Title: Facial injections of pruritogens and algogens elicit distinct scratching and wiping responses and excite partly overlapping populations of primary and second-order trigeminal neurons in mice

Running title: Trigeminal signaling of itch vs. pain

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Intradermal (id) cheek injection of pruritogens or algogens differentially elicits hindlimb scratching or forelimb wiping, suggesting that these behaviors distinguish between itch and pain. We investigated if pruritogens and algogens excite separate or overlapping populations of primary afferent and second-order trigeminal neurons in mice. Calcium imaging of primary sensory trigeminal ganglion (TG) cells revealed that 15.4% responded to histamine, 5.8% to the protease-activated receptor (PAR)-2 agonist, 13.4% to allyl isothiocyanate (AITC) and 36.7% to capsaicin. AITC and/or capsaicin activated the vast majority of histamine- and PAR-2 agonist-sensitive TG cells. A chemical search strategy identified second-order neurons in trigeminal subnucleus caudalis (Vc) responsive to histamine, the PAR-2 agonist or AITC. A minority of histamine- or PAR-2 agonist-responsive Vc neurons responded to the other pruritogen, while a large majority of puritogen-responsive Vc neurons responded to capsaicin and/or AITC. A minority of AITC-responsive Vc neurons responded to pruritogens while most responded to capsaicin. These data indicate that most primary and higher-order trigeminal sensory neurons are activated by both pruritic and algesic stimuli although a minority exhibit selectivity. The results are discussed in terms of population codes for itch and pain that result in distinct behavioral responses of hindlimb scratching and forelimb wiping that are mediated at lumbar and cervical segmental levels, respectively.
Acute itch sensation elicits a desire to scratch, which serves a protective function to remove insects or irritant substances that have invaded the skin surface. Chronic itch is associated with a variety of systemic diseases and skin conditions, and also elicits scratching which, instead of protecting the skin, can cause inflammation through a vicious itch-scratch cycle (Carstens, 2009). Most types of chronic itch are resistant to antihistamine or other treatment modalities, and there is a general lack of understanding of basic itch mechanisms. Behavioral assessment of itch usually involves quantifying hindlimb scratch bouts directed to a site of pruritic stimulation, usually at the nape of the neck (Carstens & Kuraishi, 2004). However, this is the only response available and it is thus uncertain if such scratching is specific for itch or also reflects pain or other sensations. It was recently reported that intradermal (id) microinjection of histamine in the mouse cheek elicited bouts of hindlimb scratching directed to that site, whereas capsaicin elicited an ipsilateral forelimb wiping response but little or no scratching (Shimada & LaMotte, 2008). We recently confirmed and extended this (Akiyama et al., 2010) by showing that other pruritogens, including serotonin (5-HT) and agonists of the protease-activated receptor subtypes PAR-2 and PAR-4, elicit scratching and little wiping, while algogens including mustard oil (allyl isothiocyanate, or AITC) and bradykinin elicit mainly forelimb wipes but little hindlimb scratching. These findings are summarized in Fig. 1, which plots the number of hindlimb scratch bouts and ipsilateral forelimb wipes elicited by each agent. Interestingly, intradermal insertion of a spicule of cowhage (Mucuna pruriens) elicited approximately equal numbers of scratch bouts and wipes, consistent with recent reports that cowhage evokes concomitant sensations of itch and
burning or stinging (LaMotte et al., 2009; Sikand et al., 2009). The main aim of present
study was to begin to investigate possible neural circuits mediating these distinct
behavioral responses.

Recent evidence supports the existence of largely separate pathways for
histaminergic and nonhistaminergic itch. Whereas id injection of histamine elicits itch
and associated flare that is prevented by antihistamines, id insertion of cowhage spicules
elicits itch that is not accompanied by flare and is not reduced by antihistamines (Johanek
et al., 2007). Cowhage contains a substance called mucunain that elicits itch via
activation of protease-activated receptor subtypes PAR-2 and -4 (Reddy et al., 2008).
PAR-2 has been implicated in chronic itch of atopic dermatitis (Steinhoff et al., 2003),
and agonists of PAR-2 and -4 elicit scratching behavior (Shimada et al., 2006; Tsujii et
al., 2009; Akiyama et al., 2009c). Another receptor potentially involved in non-
histaminergic itch is mrgprA3 (Liu et al., 2010). This receptor is expressed in superficial
cutaneous sensory nerve endings and selectively binds chloroquine, an anti-malarial drug
that frequently induces itching. Chloroquine and histamine elicited scratching in
wildtype mice, whereas histamine, but not chloroquine, elicited scratching in mice
lacking mrgprA3 (Liu et al., 2010).

Histamine and cowhage activate largely separate populations of human primary
afferent C-fibers (Namer et al., 2008) and monkey spinothalamic tract neurons (Davidson
et al., 2007). However, most of these prurisponsive neurons additionally respond to the
algogen capsaicin, raising the issue of how the central nervous system discriminates
between itch and pain. The present study used calcium imaging and single-unit
electrophysiological recording methods was to investigate activation by pruritogens and
algogens of primary sensory trigeminal ganglion (TG) cells as well as second- or higher-order neurons in trigeminal subnucleus caudalis (Vc) which relays orofacial sensory information to the brain. We wished to determine if cheek microinjections of pruritogens or algogens, which elicit distinct behavioral responses, activate separate or overlapping populations of TG and Vc neurons.

MATERIALS AND METHODS

Behavior. Details of the cheek microinjections and video recording of scratching and wiping behavior are provided in our recent study (Akiyama et al., 2010a), from which much of the data in Fig. 1 was adapted. In the present study we additionally tested chloroquine. Briefly, following habituation to the test environment, ICR mice (n=6) received an id microinjection of chloroquine (100 µg in saline, 10 µl) in the cheek and were videotaped from below. Two reviewers blinded as to treatment reviewed the videotapes to count the numbers of ipsilateral forelimb wipes, and hindlimb scratch bouts, directed to the injection site at 5-min intervals over a 40-min period. Total numbers of wipes or scratch bouts/40 min were compared with saline controls using an unpaired t-test with p<0.05 considered to be statistically significant.

Calcium Imaging. A total of 12 adult male ICR mice (32-49 g) was used under a protocol approved by the UC Davis Institutional Animal Care and Use Committee. The animal was euthanized under sodium pentobarbital anesthesia and trigeminal ganglia were acutely dissected and 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 trituated using fire-polished glass pipettes. TG 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.

TG cells were incubated in Ringers solution (pH7.4; 140 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 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 microscope (Nikon TS100, Technical Instruments, San Francisco CA). 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 Simple PCI software (Compix Inc, Cranberry Township, PA) every 3 sec.

Solutions were delivered by a solenoid-controlled 8-channel perfusion system (ValveLink, AutoM8). Histamine (100 μM), the PAR-2 agonist SLIGRL-NH₂ (100 μM) and 5-HT (100 μM) were delivered in randomized order. The 100 μM concentration selected for each pruritogen was the same as used in prior studies (histamine: Han et al., 2006; Nicholson et al., 2007; Shim et al., 2007; PAR-2 agonist: Steinhoff et al., 2000; Amadesi et al., 2004; 5-HT: Nicholson et al., 2002). This was then followed by AITC (100 μM), capsaicin (1 μM) and lastly 144 mM potassium in the same order for all experiments. Stimulus duration was 30 sec (10 sec for capsaicin). Ratios were normalized to baseline. Cells were judged to be sensitive if the ratio value increased by
more than 10% of the resting level following chemical application. Only cells responsive to high-K+ were included for analysis. Between-group differences were compared by t-test and Fisher’s exact probability test.

Single-unit recordings from Vc. Experiments were performed using 55 adult male ICR mice (Harlan, Oxnard, CA) (32-50 g) under a protocol approved by the UC Davis Animal Care and Use Committee. The methods were similar to those reported in our recent study (Akiyama et al., 2009a) and are briefly summarized as follows. Anesthesia was induced with sodium pentobarbital (60 mg/kg ip) and maintained by intermittent supplemental injections to achieve a level of approximately 10-20 mg/kg/hr. The upper cervical (C1-C2) spinal cord and caudal medulla were exposed by laminectomy and a tungsten microelectrode (FHC, Bowdoin, ME) recorded extracellular single-unit activity. A chemical search strategy was used to isolate units in superficial laminae of Vc. Using a 30.5 gauge needle connected to a Hamilton microsyringe, a small volume (~0.25 µl, approx. 12 µg) of either histamine, SLIGRL-NH2 or AITC was microinjected in the cheek id and the recording electrode was driven into the ipsilateral medulla to isolate an action potential in the superficial medullary dorsal horn (<300 µm from surface) that exhibited ongoing activity. If no unit was isolated, the procedure was repeated on the opposite cheek. We then waited at least 10 min until firing decreased to a steady low level. Then, the same chemical used to isolate the unit was reinjected in a volume of 1 µl. Responsive units (i.e., >30% increase above baseline) were studied further. The chemically-evoked response was recorded for at least 20 min, after which a second 30.5 g needle containing a different chemical was inserted in the cheek id. The order of chemical injections was counterbalanced, with histamine (50 µg) first followed by
SLIGRL-NH₂ (50 µg), 5-HT (10 µg), vehicle, AITC (10 µg) and capsaicin (30 µg) (n=25 units), SLIGRL-NH₂, histamine, 5-HT, vehicle, AITC and capsaicin (15 units), or AITC, histamine, SLIGRL-NH₂, 5-HT, vehicle and capsaicin (18 units). The second chemical was then injected and unit activity recorded for another 20-30 min. After the first 3 chemicals, we recorded unit responses to light brushing with a cotton wisp and pinching using forceps. Units were classified as wide dynamic range (WDR) if they responded at higher firing rate to pinch than light touch, nociceptive specific (NS) if they responded to pinch but not light touch, or mechanically-insensitive if the responded to neither. Only one unit was studied per cheek in a given animal.

Action potentials were recorded to a computer and counted using Chart software (AD Instruments, Colorado Springs CO). Unit activity was usually quantified as number of action potentials/ min and displayed in peristimulus-time histogram (PSTH) format with 1 sec bins. Group responses at 1-min intervals following a given stimulus were compared with activity 1 min prior to the stimulus by paired t-test or repeated-measures ANOVA (SPSS 9.0, Chicago IL), with p< 0.05 set as significant.

At the end of the experiment an electrolytic lesion was made through the microelectrode. The medulla was fixed in 10% buffered formalin, and 50 µm sections were cut and mounted on slides for microscopic verification of the lesion site.

RESULTS

Behavior

Behavioral data are summarized in Fig. 1 which plots mean numbers of scratch bouts vs. forelimb wipes/ 40 min observation period. Chloroquine elicited significantly more scratch bouts (p<0.05), but not ipsilateral forelimb wipes, compared to vehicle
(saline) controls, comparable with the effects of histamine and the PAR-2 and -4 agonists.

In contrast, algogens bradykinin, capsaicin and AITC elicited significantly more wipes (p<0.05 for all), but not scratch bouts, compared to vehicles as previously reported (Akiyama et al, 2010a). 5-HT and formalin elicited more scratch bouts than wipes, while insertion of a cowhage spicule elicited equivalent numbers of scratch bouts and wipes.

*Calcium imaging of TG cells*

A total of 853 TG cells was imaged for responsiveness to histamine, SLIGRL-NH2, AITC and capsaicin. Examples of TG cell responses are shown in Fig. 2. The cell indicated by the red trace responded to SLIGRL-NH2, AITC and capsaicin but not histamine, while the cell indicated by the blue trace responded to both histamine and SLIGRL-NH2 but not to AITC or capsaicin.

Overall, 15.4% of TG cells responded to histamine, 5.8% to the PAR-2 agonist, 11% to 5-HT, 13.4% to AITC and 36.7% to capsaicin, when tested in that order. The percentages of TG cells activated by these chemicals were similar, regardless of whether histamine, the PAR-2 agonist, or 5-HT was tested first (Table 1), arguing against order effects.

The mean diameter of all 853 TG cells tested was 20.0 µm +/- 0.22 (SEM). There were no marked differences in mean diameter among cells responsive to histamine (18.3 +/- 0.4 µm; n=133), the PAR-2 agonist (15.7 µm +/- 0.5, n=46), 5-HT (15.3 µm +/- 0.35; n=81), capsaicin (18 µm +/- 0.25; n=315) and AITC (17.3 µm +/- 0.37; n=113). The mean diameter of TG cells unresponsive to any of the tested chemicals was 24.03 µm +/- 0.34 (n=384).
Fig. 3A shows that 9.6% of TG cells responded only to histamine, 1.9% only to the PAR-2 agonist, and 6.9% only AITC, while the remainder responded to two or all three agents (proportions indicated within overlap regions in Fig. 3A). Capsaicin activated 36.7% of all TG cells (Fig. 3B), and 40% of cells were excited by capsaicin and/or AITC (Fig. 3C). Of the capsaicin-sensitive TG cells, 29% also responded to histamine, 10.2% also responded to the PAR-2 agonist, and some responded to both, while 63% did not respond to either pruritogen (Fig. 3B). In addition, 20.5% of capsaicin-sensitive TG cells were also activated by 5-HT. The percentages of coincident responses were similar when capsaicin- and AITC-sensitive TG cell populations were combined (Fig. 3C). Overall, 3.7% of the TG cells only responded to histamine and 1% only responded to the PAR-2 agonist, with the remainder also responding to AITC and/or capsaicin (Fig. 3C).

Eleven percent of TG cells responded to 5-HT. 5-HT activated 23% of histamine-responsive TG cells and 36% of PAR-2 agonist-responsive TG cells (Table 2). Histamine, the PAR-2 agonist, AITC and capsaicin activated 32%, 19%, 32% and 72%, respectively, of 5-HT-sensitive TG cells.

**Vc Electrophysiology**

Data were obtained from a total of 58 Vc units identified by histamine (n=25), PAR-2 agonist (n=15) or AITC (n=18) search stimuli. Most units (88%) had a mechanosensitive receptive field on the cheek including the id injection site with 62% classified as WDR and 26% as nociceptive-specific (NS), while 12% were classified as mechanically-insensitive. It should be noted, however, that mechanical sensitivity was
always tested following application of chemical stimuli which may have altered neurons’
mechanically-evoked responses.

Unit recording sites were typically located superficially in the dorsal aspect of Vc
at or near the level of the pyramidal decussation in the caudal medulla. The mean depth
of recordings was 148.7 um +/- 110.4 (SE) from the medullary surface. Histologically
recovered lesion sites are shown in Figs. 4 and 5.

Histamine search. Twenty-five units isolated using the histamine search strategy
were studied. An example is shown in Fig. 4A. This superficially-located NS unit
responded to id histamine and pinch but not brush and was generally unresponsive to
other stimuli. Averaged responses of the 25 histamine-sensitive units are shown in Fig.
5A. The mean histamine-evoked response was significantly greater than the preceding
baseline out to 9 min post-injection. Of these, 4/14 tested also responded to id cheek
injection of the PAR-2 agonist, 8/11 to 5-HT, 8/17 to capsaicin, and 12/14 to AITC.
None of the 13 units responded to id saline (vehicle control). Four units were classified
as mechanically insensitive; each of these responded to capsaicin and/or AITC.
Histologically-recovered recording sites are compiled on a representative medullary
section, with most located mainly superficially in dorsal and dorsolateral Vc within the
region of cheek representation (Fig. 5A, inset).

PAR-2 agonist search. Fifteen units were isolated using SLIGRL-NH2 as a
search stimulus. Fig. 4B shows a typical example of a unit in superficial Vc that
responded to SLIGRL-NH2, histamine and 5-HT, as well as AITC and capsaicin. Fig.
5B shows averaged responses of the 15 units. The mean response to SLIGRL-NH2 was
significantly above preinjection baseline for 3 min. Of the units tested, 5/14 responded to
histamine, 7/13 to 5-HT, 9/14 to ATIC, and 12/14 to capsaicin. None of the 14 units tested responded to id saline or Tween-80 (vehicle controls). Recording sites in superficial Vc are shown in the inset to Fig. 5B.

**AITC search.** Eighteen units were isolated using an AITC search stimulus. An example is shown in Fig. 4C. This superficial Vc unit responded to id cheek injection of AITC, and also gave response to histamine and a multiphasic response to capsaicin. Averaged responses of the 18 units are shown in Fig. 5C. Of units tested, 7/17 responded to histamine, 2/15 to SLIGRL-NH2, 3/13 to 5-HT, and 12/14 to capsaicin. Units were unresponsive to vehicle (saline or Tween-80). Recordings sites are shown in the inset to Fig. 5C.

**Cross-interactions.** We recorded from a sufficient number of AITC-responsive neurons to investigate the effect of prior pruritogenic stimulation on neuronal responses to this algogen. The mean baseline-corrected response when AITC was tested following application of histamine, SLIGRL-NH2 and 5-HT (Figs. 5A, B; mean 184.5 impulses/min +/- 53.5 [SEM], n=17) was significantly greater (p=0.049, unpaired t-test) compared to the response to AITC when tested first (50.7 +/- 12.9; n=12). This suggests that one or a combination of the pruitogens sensitized neuronal responses to subsequent application of AITC.

**Comparison of TG and Vc cells.** Table 2 lists the percentages of histamine-, SLIGRL-NH2- and AITC-responsive TG and Vc cells that additionally responded to other agents. The Vc cells are sorted according to the chemical search stimulus (which activated 100% of the cells). In general, TG and Vc cells exhibited similar coincidences of activation by different agents, with no significant difference between TG and Vc cells.
The only exception was that histamine-responsive Vc neurons exhibited a significantly higher percentage of coactivation by 5-HT and AITC compared to histamine-responsive TG cells (Table 2, *: p<0.05, Fisher’s exact test). This suggests that histamine-sensitive Vc neurons may have received convergent input histamine-sensitive as well as 5-HT- and AITC-sensitive primary afferents.

DISCUSSION

The present study was predicated on the observation that id cheek injections of pruritogens or algogens elicit distinct behavioral responses, i.e., hindlimb scratches or forelimb wipes, respectively (Shimada & LaMotte, 2008). We recently extended this by showing that other pruritogens (PAR-2 and PAR-4 agonists; 5-HT; chloroquine) also elicit facial scratching but little wiping, while algogens (AITC, bradykinin) elicit mainly forelimb wiping with little hindlimb scratching (Akiyama et al., 2010a). The scratching elicited by cheek injection of histamine or the PAR-2 agonist SLIGRL-NH2 was significantly attenuated by naltrexone but not morphine, while capsaicin-evoked wiping was dose-dependently reduced by morphine but unaffected by naltrexone (Akiyama et al., 2010a), supporting the view that scratching and wiping reflect itch and pain, respectively.

The PAR-2 and -4 agonists and chloroquine elicited a significant increase in number of hindlimb scratch bouts, while the number of ipsilateral forelimb wipes was not different from vehicle controls (Fig. 1). This supports the argument that these agents are selective pruritogens. In contrast, capsaicin, AITC and bradykinin elicited significant wiping but did not elicit scratching above vehicle control levels (Fig. 1), supporting the argument that these agents are selective algogens. 5-HT elicited significant scratching, but also more forelimb wipes compared to vehicle controls. This suggests that 5-HT may
elicit pain in addition to itch, consistent with 5-HT being an inflammatory mediator (Hong & Abbott, 1994) as well as pruritogen. Formalin also elicited more scratching than wiping, suggesting the possibility that it may elicit itch as well as pain in rodents. Cowhage spicules elicited equivalent numbers of scratch bouts and wipes (Fig. 1), consistent with recent human psychophysical studies showing that id insertion of a single cowhage spicule simultaneously elicits itch and nociceptive (stinging, burning) sensations (LaMotte et al., 2009; Sikand et al., 2009).

### TG Cell Imaging

We presently observed that 15.6% of TG cells responded to histamine. This is similar to reported proportions of histamine-sensitive DRG cells (13.4% and 15.9%) from mice (Akiyama et al., 2010b; Han et al., 2006) and rats (15%; Nicholsen et al., 2002). Oh’s group has reported higher percentages of histamine-sensitive DRG cells (62.7%; Kim et al., 2004; Shim et al., 2007). Presently only a small percentage (5.8%) of TG cells responded to the PAR-2 agonist, similar to the small percentage (2.7%) of mouse DRG cells that were activated by SLIGRL-NH2 (Akiyama et al., 2010b). In contrast, much higher proportions (32-50%) of rat DRG cells responded to PAR-2 agonists (Steinhoff et al., 2000; Amadesi et al., 2004). Eleven percent of TG cells responded to 5-HT, a value intermediate between that of mouse (6.5%; Ohta et al., 2006) and rat (21%; Nicolson et al., 2002) DRG cells. The majority (64-70%) of histamine- and SLIGRL-NH2-sensitive TG cells also responded to capsaicin, similar to proportions of histamine-sensitive (40-66%) and PAR-2 agonist-sensitive (79.3%) DRG cells that also responded to capsaicin (Shim et al., 2007; Nicolsen et al., 2002; Amadesi et al., 2004; Akiyama et al., 2010b).
Overall, <5% of TG cells responded selectively to pruritogens while the vast majority additionally responded to capsaicin and/or AITC. This is consistent with human microneurographic studies showing that histamine-sensitive, mechanically-insensitive C-fiber afferents, as well as cowhage-sensitive C-fiber polymodal nociceptors, also responded to capsaicin (Schmelz et al., 1997; Namer et al., 2008), although capsaicin excited a much lower percentage of monkey C-fiber polymodal nociceptors (Johanek et al., 2008). About 1/3 of SLIGRL-NH2-sensitive TG cells also responded to histamine while 14% of histamine-sensitive TG cells responded to SLIGRL-NH2, indicating that the majority of TG cells are selective for one but not both pruritogens. This is consistent with a recent study showing that histamine but not cowhage preferentially excited a subpopulation of mechanically-insensitive C-fiber afferents, while cowhage but not histamine excited mechano-sensitive C-fiber polymodal nociceptors in humans (Namer et al., 2008). In monkeys, a higher percentage of C-fiber polymodal nociceptors responded to both cowhage and histamine although cowhage-evoked responses were considerably larger (Johanek et al., 2008).

Vc neurons. Our chemical search strategy maximized the chances of finding chemoresponsive neurons in superficial laminae of Vc. Only 28.6% of histamine- and 36% of SLIGRL-NH2-responsive Vc neurons also responded to the other pruritogen. This differs from superficial lumbar dorsal horn neurons identified using a similar hindpaw chemical search strategy; these neurons exhibited a much higher incidence of responsiveness (76-85%) to both pruritogens (Akiyama et al., 2009). The segregation of histamine- and PAR-2 agonist-responsive Vc neurons is consistent with lumbar primate spinothalamic tract neurons that have been shown to respond either to histamine or to
cowhage, but rarely both (Davidson et al., 2007). Many histamine- and SLIGRL-NH2-responsive Vc units also responded to 5-HT. Interestingly, Vc neurons identified by the histamine search stimulus exhibited a significantly greater incidence of responsiveness to 5-HT and AITC compared to histamine-sensitive TG cells (Table 2), suggesting that these Vc neurons receive convergent input from histamine-, 5-HT- and/or AITC-sensitive trigeminal afferents. The incidence of responses of SLIGRL-NH2- and AITC-sensitive Vc and TG cells to other agents did not differ (Table 2). This suggests that input from SLIGRL-NH2-sensitive primary afferents fully accounts for the chemical tuning of the SLIGRL-NH2-sensitive Vc neurons. The same logic applies to AITC-sensitive TG and Vc neurons.

Given that AITC enhances responses of nociceptive trigeminal neurons (Hu et al., 1992), it is possible that our use of an AITC search stimulus may have sensitized Vc neurons to increase the proportion that responded to subsequent chemical stimuli. Nevertheless, only a minority of Vc neurons identified by the AITC search stimulus responded to pruritogens, in proportions similar to TG cells (Table 2). This argues against the possibility that AITC markedly enhanced Vc neuronal sensitivity to pruritogens. On the other hand, the mean response of AITC-sensitive Vc neurons was smaller when AITC was tested first, compared to when it was tested following sequential application of pruritogens. This suggests that one or more of the pruritogens may have sensitized responses to AITC, consistent with previous reports of hyperalgesia induced by histamine, 5-HT (Hong & Abbott, 1994) and PAR-2 agonists (Vergnolle et al., 2001). Finally, several of the present Vc units were mechanically insensitive. The present Vc units were isolated using a chemical search strategy, disallowing assessment of their
mechanical responsiveness prior to chemical application. We thus cannot rule out the possibility that prior chemical stimuli might have attenuated or otherwise affected unit mechanosensitivity. It should be noted, however, that many histamine-responsive feline lamina I spinothalamic tract neurons were mechanically insensitive (Andrew & Craig, 2001), suggesting a role for such units in signaling itch.

Neural mechanisms of itch and pain. The present data indicate that largely overlapping populations of primary and second-order trigeminal sensory are activated by pruritogens vs. algogens, raising questions as to the central transmission of itch and pain. The gastrin releasing peptide receptor (GRPR) is important for the spinal transmission of itch but not pain (Sun & Chen, 2007). Neurotoxic ablation of GRPR-expressing neurons drastically attenuated pruritogen-evoked scratching with no effect on pain sensitivity (Sun et al., 2009), suggesting neurochemically distinct central pathways for itch vs. pain. Neurotoxic ablation of substance P NK-1 receptor-expressing neurons in the superficial dorsal horn also significantly attenuated pruritogen-evoked scratching in rats (Carstens et al., 2010). This treatment reduced hyperalgesia but had little effect on acute nocifensive behavior (Mantyh et al., 2007), suggesting that substance P may also function as a spinal itch-transmitting neuropeptide transmitter. Conceivably, itch may be signaled by spinal neurons co-expressing GRPR and NK-1 receptors.

We presently identified small subpopulations of TG and Vc sensory neurons that selectively responded to either pruritic or algesic stimuli, providing limited evidence for itch- and pain-selective pathways. However, the large majority of pruritogen-responsive TG and Vc neurons responded to algogens. This is partly consistent with previous studies of prurisponsive primary afferents (see above), and fully consistent with reports
that a majority of prurisponsive lumbar dorsal horn neurons also responds to capsaicin and/or AITC (Akiyama et al., 2009a,b; Davidson et al., 2007; Simone et al., 2004; Andrew & Craig, 2001; Jinks & Carstens, 2002). However, this raises the question as to how the nervous system discriminates between itch and pain. One possibility is that a small sub-population of TG and Vc neurons that responds selectively to pruritogens is sufficient to signal itch. Likewise, the subpopulation of TG and Vc neurons responsive to algogens but not pruritogens may signal pain. A second possibility involves a population code. Itch mediators activate pruritogen-selective TG and Vc neurons as (Fig. 6; white) as well as a larger population that responds to both pruritogens and algogens (Fig. 6; light gray), with activity in both neuronal populations postulated to signal itch. Noxious chemicals activate algogen-selective neurons (Fig. 6; dark gray) that are proposed to signal pain. It is possible that both mechanisms, i.e. selectivity and population coding, contribute to itch and pain signaling.

A third possibility is that the population of TG and Vc neurons that responds to both pruritogens and algogens (Fig. 6, light gray) signals both itch and pain simultaneously. This concept is supported by recent psychophysical evidence that id insertion of spicules of cowhage, or inactivated cowhage spicules loaded with histamine or capsaicin, all elicit similar complex sensations consisting of itch accompanied by nociceptive sensations of pricking-stinging and/or burning (LaMotte et al., 2009; Sikand et al., 2009). The degree to which itch or nociceptive sensation dominates may depend on the degree of activation of pruritogen- or algogen-selective neuronal populations in combination with the non-selective neurons. Fig. 6 presents a model for the differential behavioral effects of pruritogens and algogens. Pruritogens elicited hindlimb scratches
directed to the cheek injection site, implying that the trigeminal sensory stimulus
ultimately engages networks in the lumbosacral spinal cord that govern extensor and
flexor motoneurons driving the rhythmic back-and-forth hindlimb scratch movements. In
contrast, algogens elicited ipsilateral forelimb cheek-wipes, implying that the sensory
stimulus ultimately engages a neural circuit in the ipsilateral cervical enlargement that
governs motoneurons driving the forelimb motion. Future studies are needed to elucidate
the detailed neural circuitry that underlies the ability of pruritic and algesic trigeminal
stimulation to differentially elicit these distinct motor responses.
REFERENCES


Table 1. Percentages of TG cells responsive to each chemical as a function of which chemical was tested first (order). The order of testing was as follows:

- histamine, PAR-2 agonist, 5-HT, AITC, capsaicin (n=589);
- PAR-2 agonist, histamine, 5-HT, AITC, capsaicin (n=116);
- 5-HT, histamine, PAR-2 agonist, AITC, capsaicin (n=55).

<table>
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<tr>
<th>Order</th>
<th>Histamine</th>
<th>PAR-2 agonist</th>
<th>5-HT</th>
<th>AITC</th>
<th>Capsaicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine 1st</td>
<td>15.8</td>
<td>5.4</td>
<td>8.2</td>
<td>12.8</td>
<td>36.5</td>
</tr>
<tr>
<td>PAR-2 agonist 1st</td>
<td>11.2</td>
<td>2.6</td>
<td>19</td>
<td>12.9</td>
<td>36.2</td>
</tr>
<tr>
<td>5-HT 1st</td>
<td>18.2</td>
<td>9.1</td>
<td>12.7</td>
<td>21.8</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 2. Co-incident activation of TG and Vc cells by pruritogens and algogens. Left column indicates the chemical that excited the TG or Vc cells. For Vc cells, the chemical was used as a search stimulus to identify responsive units. The 2nd through 6th columns indicate the percentages of TG and Vc cells that were additionally excited by the given chemical. 100%: all cells responded to that chemical. *: significant difference between TG and Vc incidence (p<0.05, Fisher’s exact test).

<table>
<thead>
<tr>
<th>Excited by</th>
<th>% co-activated by:</th>
<th>Histamine</th>
<th>SLIGRL</th>
<th>5-HT</th>
<th>AITC</th>
<th>Capsaicin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TG</td>
<td>Vc</td>
<td>TG</td>
<td>Vc</td>
<td>TG</td>
<td>Vc</td>
</tr>
<tr>
<td>Histamine</td>
<td>100</td>
<td>100</td>
<td>13.8</td>
<td>28.6</td>
<td>23.3</td>
<td>72.7*</td>
</tr>
<tr>
<td>SLIGRL</td>
<td>35.6</td>
<td>35.7</td>
<td>100</td>
<td>100</td>
<td>35.6</td>
<td>40</td>
</tr>
<tr>
<td>AITC</td>
<td>34.2</td>
<td>41.2</td>
<td>21.2</td>
<td>13.3</td>
<td>26.9</td>
<td>23.1</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Fig. 1. Behavioral scratching and wiping elicited by cheek injection of pruritogens and algogens. Scatter plot shows mean numbers of scratch bouts and ipsilateral forelimb wipes (error bars: SEM) elicited by each pruritic and/or algesic agent shown. Each chemical was injected id in volume of 10 µl. Doses (in nmol) were: histamine 272, PAR-2 agonist 76; PAR-4 agonist 73.4, chloroquine 194; 5-HT 47; capsaicin 98.2; AITC 100.9; bradykinin 1.0. The formalin dose was 16.7 µmol. For cowhage, a single spicule was inserted id, left in for 5 sec, and then removed. Dashed line: equal numbers of wipes and scratch bouts. Data for histamine, PAR-2 and -4 agonists, 5-HT, capsaicin, AITC, bradykinin and cowhage modified from Akiyama et al., 2010.

Fig. 2. Examples of 3 TG cell responses to itch mediators. Graph plots 340/380 nm ratio as a function of time. Black bars indicate time of application of each indicated chemical.

Fig. 3. Venn diagrams showing coincident responses of TG cells to pruritogens and algogens. A: TG cells selected based on their response(s) to AITC, histamine and/or the PAR-2 agonist SLIGRL-NH2. Numbers within each sector indicate the percentage of cells that responded. B: as in A for cells responsive to capsaicin, histamine and/or SLIGRL-NH2. C: as in B except that cells responsive to capsaicin and AITC are pooled.

Fig. 4. Examples of Vc neurons identified by chemical search. A: Unit identified by histamine search. Shown are peristimulus-time histograms (PSTHs; bin width: 1 sec) of unit responses to presentation (at arrow) of each indicates stimulus. This responded to id
injection of histamine in the ipsilateral cheek, but not to any other stimulus except cheek
pinch. Inset at upper left shows recording site in superficial Vc on drawing of section
through caudal medulla/C2. Abbreviations: NPS, nucleus of solitary tract; PX, pyramidal
decussation; Vc, trigeminal subnucleus caudalis.

B: Unit identified using SLIGRL-NH2 search. This unit was located in superficial Vc
and responded to id injection of SLIGRL-NH2, histamine, 5-HT and AITC.

C: Unit identified by AITC search. This unit was located in superficial Vc and responded
to AITC and briefly to histamine and capsaicin.

Fig. 5. Averaged responses of Vc units isolated by chemical search strategy. A:

Histamine. Averaged peristimulus-time histograms (PSTHs; bin width: 1 sec) of 25
units isolated using histamine search strategy. Gray error bars: SEM. Average firing
following id histamine (left-hand PSTH) was significantly greater than prestimulus
baseline out to 9 min post-injection (p<0.05, paired t-test). PSTHs to right show
averaged responses of units that responded to indicated stimuli. Numbers in parentheses
indicate the number that responded (>30% increase) of all units tested. The mean
response of the 4 units to SLIGRL-NH2 1-min post-injection did not reach a statistically
significant difference compared to pre-injection baseline (p=0.052). The same was true
for the mean response of the 8 units to 5-HT (p=0.053) and the 8 units to brush (p=0.057).
The mean response to pinch was significantly different compared to pre-stimulus baseline
(p<0.05). The mean response to capsaicin was significantly different from baseline at the
first min post-injection (p<0.05), while the mean response to AITC was significantly
different out to the 6th min post-injection (p<0.05 for minutes 1-6). The mean firing rate
post-saline injection was not significantly different from pre-stimulus baseline at any
time (p>0.5). Inset to left shows histologically identified lesion sites (●) plotted on
representative drawing of caudal medulla at the level of the pyramidal decussation.

Abbreviations: Vc, trigeminal subnucleus caudalis; NTS, nucleus of the solitary tract; PX,
pyramidal decussation.

B: PAR-2 agonist SLIGRH-NH2 (format as in A). Mean response to SLIGRL-NH2 was
significantly greater than pre-stimulus baseline out to min 3 (p<0.05). The mean
response to histamine was significantly different for 1st min post-injection (p<0.05). The
mean response 1 min post-5-HT was not significantly different from baseline (p=0.07).
The mean brush-evoked response was significantly different from baseline (p<0.01). The
mean pinch-evoked response was significantly different from baseline (p<0.001). The
mean response to AITC was significantly different from baseline at min 2 (p<0.05) but
not 1 (p=0.054) post-injection. The mean response to capsaicin was significantly
different from baseline at the 1st min (p<0.01). Mean responses to saline and Tween-80
were not significantly different from baseline (p>0.4 for both). Inset: recording sites
(format and abbreviations as in A).

C: AITC (format as in A, B). Mean response to AITC significant at the 1st min post-
injection (p<0.05). The mean response to histamine was significantly different from
baseline out to the 11th min post-injection (p<0.05 for min 1-11). The mean responses of
the few units to SLIGRL-NH2 or 5-HT were not significantly different from baseline
(p>0.3 for both). The mean response to brush was significantly greater than baseline
(p<0.01), as was the mean response to pinch (p<0.05). The mean response to capsaicin
was also not significantly different from baseline (p=0.15). Neither mean responses to
saline nor Tween-80 were significantly different from baseline (p>0.5 for both). Inset to
left: recording sites (format and abbreviations as in A).

Fig. 6. Schematic diagram of partially overlapping itch- and pain-signaling pathways (see text for explanation).