

# Scratching Behavior and Fos Expression in Superficial Dorsal Horn Elicited by Protease-Activated Receptor Agonists and Other Itch Mediators in Mice

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Received February 10, 2009; accepted March 16, 2009

## ABSTRACT

Protease-activated receptor (PAR)-2 and PAR-4 are implicated in nonhistaminergic itch. We investigated dose dependence, tachyphylaxis, and cross-tachyphylaxis of itch-associated scratching elicited by intradermal injections of PAR-2 and PAR-4 agonists, serotonin (5-hydroxytryptamine, 5-HT), and histamine in ICR mice, as well as  $\mu$ -opioid modulation of PAR-2 agonist-evoked scratching. Each agent elicited dose-related increases in scratch bouts. Scratching elicited by the PAR-4 agonist and histamine both exhibited significant tachyphylaxis but no cross-tachyphylaxis with each other. Scratching evoked by 5-HT did not exhibit significant tachyphylaxis but did exhibit significant cross-tachyphylaxis to scratching evoked by the

PAR-2 and PAR-4 agonists and histamine. Naltrexone and high-dose morphine (10 mg/kg) attenuated PAR-2 agonist-evoked scratching, whereas lower dose morphine (1 mg/kg) had no effect. High-dose morphine also significantly increased circling behavior, which may have interfered with scratching. The PAR-2 agonist and 5-HT produced overlapping distributions of Fos-like immunoreactivity in the superficial dorsal horn. These results indicate that PAR-2 and PAR-4 agonists, histamine, and 5-HT elicit itch-related scratching and activate superficial dorsal horn neurons that may participate in scratch reflex and ascending itch signaling pathways.

Chronic itch occurs in a variety of dermatologic conditions and systemic diseases and is often resistant to antihistamine treatment (Twycross et al., 2003; Ikoma et al., 2006). The protease-activated receptor (PAR)-2 has recently been implicated in pain and inflammation (Cottrell et al., 2003) and itch (Steinhoff et al., 2003). Intradermal injection of PAR-2 agonists elicits dose-related scratching in mice (Shimada et al., 2006; Ui et al., 2006; Tsujii et al., 2008), and PAR-2 may represent a nonhistaminergic transduction mechanism for itch. Pods from the bean plant *Mucuna pruriens* (cowhage) have spicules or trichomes that contain the protease mucunain, which elicits itch with no accompanying flare when inserted into the epidermis (Johanek et al., 2007). Mucunain was recently characterized and shown to evoke itch via PAR-2 and PAR-4 (Reddy et al., 2008) and intradermal injections of PAR-1, PAR-2, and PAR-4 agonists elicit scratching in ICR mice (Tsujii et al., 2008). Cowhage excites C- and A-fiber mechanosensitive nociceptors (Johanek et al., 2008;

Namer et al., 2008; Schepers et al., 2008). In contrast, intradermal histamine induces a local flare and itch sensation by exciting a different population of mechanically insensitive C-fiber afferents (Schmelz et al., 1997), as well as mechanosensitive C-fiber nociceptors to varying degrees (Handwerker et al., 1991; Johanek et al., 2008). Histamine also elicits dose-related scratching behavior in ICR mice and variable (or no) scratching in other mouse strains (Kuraishi et al., 1995; Inagaki et al., 2001; Green et al., 2006; Shimada and LaMotte, 2008). Histamine excites lamina I spinothalamic tract neurons in cats over a time course consistent with itch sensation (Andrew and Craig, 2001). It is noteworthy that histamine and cowhage were recently shown to excite largely separate populations of primate spinothalamic tract neurons (Davidson et al., 2007), suggesting that there may be distinct pathways for transmission of histaminergic and nonhistaminergic itch.

Serotonin (5-hydroxytryptamine, 5-HT) is another inflammatory mediator that elicits weak itch sensation in humans (Fjellner and Hägermark, 1979; Schmelz et al., 2003) but robust dose-dependent scratching behavior in rats (Thomsen et al., 2001; Jinks and Carstens, 2002; Nojima and Carstens, 2003; Nojima et al., 2003) and mice (Yamaguchi et al., 1999; Inagaki et al., 2001; Cuellar et al., 2003). 5-HT excites su-

This work was supported by the National Institutes of Health Institute of Dental and Craniofacial Research [Grant DE013685]; and by the National Institutes of Health National Institute of Arthritis and Musculoskeletal and Skin Diseases [Grant AR057194].

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.  
doi:10.1124/jpet.109.152256.

**ABBREVIATIONS:** PAR, protease-activated receptor 2; 5-HT, 5-hydroxytryptamine; ANOVA, analysis of variance; FLI, Fos-like immunoreactivity.

perforial dorsal horn neurons in the rat over a prolonged time course consistent with 5-HT-evoked scratching behavior (Jinks and Carstens, 2002), suggesting a role in itch. In the present study, we wanted to directly compare the scratch-evoking capacity of 5-HT, histamine, and PAR-2 and PAR-4 agonists and to investigate whether they exhibit tachyphylaxis and cross-tachyphylaxis. Because PAR-2 represents a particularly attractive target for development of nonhistaminergic antipruritics, we wanted to further investigate whether scratching elicited by the PAR-2 agonist SLIGRL-NH<sub>2</sub> is affected by opioids in a manner consistent with itch (i.e., reduced by  $\mu$ -opioid antagonists but not agonists) (Nojima et al., 2003). Finally, we used the method of Fos immunohistochemistry to investigate whether the PAR-2 agonist and 5-HT activate overlapping populations of neurons in the mouse superficial dorsal horn consistent with the proposed role for such neurons in signaling itch (Andrew and Craig, 2001; Jinks and Carstens, 2002).

## Materials and Methods

Experiments were conducted using adult male ICR mice (Harlan, Oxnard, CA) under a protocol approved by the University of California Davis Animal Care and Use Committee.

**Chemicals.** Drugs used were PAR-2 agonist SLIGRL-NH<sub>2</sub> (Quality Controlled Biochemicals, Hopkinton, MA, and GenScript, Piscataway, NJ; Shimada et al., 2006), PAR-4 agonist AYPGKF-NH<sub>2</sub> (GenScript) (Tsuji et al., 2008), 5-HT HCl (Sigma-Aldrich, St. Louis, MO), histamine (Sigma-Aldrich), capsaicin (Sigma-Aldrich), morphine sulfate (Sigma-Aldrich), and naltrexone (DuPont, Garden City, NJ). All chemicals were dissolved in sterile isotonic saline except capsaicin, which was dissolved in 7% Tween 80.

TABLE 1

Experimental groups to investigate dose dependency, tachyphylaxis, and cross-tachyphylaxis, and opioid modulation of scratching behavior. Column denoted "first" indicates which drug was applied first, and column denoted "second" indicates which agent (if any) was applied next in the same session. For groups 7 to 22, after the first agent was injected, mice were videotaped for 40 min, at which time a second injection of the same or a different mediator was made at the same site and mice were videotaped for another 40 min. For groups 23 to 29, the first agent was administered systemically (i.e., intraperitoneally or subcutaneously) 10 min before intradermal injection of the second agent. All treatments are intradermal unless otherwise specified.

Group	First	Dose/Route	Second	Dose/Route
1	None (spontaneous)		None	
2	Vehicle (saline)	10 $\mu$ l	None	
3a, b, c	SLIGRL-NH <sub>2</sub>	35, 50, or 100 $\mu$ g/10 $\mu$ l	None	
4a, b, c	AYPGKF-NH <sub>2</sub>	35, 50, or 100 $\mu$ g/10 $\mu$ l	None	
5a, b, c	5-HT	14, 47, and 141 nmol/10 $\mu$ l	None	
6	Histamine	35, 50, 100 $\mu$ g/10 $\mu$ l	None	
7	SLIGRL-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l	SLIGRL-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l
8	SLIGRL-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l	AYPGKF-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l
9	SLIGRL-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l	Histamine	50 $\mu$ g/10 $\mu$ l
10	SLIGRL-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l	5-HT	47 nmol/10 $\mu$ l
11	AYPGKF-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l	AYPGKF-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l
12	AYPGKF-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l	SLIGRL-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l
13	AYPGKF-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l	Histamine	50 $\mu$ g/10 $\mu$ l
14	AYPGKF-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l	5-HT	47 nmol/10 $\mu$ l
15	Histamine	50 $\mu$ g/10 $\mu$ l	Histamine	50 $\mu$ g/10 $\mu$ l
16	Histamine	50 $\mu$ g/10 $\mu$ l	SLIGRL-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l
17	Histamine	50 $\mu$ g/10 $\mu$ l	AYPGKF-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l
18	Histamine	50 $\mu$ g/10 $\mu$ l	5-HT	47 nmol/10 $\mu$ l
19	5-HT	47 nmol/10 $\mu$ l	5-HT	47 nmol/10 $\mu$ l
20	5-HT	47 nmol/10 $\mu$ l	SLIGRL-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l
21	5-HT	47 nmol/10 $\mu$ l	AYPGKF-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l
22	5-HT	47 nmol/10 $\mu$ l	Histamine	50 $\mu$ g/10 $\mu$ l
23	Saline	0.1 ml/kg i.p.	SLIGRL-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l
24	Naltrexone	1 mg/kg s.c.	SLIGRL-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l
25	Morphine	1 mg/kg i.p.	SLIGRL-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l
26	Morphine	3 mg/kg i.p.	SLIGRL-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l
27	Morphine	10 mg/kg i.p.	SLIGRL-NH <sub>2</sub>	50 $\mu$ g/10 $\mu$ l
28	Saline	0.1 ml/kg i.p.	Capsaicin	10 $\mu$ g/10 $\mu$ l
29	Naltrexone	1 mg/kg s.c.	Capsaicin	10 $\mu$ g/10 $\mu$ l

i.d., intradermal.

**Behavioral Scratching Studies.** Scratching experiments followed procedures described in our previous report (Cuellar et al., 2003). In brief, the fur on the rostral back was shaved, and mice were habituated to the Plexiglas recording arena 1 week before testing. Microinjections were made intradermally in the nape of the neck using a 30-gauge needle attached to a Hamilton microsyringe (Hamilton Co., Reno, NV) by polyethylene-50 tubing. The injection site was marked so a second injection could be made at the same location in experiments testing for tachyphylaxis and cross-tachyphylaxis (Table 1, groups 7–22). Immediately after the injection, the mouse was placed into the arena and videotaped from above for 40 to 60 min. In general, three to four mice were injected and videotaped simultaneously. Immediately after commencing videotaping, all investigators left the room.

The various treatment conditions are listed in Table 1. Groups 1 to 6 were studies of dose-related scratching behavior elicited by each pruritogen (5–8 mice/group). For studies of tachyphylaxis and cross-tachyphylaxis, a 4  $\times$  4 design was used to test the effects of an injection of each mediator followed by subsequent injection of that same mediator or one of the other three mediators, 40 min after the first injection. The 16 combinations are represented in groups 7 to 22 in Table 1. For studies of opioid modulation of scratching (Table 1, groups 23–29), either vehicle (saline; groups 23 and 28), naltrexone (groups 24 and 29), or morphine at three different doses (groups 25–27) was administered systemically 10 min before intradermal injection of either SLIGRL-NH<sub>2</sub> (groups 23–27) or capsaicin (groups 28 and 29). For some studies (e.g., dose-related scratching elicited by a given agent), the same cohort of mice (6–8/group) was used, with a minimum of 4 days between successive test sessions to avoid any carryover effects.

Videotapes were reviewed by investigators blinded as to treatment, and the number of scratch bouts was recorded at 5-min intervals. A scratch bout was defined as one or more rapid back-and-forth

hind paw motions directed toward and contacting the injection site and ending with licking or biting of the toes and/or placement the hind paw on the floor. Hind paw movements directed away from the injection site (e.g., ear-scratching) and grooming movements were not counted. Dose-related scratching for each agent (including the vehicle trial) was analyzed by analysis of variance, with dose as the main effect. For studies of tachyphylaxis, a paired *t* test was used to compare the total number of scratch bouts/40 min elicited by the first versus the second injection. For studies of cross-tachyphylaxis, scratch counts over the four sessions in which animals received a given mediator first were compared with scratch counts evoked by the same mediator when it was applied second using *t* tests. ANOVA was used to compare the total number of scratch bouts across morphine and naltrexone treatment groups. Animals receiving morphine (Table 1, groups 25–27) exhibited rotational (circling) behavior so we additionally counted the total number of 360° rotations over the 45-min period. ANOVA followed by post hoc least significant difference test was used to compare numbers of rotations across morphine concentrations. In all cases,  $p < 0.05$  was considered to be significant.

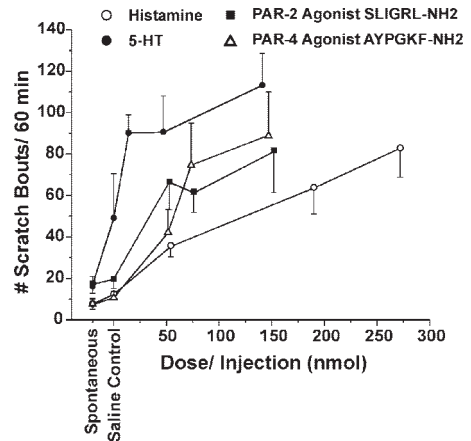
**Fos Immunohistochemistry.** These experiments used mice that had completed behavioral testing. They were anesthetized with sodium pentobarbital (80 mg/kg i.p.) and received an intradermal microinjection of SLIGRL-NH<sub>2</sub> (50 μg/5 μl), 5-HT (47 nmol/5 μl), or saline in the nape of the neck as in the behavioral experiments. After 2 h, the mice were perfused transcardially with phosphate-buffered saline followed by 4% paraformaldehyde as described previously (Merrill et al., 2006). The cervical spinal cord was postfixed, transferred to 30% sucrose, and cut in 50-μm sections that were collected in 24-well containers. The sections were processed for Fos immunohistochemistry as described previously (Merrill et al., 2006). In brief, every third section (150-μm intervals) was blocked in goat serum (3%) and then incubated in primary c-fos antibody (1:50,000) for 2 days. Sections were subsequently exposed to a secondary biotinylated (goat anti-rabbit) antibody and then subjected to an avidin-biotin-peroxidase complex reaction enhanced with biotinyl tyramide/H<sub>2</sub>O<sub>2</sub>. The reaction product was visualized as black through a nickel-enhanced diaminobenzidine reaction. Finally, sections were collected onto glass microscope slides, covered with a coverslip, and examined under the light microscope by an investigator blinded as to treatment. The number of cell nuclei in superficial laminae of the cervical dorsal horn exhibiting Fos-like immunoreactivity (FLI) was counted in five representative sections from each mouse. Between-treatment counts of FLI were analyzed by ANOVA.

## Results

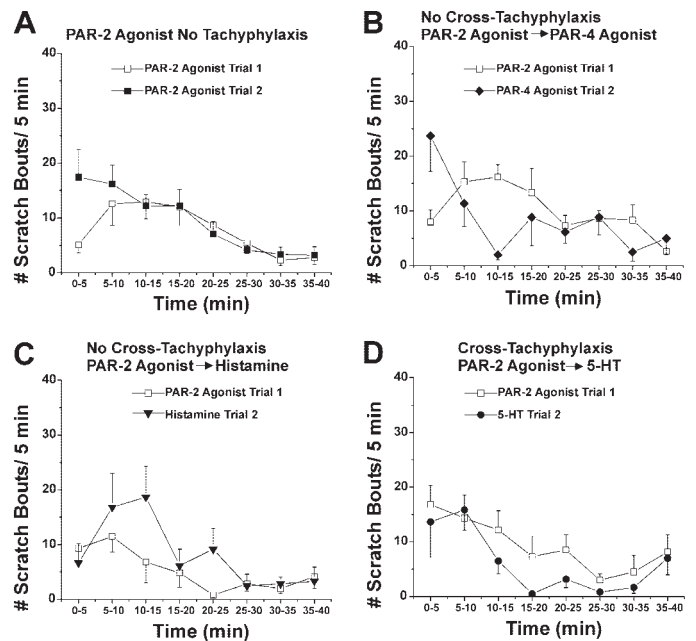
**Dose-Dependent Scratching.** Intradermal injection of 5-HT, histamine, and agonists of PAR-2 and PAR-4 each evoked dose-related scratching, as summarized in Fig. 1 for the absolute doses in nanomoles. For each agent, there was a significant overall effect of dose (ANOVA;  $p < 0.05$ ).

The time course of scratching elicited by the initial intradermal injection is shown in Figs. 2 to 5 for the PAR-2 agonist SLIGRL-NH<sub>2</sub>, the PAR-4 agonist AYPGKF-NH<sub>2</sub>, histamine, and 5-HT, respectively (open symbols). In each case, maximal scratching occurred within the initial 5 to 10 min after the injection and decreased within 20 to 30 min.

**Tachyphylaxis.** After the initial intradermal injection of a given agent, it was reinjected at the identical site 40 min later to test for tachyphylaxis. Figure 2A shows that the first and second injections of SLIGRL-NH<sub>2</sub> (50 μg/10 μl) elicited an equivalent amount of scratching over a similar time course. The summary data in Fig. 6A show that the total number of scratch bouts/40 min did not differ significantly



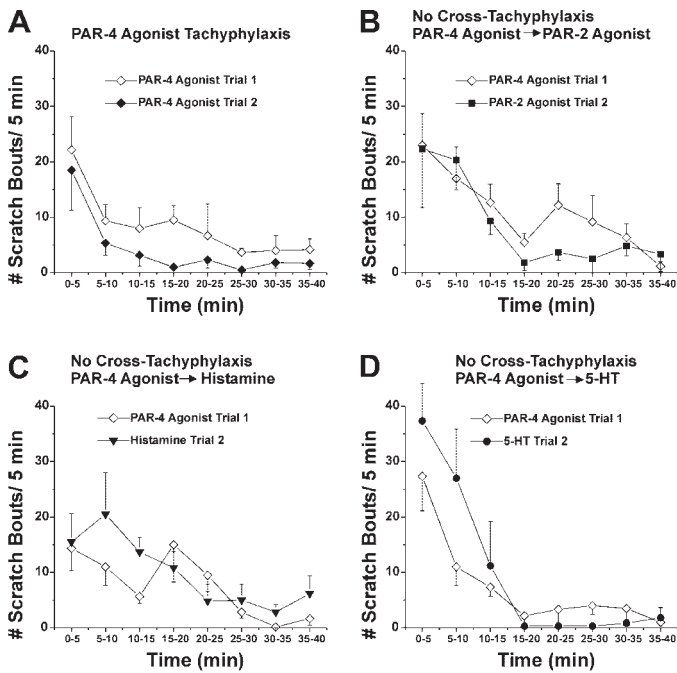
**Fig. 1.** Dose-dependent scratching elicited by various itch mediators. Graph plots mean number of scratch bouts/60 min versus dose of PAR-2 agonist SLIGRL-NH<sub>2</sub>, PAR-4 agonist AYPGKF-NH<sub>2</sub>, 5-HT, and histamine. Total injected dose calculated as nanomoles in a 10-μl volume ( $n = 6–8$ /group). There was a significant overall effect of dose for each agent (ANOVA;  $p < 0.05$ ). Error bars are S.E.M.



**Fig. 2.** Time course, tachyphylaxis, and cross-tachyphylaxis for hind limb scratching elicited by intradermal microinjection of PAR-2 agonist. In each panel, the PAR-2 agonist was injected first, and scratch bouts (□) were counted over 40 min, followed by injection of a second agent (filled symbols), and scratch bouts were counted again over 40 min. A, graph plots mean number of scratch bouts/5-min intervals after first injection of PAR-2 agonist SLIGRL-NH<sub>2</sub> (□; trial 1; 50 μg/10 μl) and after second injection of the same dose of SLIGRL-NH<sub>2</sub> at the same site (■; trial 2). B, as in A for PAR-2 agonist followed by PAR-4 agonist AYPGKF-NH<sub>2</sub> (50 μg/10 μl). C, as in A for PAR-2 agonist followed by 5-HT (47 mM/10 μl). D, as in A for PAR-2 agonist followed by histamine (50 μg/10 μl). Error bars are S.E.M. ( $n = 5–8$ /group).

between the first and second injections (Fig. 6A, ■ versus □), indicating lack of tachyphylaxis.

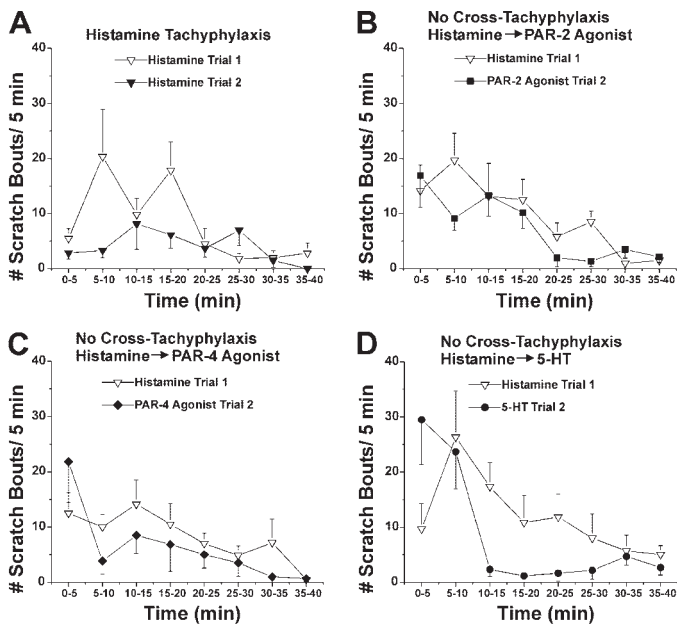
A second injection of the PAR-4 agonist AYPGKF-NH<sub>2</sub> elicited less scratching compared with the first (Fig. 3A), with the total scratch count being significantly less after the second injection compared with the first injection (Fig. 6B, ■ versus □). Histamine-evoked scratching also exhibited tachyphylaxis, with the second injection evoking a signifi-



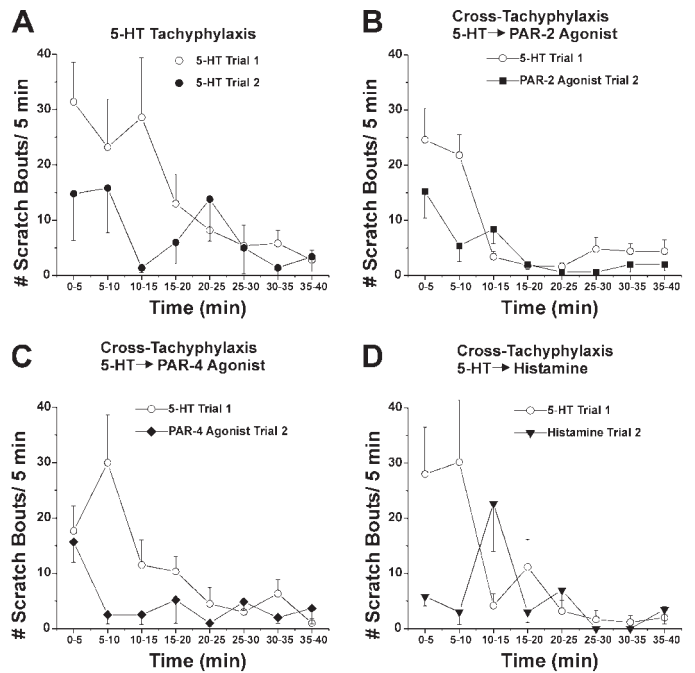
**Fig. 3.** PAR-4 agonist. Graphs as in Fig. 2 for experiments in which the PAR-4 agonist AYPGKF-NH<sub>2</sub> was injected first, followed 40 min later by injection of either the PAR-4 agonist again (A), the PAR-2 agonist (B), histamine (C), or 5-HT (D).

cantly lower total scratch count compared with the first injection (Figs. 4A and 6C).

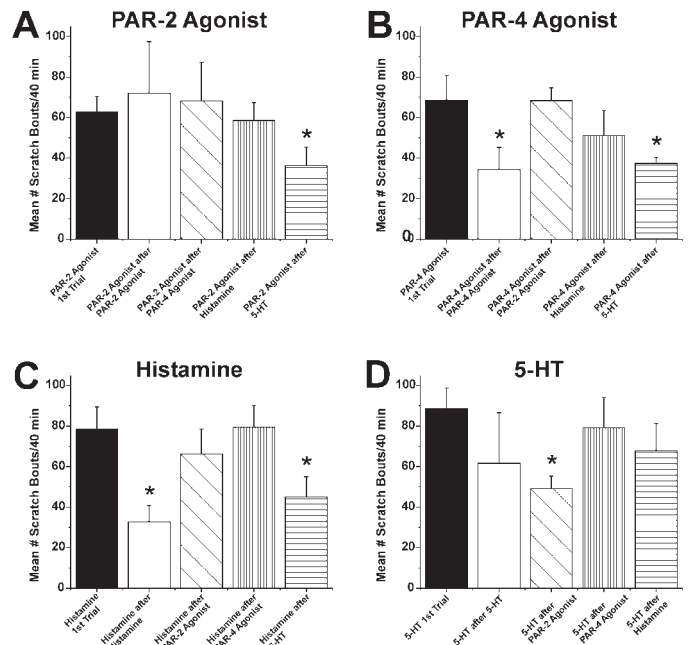
For 5-HT, the second injection elicited significantly ( $p < 0.01$ ) less scratching compared with the first (Fig. 5A), although this value did not differ significantly compared with the mean number of 5-HT-evoked scratch bouts averaged across all four experiments in which 5-HT was tested first (Fig. 6D, ■ versus □).



**Fig. 4.** Histamine. Graphs as in Fig. 2 for experiments in which histamine was injected first, followed 40 min later by injection of either histamine again (A), the PAR-2 agonist (B), the PAR-4 agonist (C), or 5-HT (D).



**Fig. 5.** 5-HT. Graphs as in Figs. 2 to 4 for experiments in which 5-HT was injected first, followed 40 min later by injection of either 5-HT again (A), the PAR-2 agonist (B), the PAR-4 agonist (C), or histamine (D).



**Fig. 6.** Summary of tachyphylaxis and cross-tachyphylaxis results. A, bar graph plots mean number of scratch bouts/40 min elicited by intradermal microinjection of the PAR-2 agonist when it was given first (left-hand bar) and when it was injected 40 min after a prior injection of the same PAR-2 agonist, the PAR-4 agonist, histamine, or 5-HT (second-fifth bars to right). \*,  $p < 0.05$ , significantly different from first trial of PAR-2 agonist. B, as in A for PAR-4 agonist. \*,  $p < 0.05$ , significantly different from first trial of PAR-4 agonist. C, as in A for scratch bouts elicited by histamine. \*,  $p < 0.05$ , significantly different from first trial of histamine. D, as in A for 5-HT. \*,  $p < 0.05$ , significantly different from first trial of 5-HT.

**Cross-Tachyphylaxis.** We also investigated the effect of each mediator to reduce (cross-tachyphylaxis) or otherwise affect scratching elicited by subsequent application of a different mediator. The PAR-2 agonist SLIGRL-NH<sub>2</sub> did not



significantly affect scratching elicited by subsequent injection of the PAR-4 agonist AYPGKF-NH<sub>2</sub> (Figs. 2B and 6B, ▨) or histamine (Figs. 2C and 6C, ▨) but resulted in a reduction in scratching elicited by subsequent injection of 5-HT (Figs. 2D and 6D, ▨). Although the PAR-4 agonist AYPGKF-NH<sub>2</sub> exhibited tachyphylaxis, it did not exhibit cross-tachyphylaxis to any other mediator (Figs. 3, B–D; 6A, ▨; and C and D, ▨).

Histamine also exhibited tachyphylaxis but no cross-tachyphylaxis to the other mediators (Figs. 4, B–D; and 6, A and B, ▨; and D, ▨).

5-HT exhibited significant cross-tachyphylaxis to each of the other mediators (Figs. 5, B–D; and 6, A–C, ▨).

**μ-Opioid Modulation of Scratching.** The μ-agonist morphine (1 mg/kg) had no effect on scratching elicited by intradermal injection of the PAR-2 agonist SLIGRL-NH<sub>2</sub> (Fig. 7A, ▨ versus ▨), whereas naltrexone significantly attenuated scratching (Fig. 7A, □). Figure 7B plots mean scratching versus dose of morphine, which significantly reduced scratching only at the highest dose (10 mg/kg). In addition, we tested capsaicin, which was recently reported to elicit hind limb scratching in ICR mice (Shimada and LaMotte, 2008), although not in ddY mice (Kuraishi et al., 1995). The present data confirm that intradermal capsaicin elicits scratching in ICR mice (Fig. 7A, ▨) comparable with scratching elicited by pruritogens (Fig. 1). Naltrexone did not significantly reduce capsaicin-evoked scratching (Fig. 7A, ▨ versus ▨).

Observation of mice receiving morphine revealed that they exhibited rotational (circling) behavior. Counts of rotations increased with morphine dose (Fig. 7C), confirming a previous study (Morihisa and Glick, 1977).

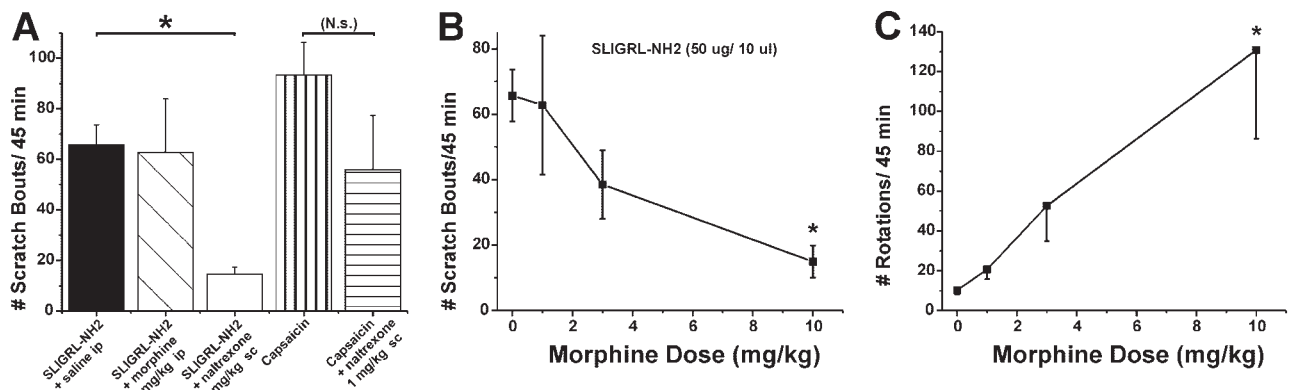
**Fos Expression.** Intradermal microinjection of the PAR-2 agonist SLIGRL-NH<sub>2</sub> resulted in FLI that was distributed in the lateral aspect of the cervical superficial dorsal horn (Fig. 8A) similar to that observed previously (Nojima et al., 2003; Nakano et al., 2008). 5-HT resulted in a similar, overlapping distribution of FLI (Fig. 8B). Both SLIGRL-NH<sub>2</sub> and 5-HT resulted in a significant increase in FLI compared with intradermal vehicle (saline) injections (Fig. 8C).

## Discussion

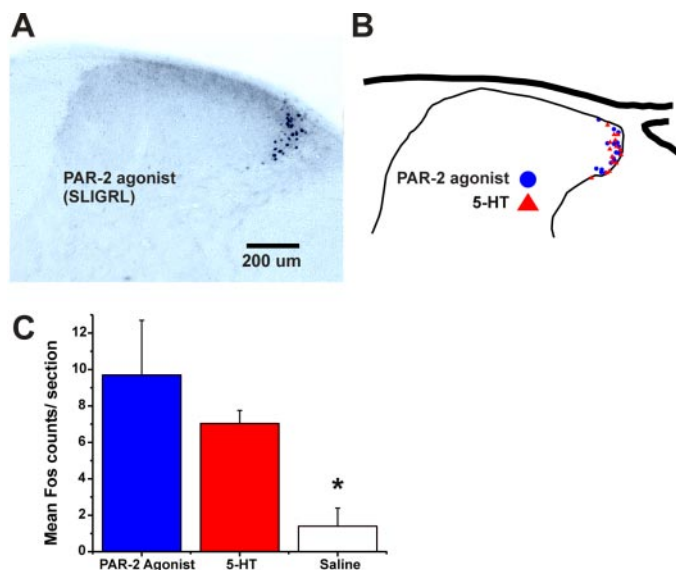
The present results confirm dose-dependent scratching behavior and show tachyphylaxis and cross-tachyphylaxis of scratching for some of the itch mediators tested. Scratching elicited by the PAR-2 agonist was not decreased by low or intermediate doses of morphine but was attenuated by the μ-opioid antagonist, consistent with itch sensation. Moreover, the PAR-2 agonist and 5-HT activated neurons in the superficial dorsal horn in an overlapping distribution. The results are discussed in terms of roles for each of these mediators in itch and the central mechanisms involved.

**PARs.** Both PAR-2 and PAR-4 agonists elicited equivalent dose-related scratching (Fig. 1). Our data confirm recent reports that the PAR-2 agonist SLIGRL-NH<sub>2</sub> evoked dose-related scratching in ICR mice over a 10- to 100-μg range (Shimada et al., 2006; Tsujii et al., 2008), as did the PAR-4 agonist AYPGKF-NH<sub>2</sub> (Tsujii et al., 2008). Tryptase, another PAR-2 agonist, elicited scratching in a manner that was antagonized by a PAR-2 antagonist and antibody (Ui et al., 2006). PAR-2 agonist-evoked scratching was not reduced by antihistamines (Shimada et al., 2006; Tsujii et al., 2008). We did not presently observe cross-tachyphylaxis between the PAR-2 agonist and histamine, in further support that these mediators do not share a common transduction mechanism.

Opioid antagonists reduce experimentally evoked itch in humans (Heyer et al., 1997), and μ-opioid agonists are commonly known to induce itch while reducing pain. It is therefore assumed that μ-opioid antagonists should reduce, whereas μ-agonists should enhance or not affect, itch-related scratching. This was presently borne out, because PAR-2 agonist-evoked scratching was significantly attenuated by naltrexone but was not significantly affected by morphine at 1- and 3-mg/kg doses (Fig. 7). This confirms our previous findings showing suppression of 5-HT-evoked scratching in rats by naltrexone but not morphine (Nojima et al., 2003). At the highest morphine dose tested (10 mg/kg), PAR-2 agonist-evoked scratching was significantly attenuated. However, mice exhibited significant dose-dependent circling behavior (Fig. 7C). This confirms a previous study showing dose-dependent circling behavior in mice that was virtually identical



**Fig. 7.** Opioid modulation of PAR-2 agonist-evoked scratching. A, bar graph plots mean number of scratch bouts/45 min. First three bars show, from left to right, number of scratch bouts evoked PAR-2 agonist (SLIGRL-NH<sub>2</sub>; 50 μg/10 μl) when preceded by intraperitoneal saline (▨; control), morphine (1 mg/kg; ▨), or naltrexone (1 mg/kg; □), respectively. \*,  $p < 0.01$ , significant difference between saline and naltrexone groups ( $n = 5-6$ /group). Bars to right show scratching elicited by intradermal capsaicin (10 μg/10 μl; ▨) and lack of significant effect of naltrexone (▨). B, graph plots total number of scratch bouts/45 min elicited by the PAR-2 agonist SLIGRL-NH<sub>2</sub> versus dose of morphine. The number of scratch bouts at 1- and 3-mg/kg doses of morphine was not significantly different from vehicle but was significantly lower (\*,  $p < 0.05$ ) at the highest morphine dose (10 mg/kg). C, graph plots mean number of rotations (circling)/45 min versus dose of morphine. \*,  $p < 0.05$  for all, significantly different from 0-, 1-, and 3-mg/kg doses.



**Fig. 8.** Overlapping distributions of FLI in cervical spinal cord after intradermal injections of SLIGRL-NH<sub>2</sub> (50  $\mu$ g/5  $\mu$ l) or 5-HT (47 nmol/5  $\mu$ l) in nape of neck. **A**, photomicrograph of midcervical section showing FLI elicited by PAR-2 agonist. **B**, drawing of cervical dorsal horn compiling FLI from one section each from mice injected with PAR-2 agonist (●) or 5-HT (▲) in nape of neck. **C**, mean counts of FLI from mice receiving intradermal PAR-2 agonist (blue bar;  $n = 6$ ), 5-HT (red bar;  $n = 6$ ), or saline (white bar;  $n = 4$ ). \*,  $p < 0.05$  significantly different versus groups receiving PAR-2 agonist and 5-HT (counts for PAR-2 agonist versus 5-HT not significantly different).

to our data over the 1- to 10-mg/kg dose range (Morihisa and Glick, 1977). We speculate that the marked circling behavior at the 10 mg/kg morphine dose may have interfered with scratching behavior. It is conceivable that the strong locomotor drive induced by morphine may have prevented scratching by overriding the urge of the animals to stop and scratch at the PAR-2 agonist injection site.

Capsaicin, which normally elicits burning pain sensation in humans (LaMotte et al., 1991), presently elicited robust scratching that was not significantly affected by naltrexone (Fig. 7A). Capsaicin-evoked scratching might conceivably reflect itch or some irritant sensation that compels the animal to scratch. In support of this idea, topical capsaicin was reported to elicit a sensation of itch in >50% of human subjects tested (Green and Shaffer, 1993). Alternatively, capsaicin may evoke burning pain that induces scratching as a means of counterirritation, despite the general assumption that scratching would exacerbate the pain and thus be avoided. It has been shown that capsaicin injections into the cheek elicited an ipsilateral forelimb wiping response but little or no hind limb scratching directed to the injection site, whereas injection of histamine evoked hind limb scratching but little forelimb wiping (Shimada and LaMotte, 2008). The authors suggested that hind limb scratching and forelimb wiping responses reflect facial itch and pain, respectively. In behavioral studies of itch, chemicals are usually injected in the nape of the neck. However, the response repertoire of the animal is biomechanically limited because the injection site can only be accessed by hind limb scratch movements. We believe that naltrexone-sensitive, morphine-insensitive scratching evoked by the PAR-2 agonist probably reflected itch sensation, whereas naltrexone-insensitive scratching elicited by capsaicin probably reflected pain.

The present data with the PAR-4 agonist AYPGKF-NH<sub>2</sub> confirm a recent report that this agent elicited dose-related scratching in ICR mice over a 10- to 100- $\mu$ g dose range (Tsuji et al., 2008). In the latter study, the PAR-4 agonist was less efficacious than the PAR-2 agonist, whereas we observed an equivalent degree of scratching in response to these two agonists (Fig. 1). It is noteworthy that the antihistamine terfenadine significantly reduced scratching elicited by the PAR-4 but not PAR-2 agonist, suggesting that the former might act partly via degranulation of cutaneous mast cells to release histamine (Tsuji et al., 2008). However, we did not presently observe cross-tachyphylaxis of scratching elicited by histamine when given after a prior injection of the PAR-4 agonist. We did observe significant tachyphylaxis to PAR-4, but not PAR-2, agonist-evoked scratching, and no cross-tachyphylaxis between them, suggesting that these two agents may not share a common mechanism of action. A PAR-1 agonist also evoked histamine-dependent scratching (Tsuji et al., 2008), suggesting two or more mechanisms of action for the participation of PAR-1, -2, and -4 in itch.

**Histamine.** Histamine elicited dose-dependent scratching (Fig. 1), confirming previous studies showing robust scratching in ICR mice with variable efficacy in other strains (Inagaki et al., 2001; Green et al., 2006; Shimada and LaMotte, 2008) that is mediated via H1 and H4 histamine receptors (Ohtsuka et al., 2001; Bell et al., 2004). Histamine-evoked scratching exhibited significant tachyphylaxis, consistent with reduced itch sensation upon repeated challenge with histamine in humans (Stähle-Bäckdahl et al., 1988).

**5-HT.** 5-HT elicits itch or sometimes pain in human skin when injected (Fjellner and Hägermark, 1979) or applied by iontophoresis (Weisshaar et al., 1997) or microdialysis (Schmelz et al., 2003). 5-HT-evoked scratching in mice was attenuated by naloxone (Yamaguchi et al., 1999). The degree of 5-HT-evoked scratching observed presently in ICR mice was comparable with that reported previously in this and other mouse strains (Yamaguchi et al., 1999; Inagaki et al., 2001; Cuellar et al., 2003). Pharmacological studies indicate that scratching is mediated via peripheral 5-HT<sub>2</sub> receptors in mice (Yamaguchi et al., 1999) and rats (Nojima and Carstens, 2003). 5-HT-evoked scratching did not exhibit significant tachyphylaxis, although there was a numeric reduction in the number of scratch bouts evoked by the second compared with the first 5-HT injection. It is noteworthy that 5-HT induced significant cross-tachyphylaxis to scratching elicited by histamine and the PAR-2 and PAR-4 agonists (Figs. 5 and 6), suggesting that 5-HT may exert a depressant effect on peripheral transduction of these two mediators. If itch sensations mediated via PAR-2/4 versus histamine H1/H4 receptors are signaled separately by C-fiber polymodal nociceptors and mechanically insensitive C-afferents, respectively (see Introduction), then it may be speculated that 5-HT acts at sensory nerve endings of both types of C-fiber. It would be of interest to determine the cellular mechanisms of 5-HT cross-interactions with C-fiber responses to histamine or the PAR-2/4 agonists.

**Central Transmission of Itch.** Intradermal injection of both the PAR-2 agonist and 5-HT elicited overlapping distributions of FLI in the lateral aspect of the cervical superficial dorsal horn (Fig. 8). The distribution of 5-HT-evoked FLI was similar to that reported previously in the rat (Nojima et al., 2003). Intradermal injection of the PAR-2 agonist SLIGRL-

NH<sub>2</sub> was recently reported to evoke FLI mainly in laminae I and outer II of the cervical dorsal horn in ICR mice, whereas histamine elicited FLI in inner lamina II within an isolectin-B4 positive region (Nakano et al., 2008), suggesting that synaptic input from primary afferents activated by these two agents may be spatially segregated. Additional studies are needed to determine whether 5-HT and histamine elicit overlapping distributions of FLI.

A limitation of anatomical Fos studies is that they do not reveal whether a given neuron is excited by multiple agents, and an electrophysiological approach is needed to answer this question. In rats, the majority of superficial dorsal horn neurons were excited by both histamine and 5-HT, as well as algogens including capsaicin and mustard oil (Jinks and Carstens, 2002). Superficial dorsal horn neurons in mouse responded to intradermal injection of the PAR-2 agonist SLIGRL-NH<sub>2</sub>, and most of these additionally responded to 5-HT, capsaicin, and mustard oil (Akiyama et al., 2009). These data are consistent with the distributions of FLI elicited by the PAR-2 agonist and 5-HT. However, cowhage and histamine seem to excite largely separate populations of C-fiber afferents (Johanek et al., 2008; Namer et al., 2008) and primate spinothalamic tract neurons (Davidson et al., 2007). We are currently investigating whether the PAR-2 agonist SLIGRL-NH<sub>2</sub> and histamine excite separate populations of superficial dorsal horn neurons in the mouse, as would be consistent with the differing distributions of FLI elicited by these agents (Nakano et al., 2008). If this turns out to be the case, it will be of further interest to determine whether these separate itch-signaling pathways use different neurotransmitters such as substance P or gastrin-releasing peptide and its receptor that has recently been implicated in the central transmission of itch (Sun and Chen, 2007).

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