient force compared with the hindpaw. Scratch bouts consist of rapid (12 Hz) back-and-forth movements of the hindpaw claws lasting ~0.4 s; the number of bouts, but not bout duration or within-bout scratch frequency, varies with stimulus intensity (9). In contrast, noxious stimuli usually elicit withdrawal of the stimulated limb or sometimes rubbing of the injury site. Algogens injected in the cheek reliably elicited ipsilateral forelimb wiping, rather than hindlimb scratches. Wipes consisted of brief singular caudal-to-rostral movements of the inner forearm across the injected cheek, similar to a rubbing motion. The duration of individual wiping movements was about one-half that of individual scratch bouts. Despite these differences in
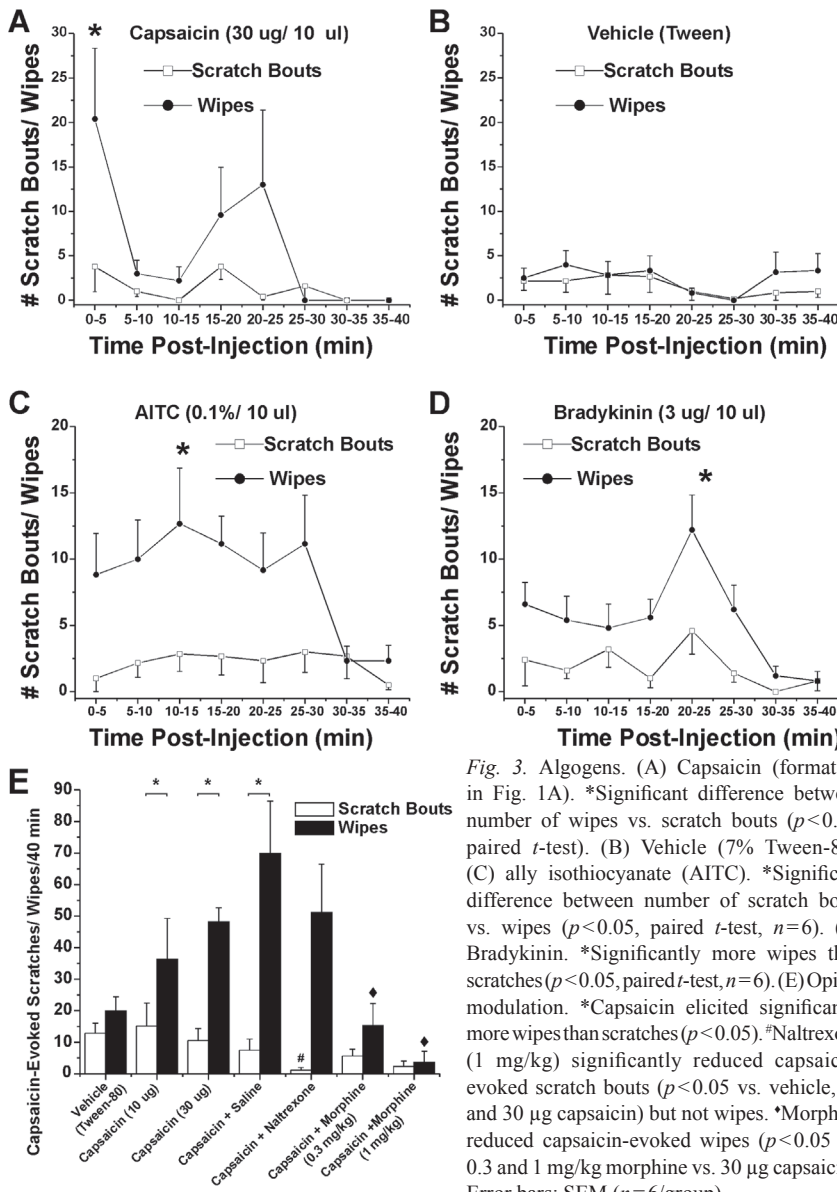
Fig. 3. Algogens. (A) Capsaicin (format as in Fig. 1A). *Significant difference between number of wipes vs. scratch bouts (*p*<0.05, paired *t*-test). (B) Vehicle (7% Tween-80). (C) ally isothiocyanate (AITC). *Significant difference between number of scratch bouts vs. wipes (*p*<0.05, paired *t*-test, *n*=6). (D) Bradykinin. *Significantly more wipes than scratches (*p*<0.05, paired *t*-test, *n*=6). (E) Opioid modulation. *Capsaicin elicited significantly more wipes than scratches (*p*<0.05). #Naltrexone (1 mg/kg) significantly reduced capsaicin-evoked scratch bouts (*p*<0.05 vs. vehicle, 10 and 30 μg capsaicin) but not wipes. ♦Morphine reduced capsaicin-evoked wipes (*p*<0.05 for 0.3 and 1 mg/kg morphine vs. 30 μg capsaicin). Error bars: SEM (*n*=6/group).

tive sensations was somewhat more prolonged for capsaicin- compared with histamine-loaded spicules (15). We showed that insertion of a single inactivated cowhage spicule loaded with histamine elicited significantly more scratches than wipes, whereas the capsaicin-loaded spicule elicited significantly more wipes than scratches (Fig. 4). This suggests that the mouse cheek model may provide a more objective behavioral measure of itch and pain sensations, compared with the subjective ratings of itch and nociceptive sensory qualities provided in human psychophysical studies. Moreover, the degree of scratching elicited by the histamine-loaded spicule (Fig. 4C) was comparable to that of histamine injected i.d. (Fig. 1A), supporting the efficacy of the delivery method by spicule.

Mucunain, the chemical constituent of cowhage spicules, was recently characterized and shown to evoke itch and nociceptive sensations via activation of PAR-2 and PAR-4 (27). Presently, cheek injection of PAR-2 and PAR-4 agonists evoked scratches but very few wipes, suggesting that these protease receptors primarily signal itch rather than pain.

Neural mechanisms

Peripherally, there are at least two distinct candidate populations of primary afferent fibers for signaling itch. Cowhage activates polymodal C-fiber nociceptors, some of which also respond more weakly to histamine and capsaicin (28, 29). Histamine activated a subset of mechanically insensitive C-fiber afferents, many of which also responded to capsaicin (29, 30) but not cowhage (29). Centrally, subpopulations of primate lumbar spinothalamic tract neurons responded to either cowhage or histamine, but rarely both, and all additionally responded to capsaicin (31). In cats, 50% of histamine-responsive lumbar lamina I spinothalamic tract neurons also responded to AITC (32). In mice, a large majority of pruritogen-responsive lumbar superficial dorsal horn neurons responded to both histamine and the PAR-2 agonist SLIGRL-NH2, as well as to capsaicin and AITC (33, 34). If it is assumed that pruritogen-responsive spinal dorsal horn neurons signal itch, then it is understandable how itch could be elicited by algogens such as capsaicin or AITC that also excite
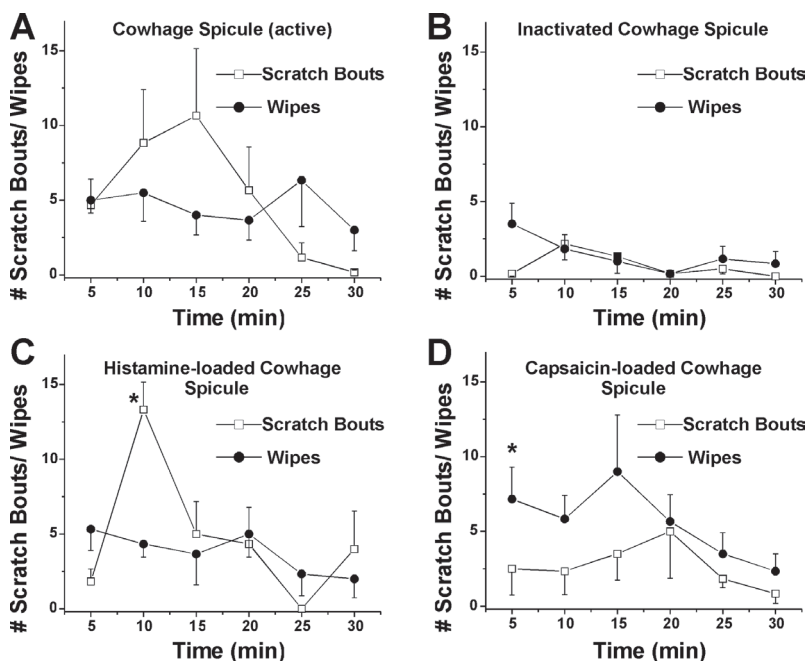
individual movement parameters, comparison of total counts of scratch bouts and wipes revealed significant differences in responses to pruritogens vs. algogens.

Cowhage

In humans, i.d. insertion of cowhage spicules elicited itch that was almost always accompanied by nociceptive sensations of pricking/stinging and/or burning (14, 15). Interestingly, insertion of a single native cowhage spicule into the mouse's cheek elicited both scratches and wipes that did not significantly differ in number, suggesting a mixed itch and nociceptive sensation consistent with the human data. In humans, insertion of inactivated cowhage spicules loaded with either capsaicin or histamine elicited mixed itch and nociceptive qualities similar to native cowhage (15). The magnitude and duration of itch was similar, while the duration of nocicep-

Fig. 4. Scratching and wiping elicited by a cowhage spicule. (A) Scratch bouts and wipes vs. time following intradermal cheek insertion of a single spicule of native cowhage (format as in Fig. 1). There was no significant difference between number of scratch bouts vs. wipes, both of which were significantly greater ($p < 0.05$) compared with inactivated cowhage spicule. (B) Inactivated cowhage spicule. (C) Histamine-loaded inactivated cowhage spicule elicited significantly more scratch bouts than wipes (*$p < 0.05$). (D) Capsaicin-loaded inactivated cowhage spicule elicited significantly more wipes than scratch bouts (*$p < 0.05$). Error bars: SEM ($n = 6$–9/group).
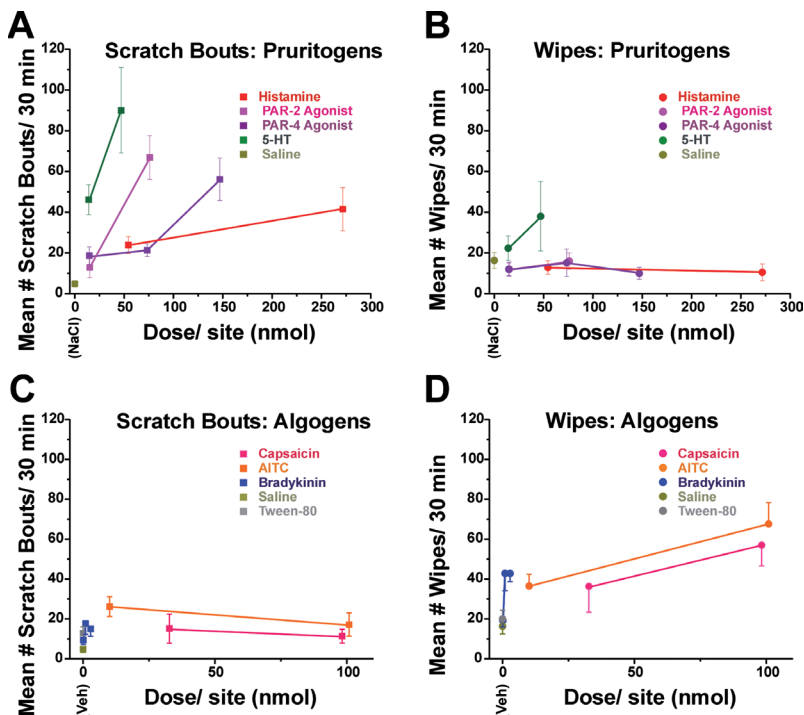

Fig. 5. Summary. (A) Pruritogen-evoked scratching. Mean number of scratch bouts vs. injected dose of histamine, PAR-2 agonist, PAR-4 agonist and 5-HT. Each mediator evoked significant ($p < 0.05$) dose-related increases in number of scratch bouts. (B) Pruritogen-evoked wiping. Each mediator elicited a low number of wipes that did not increase significantly with dose. (C) Algogen-evoked scratching. Capsaicin, ally isothiocyanate (AITC) and bradykinin elicited few scratch bouts that did not increase with dose. (D) Algogen-evoked wiping. AITC- and bradykinin-evoked wipes (but not capsaicin) increased significantly with dose ($p < 0.05$). Error bars: SEM ($n = 6$/group).

many pruriceptive neurons. Presumably, algogenic stimuli simultaneously excite a separate population of spinal neurons that do not respond to pruritogens, and that may selectively signal nociceptive sensory qualities. Finally, some spinal neurons responsive to both pruritogens and algogens may signal pain rather than itch, accounting for the ability of pruritogens to simultaneously elicit itch and nociceptive sensory qualities. It will be of future interest to determine whether there are separate populations of neurons in trigeminal subnucleus caudalis that respond selectively to pruritogens or algogens injected in the cheek, commensurate with the distinct scratching and wiping behaviors elicited by these respective classes of chemical stimuli.

ACKNOWLEDGMENTS

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