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RESEARCH ARTICLE | *Sensory Processing*

Innocuous warming enhances peripheral serotonergic itch signaling and evokes enhanced responses in serotonin-responsive dorsal horn neurons in the mouse

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Akiyama T, Nagamine M, Davoodi A, Ivanov M, Carstens MI, Carstens E. Innocuous warming enhances peripheral serotonergic itch signaling and evokes enhanced responses in serotonin-responsive dorsal horn neurons in the mouse. *J Neurophysiol* 117: 251–259, 2017. First published October 26, 2016; doi:10.1152/jn.00703.2016.—Itch is often triggered by warming the skin in patients with itchy dermatitis, but the underlying mechanism is largely unknown. We presently investigated if warming the skin enhances histamine- or serotonin (5-HT)-evoked itch behavior or responses of sensory dorsal root ganglion (DRG) cells, and if responses of superficial dorsal horn neurons to innocuous warming are enhanced by these pruritogens. In a temperature-controlled environmental chamber, mice exhibited greater scratching following intradermal injection of 5-HT, but not histamine, SLIGRL, or BAM8-22, when the skin surface temperature was above 36°C. Calcium imaging of DRG cells in a temperature-controlled bath revealed that responses to 5-HT, but not histamine, were significantly greater at a bath temperature of 35°C vs. lower temperatures. Single-unit recordings revealed a subpopulation of superficial dorsal horn neurons responsive to intradermal injection of 5-HT. Of these, 58% responded to innocuous skin warming (37°C) prior to intradermal injection of 5-HT, while 100% responded to warming following intradermal injection of 5-HT. Warming-evoked responses were superimposed on the 5-HT-evoked elevation in firing and were significantly larger compared with responses pre-5-HT, as long as 30 min after the intradermal injection of 5-HT. Five-HT-insensitive units, and units that either did or did not respond to intradermal histamine, did not exhibit any increase in the incidence of warmth sensitivity or in the mean response to warming following intradermal injection of the pruritogen. The results suggest that 5-HT-evoked responses of pruriceptors are enhanced during skin warming, leading to increased firing of 5-HT-sensitive dorsal horn neurons that signal nonhistaminergic itch.

NEW & NOTEWORTHY Skin warming often exacerbates itch in patients with itchy dermatitis. We demonstrate that warming the skin enhanced serotonin-evoked, but not histamine-evoked, itch behavior and responses of sensory dorsal root ganglion cells. Moreover, serotonin, but not histamine, enhanced responses of superficial dorsal horn neurons to innocuous warming. The results suggest that skin warming selectively enhances the responses of serotonin-sensitive pruriceptors, leading to increased firing of serotonin-sensitive dorsal horn neurons that signal nonhistaminergic itch.

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5-HT; histamine; warming; itch; pruritus; pain; spinal neurons; mice

CHRONIC ITCH affects a substantial proportion of the population and has a significant impact on the quality of life. Chronic itch is thought to sensitize itch-signaling pathways, and current treatments for chronic itch are generally ineffective (Akiyama and Carstens 2013). Sensitization of itch-signaling pathways is manifested by spontaneously occurring itch, increased itch to normally itchy stimuli (hyperknesis), and itch in response to a nonitchy stimulus such as light touch (alloknesis) (Akiyama et al. 2012). Warming the skin often triggers or worsens itch. Skin warming is one of the most commonly reported factors that exacerbates itch in patients suffering from chronic itch (Goon et al. 2007; Yosipovitch et al. 2002a,b).

Candidate molecular transducers of innocuous warmth are TRPV3 and TRPV4, which are activated at thresholds of 33–39°C and 30–35°C, respectively (Guler et al. 2002; Peier et al. 2002; Smith et al. 2002; Watanabe et al. 2002; Xu et al. 2002). These channels are expressed in keratinocytes as well as primary sensory neurons (Alessandri-Haber et al. 2003; Chung et al. 2003, 2004; Facer et al. 2007; Guler et al. 2002; Peier et al. 2002; Suzuki et al. 2003; Xu et al. 2006). We recently demonstrated that TRPV4 is expressed in 5-HT-responsive primary sensory neurons and is required for itch evoked by 5-HT, but not histamine or chloroquine (Akiyama et al. 2016). In contrast, other studies revealed that two other thermosensitive TRP channels, TRPV1 and TRPA1, are required for histamine- and chloroquine-evoked itch behavior, respectively (Shim et al. 2007; Wilson et al. 2011). Five-HT has been reported to induce itch when iontophorecally delivered to the skin (Hosogi et al. 2006; Rausl et al. 2013; Weisshaar et al. 1997) and to be upregulated in the skin of patients with atopic dermatitis (AD) as well as psoriasis and contact dermatitis (El-Nour et al. 2007; Huang et al. 2004a,b; Nordlind et al. 2008). We thus hypothesized that 5-HT sensitizes an itch-signaling pathway that can also be activated by innocuous warm stimuli.

Intradermal injection of 5-HT elicited Fos expression in neurons in the superficial spinal dorsal horn and increased the firing rate of neurons in this location (Akiyama et al. 2009b,c). Most 5-HT-sensitive neurons additionally responded to other itch mediators as well as to algogenic stimuli (Akiyama et al.

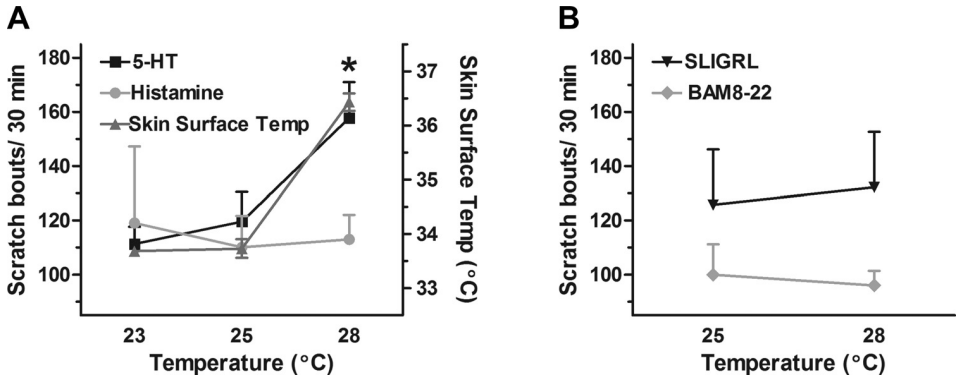

Fig. 1. Scratching elicited by 5-HT is enhanced under conditions of innocuous warming. Five-HT (10 μg/10 μl) or histamine (50 μg/10 μl) (*A*) and SLIGRL (50 μg/10 μl) or BAM8-22 (100 μg/10 μl) (*B*) were injected intradermally into the rostral back of mice. Mice were then placed into an environmental chamber where the temperature was set at 23, 25, or 28°C. Skin surface temperature of rostral back (gray triangle) was measured by thermocouple. The mean skin surface temperature was significantly higher at the 28°C chamber temperature (*$P < 0.01$, one-way ANOVA). The mean number of histamine-, SLIGRL-, or BAM8-22-evoked scratch bouts did not significantly vary with chamber temperature, while the mean number of 5-HT-evoked scratch bouts was significantly greater at 28°C (*$P < 0.05$, one-way ANOVA, $n = 6$/group).

2009b). Other pruritogen-sensitive superficial dorsal horn neurons also responded to algogenic stimuli (Akiyama et al. 2009a,b, 2010; Davidson et al. 2012; Jinks and Carstens 2002; Moser and Giesler 2014) as well as bombesin, which is thought to target itch-signaling spinal neurons (Akiyama et al. 2014b). Thus superficial dorsal horn neurons responsive to 5-HT and other itch mediators plausibly signal itch.

The present study addressed three questions. First, is itch-related scratching behavior elicited by 5-HT or histamine enhanced when the skin temperature is warmed? To investigate this question, we assessed scratching behavior of mice at different skin surface temperatures by using a temperature-controlled environmental chamber. Second, we investigated if primary sensory neuronal responses to 5-HT or histamine are affected by innocuous warming by using the method of calcium imaging of dorsal root ganglion (DRG) cells in a temperature-controlled bath. Finally, we used in vivo single-unit recording methods to investigate if the sensitivity of spinal dorsal horn neurons to innocuous warming of the skin is affected following the intradermal injection of 5-HT or histamine. The results indicate that innocuous warming selectively enhances serotonergic, but not histaminergic, itch signaling and scratching behavior.

METHODS

The procedures used in this study were approved by the UC Davis Animal Care and Use Committee. Experiments were performed using C57BL/6 mice (Simonsen, Gilroy, CA).

Behavior. Mice received intradermal microinjection of either histamine (50 μg /10 μl; Sigma Chemical, St. Louis, MO; Akiyama et al. 2009c, 2012), 5-HT (10 μg /10 μl; Sigma Chemical; Akiyama et al. 2009c, 2012), SLIGRL (50 μg /10 μl; Quality Controlled Biochemicals, Hopkinton, MA; Akiyama et al. 2009c, 2012), or BAM8-22 (100 μg /10 μl; Genemed Synthesis, San Antonio, TX; Akiyama et al. 2012) in the rostral back, and were then placed into an environmental chamber and videotaped from above for 30 min. In one set of experiments, HC067047 (10 mg/kg; Tocris Bioscience) was administered 30 min before intradermal injection of 5-HT. The environmental chamber consisted of a water-jacketed Plexiglas cylindrical chamber (ID 20 cm, height 20.5 cm). Heated water was circulated through an enclosed compartment surrounding the inner chamber that contained the mouse. The temperature of the circulating water was adjusted to maintain the air temperature in the inner chamber constant at a set level (23, 25, or 28 +/− 0.2°C) as continually monitored by electronic thermometer. Skin temperature on the rostral back of the mouse was monitored by a thermocouple attached to a digital thermometer (BAT-12, Physitemp Instruments, Clifton, NJ). The chamber was covered by a vented, clear Plexiglas top through which the mouse was videotaped. Investigators left the room during videotaping. Videotapes were reviewed offline by two reviewers blinded to treatment conditions, and the numbers of hindlimb scratch bouts directed to the injection site were counted at 5-min intervals over a 30-min period. The numbers of scratch bouts elicited by histamine or 5-HT as a function of temperature of the environmental chamber were compared by one-way analysis of variance (ANOVA) with $P < 0.05$ considered significant.

Calcium imaging. Methods were the same as previously described (Akiyama et al. 2016). Upper- to midcervical DRGs were removed, enzymatically digested, and mechanically triturated with fire-polished glass pipettes. DRG cells were plated on polylysine-D-coated glass coverslips and cultured for 16–24 h. The next day, cells were placed in a perfusion chamber whose bath temperature was precisely controlled electronically (BTC-100, Bioscience Tools), and imaged with a camera attached to an inverted fluorescence microscope (Nikon Eclipse TS100). Ratiometric measurements (340, 380 nm) were calculated at 3-sec intervals with Simple PCI software. Solutions were delivered by a solenoid-controlled eight-channel perfusion system (ValveLink, AutoM8). Histamine or 5-HT was delivered at a concentration of 100 μM for 30 sec. Potassium chloride (144 mM) was always delivered at the end of each experiment. The 100 μM concentration selected for each pruritogen was the same as used in prior studies (histamine: Han et al. 2006; Akiyama et al. 2010; 5-HT: Akiyama et al. 2010, 2016). A 10% or greater change in peak ratio after chemical application compared with the preceding baseline was considered a positive response. The bath temperature of the perfusion chamber was controlled by a temperature-controlled microscope stage

Table 1. *Number of DRG cells tested and percent responding to 5-HT and histamine at different bath temperatures*

Bath Temp, °C	*N*, 5-HT	% Responsive	*N*, Histamine	% Responsive
25	329	4.9	88	13.6
28	244	7.8	86	17.4
30	524	5.5	127	14.9
33	293	5.1	93	11.8
35	241	5.0	50	22

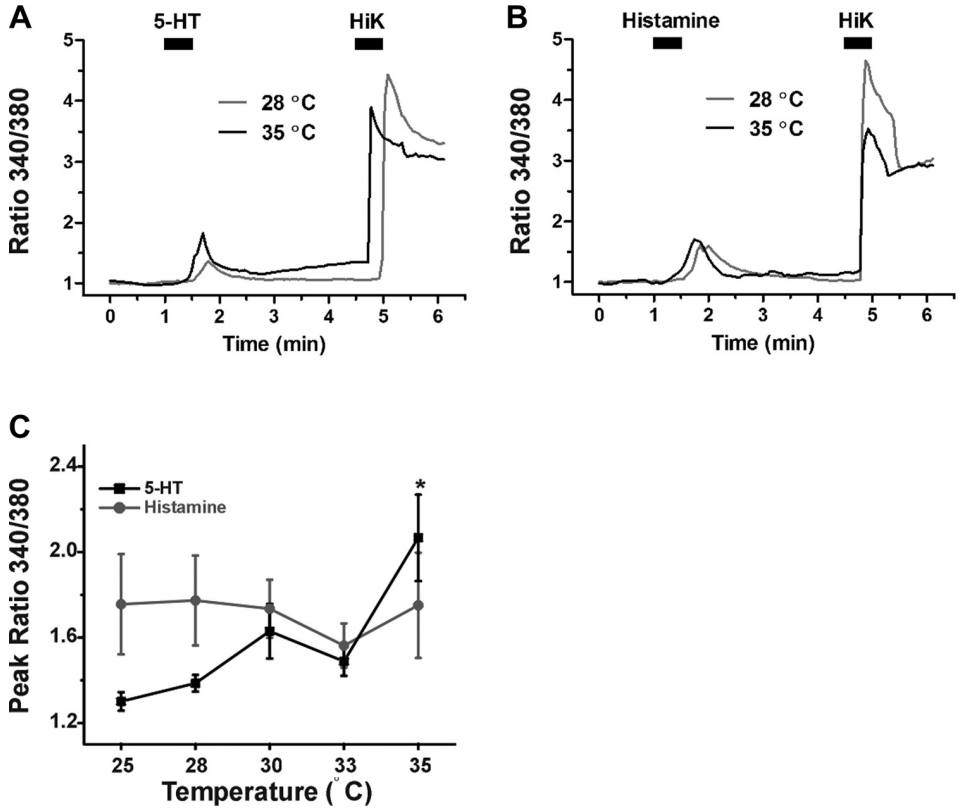

Fig. 2. Responses of DRG cells to 5-HT are enhanced by innocuous warming (35°C). *A*: representative examples of two DRG cells' responses to 5-HT (100 μM, 30 sec), followed by high potassium solution (HiK) at the indicated bath temperatures. Response was considerably larger at 35°C. *B*: representative examples of two DRG cells' responses to histamine (100 μM, 30 sec) at the indicated bath temperatures. Responses at both temperatures were of similar magnitude. *C*: mean responses of DRG cells to 5-HT (black square) or histamine (gray circle) at each tested temperature. Each data point is a separate group of DRG cells (see Table 1). Peak responses to 5-HT were significantly enhanced at 35°C relative to those at other lower temperatures (*$P < 0.05$, one-way ANOVA). In contrast, mean peak responses to histamine did not significantly differ across temperatures.

(BTC-100, Bioscience Tools). Superfusion of 5-HT or histamine was begun when the measured bath temperature stabilized at the preset level. At each bath temperature tested (25, 28, 30, 33, 35°C), separate dishes of DRG cells were tested with either 5-HT or histamine, and the peak ratios of responsive cells were averaged and compared across temperatures by ANOVA with $P < 0.05$ considered to be significant.

Single-unit recordings. Methods were similar to those described in our recent study (Akiyama et al. 2014a) and are summarized here. A total of 82 male C57BL/6 mice (18–28 g) were anesthetized with pentobarbital sodium (60 mg/kg ip), and a laminectomy was performed to expose the lumbar spinal cord for extracellular single-unit recording. The spinal cord was continually superfused

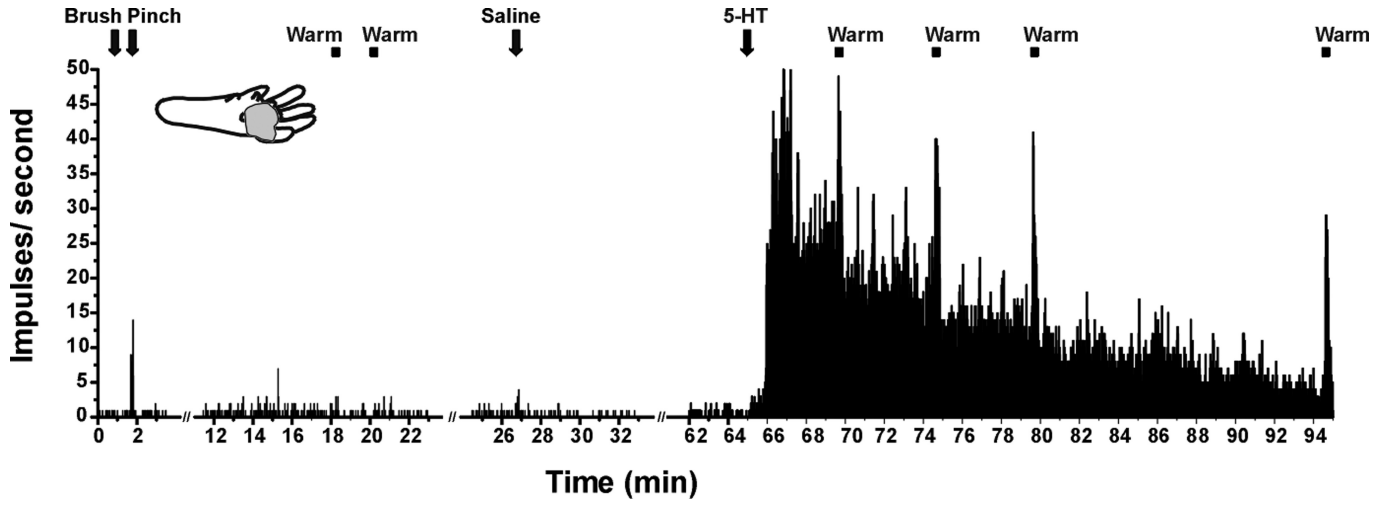

Fig. 3. Enhancement of warming-evoked response of dorsal horn neuron after 5-HT. Peristimulus-time histogram (bins: 1 sec) showing, from left to right, cotton brush and pinch stimuli, warming stimulus, intradermal injection of saline, intradermal injection of 5-HT, and warming stimuli. Bars show time of warming stimuli. Top left inset: mechanosensitive receptive field (gray) on hindpaw.

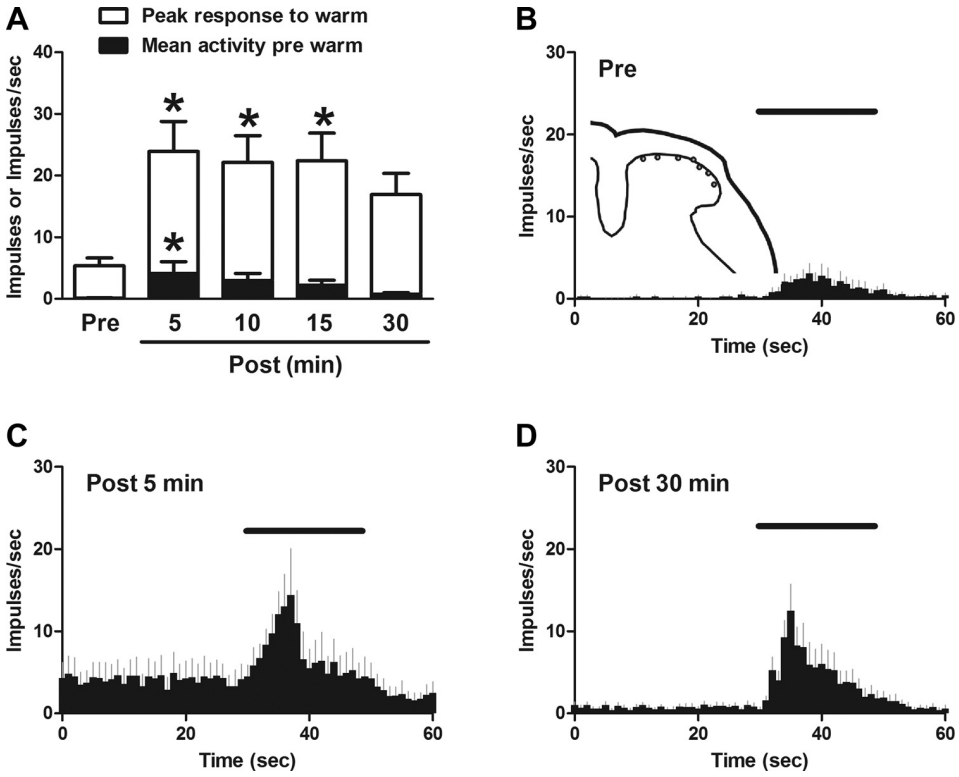

Fig. 4. Enhancement of warming-evoked responses in 5-HT-sensitive dorsal horn neurons. *A*: 5-HT-sensitive units ($n = 12$). Bar graphs indicate mean peak responses to warming stimulus (open column, number of impulses) and mean unit firing rate over 30 sec just prior to the warming stimulus (closed column, impulses per second) vs. time following intradermal injection of 5-HT (at time 0). Pre-5-HT responses are shown at the Pre time point. Error bars: SE. *Significant difference compared with corresponding responses before 5-HT ($P < 0.05$; paired *t*-test). *B*: peristimulus-time histogram (bins: 1 sec) of mean responses to warming stimulus before 5-HT. Inset: histologically recovered recording sites (dots) compiled on representative lumbar section. *C*: as in *B* for 5 min after 5-HT. *D*: as in *B* for 30 min after 5-HT.

with artificial cerebrospinal fluid consisting of (in mM) 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose, equilibrated with 95% O_2-5% CO_2 at 37°C. Extracellular action potentials were amplified and displayed with PowerLab (AD Instruments, Colorado Springs, CO) and Spike2 (CED, Cambridge, UK) software. Only one unit was recorded in a given animal. Action potentials were continually monitored during recording to ensure that the unit was still present, sorted by spike size and waveform, quantified as number of action potentials per second, and displayed in peristimulus-time histogram format with 1-sec bins. Once a mechanosensitive unit was isolated, we tested the unit's responsiveness to light brushing with a cotton wisp, followed by pinching with forceps. Units were categorized as wide dynamic range (WDR) type if they differentially responded to innocuous brush and noxious pinch, or as high threshold (HT) if they responded to pinch but not brush. Units were then tested for responsiveness to an innocuous warm stimulus (37°C) delivered to the hindpaw by computer-controlled Peltier thermode. After this, saline (vehicle) was injected intradermally within the mechanosensitive receptive field. Then, either histamine (50 $\mu g/\mu l$; Akiyama 2009a,b) or 5-HT (10 $\mu g/\mu l$; Akiyama 2009b) was injected intradermally. Unit responses to successive application of the same thermal stimulus (37°C) were determined 5, 10, 15, and 30 min after the intradermal injection. At the end of each experiment, an electrolytic lesion was made at the recording site, and the spinal cord was postfixed in 10% buffered formalin. Spinal cord sections were cut and examined by light microscope to identify lesion sites.

Unit activity was usually quantified as the number of action potentials per second or minute. Responses to the warmth stimulus were analyzed as a peak response (maximum number of impulses in a 1-sec bin). Baseline activity (prewarmth) was analyzed as the mean response over the 30 sec immediately preceding the warming stimulus. Responses to histamine or 5-HT were averaged over successive 60-sec periods. A positive response was defined as a 30% or greater increase in the total number of action potentials per 30 or 60 sec poststimulus compared with the same time interval before the stimulus. Averaged responses to the warm stimulus before and after application of the pruritogen were compared by paired *t*-test with $P < 0.05$ set as significant.

Table 2. *Comparison of peak response of superficial dorsal horn neurons to warm stimuli*

	Sensitive		Insensitive		Sensitive		Insensitive	
	Histamine		Histamine		5-HT		5-HT	
	Sensitive	Insensitive	Sensitive	Insensitive	Sensitive	Insensitive	Sensitive	Insensitive
N	4	2	11	12	7	5	5	31
Pre	7.5 ± 2.3	2.5 ± 0.5	1.1 ± 0.4	0.7 ± 0.6	7.6 ± 1.7	14.6 ± 4.2	2.0 ± 0.6	1.3 ± 0.4
Post 15 min	4.0 ± 1.6	7.5 ± 6.5	2.2 ± 0.6	0.9 ± 0.5	20.3 ± 5.9*	18.0 ± 13.4	20.0 ± 7.7*	0.5 ± 0.2

Data represent average ± SE peak responses to warm stimuli (impulses). *Significantly different from preinjection data ($P < 0.05$, paired *t*-test).

RESULTS

Behavior. Only the highest environmental chamber temperature (28°C) resulted in a significant increase in the mean temperature of skin on the rostral back (Fig. 1*A*, gray triangle). This was associated with a significant increase in the number of 5-HT-evoked scratch bouts (Fig. 1*A*, black square; 111.3 ± 6.3 in 23°C, 119.7 ± 6.3 in 25°C, 157.8 ± 13.2 in 28°C). Pretreatment with the TRPV4 antagonist HC067047 inhibited 5-HT-evoked scratching in the 28°C chamber (26.1 ± 6.6/30 min, data not shown). In contrast, histamine (Fig. 1*A*, gray circle), SLIGRL (Fig. 1*B*, black inverted triangle), or BAM8-22 (Fig. 1*B*, gray diamond)-evoked scratching did not differ significantly across chamber temperatures.

Calcium imaging. The numbers of DRG cells tested at each bath temperature with 5-HT or histamine, and percentages responding, are given in Table 1. Figure 2*A* shows representative examples of two DRG cells' responses to 5-HT; the response was larger at a bath temperature of 35°C compared with 28°C. Overall, the mean peak 5-HT-evoked response of DRG cells was significantly greater when the bath temperature was 35°C, compared with all lower (25–33°C) bath temperature (Fig. 2*C*, black squares). In contrast, the mean peak response to histamine was not significantly affected by bath temperatures (Fig. 2*C*, gray circles).

Single-unit recordings. A total of 82 lumbar dorsal horn units were identified. Of these, 48 (59%) responded to brush and pinch of the hindpaw receptive field (WDR), and 34 (41%) to pinch only (HT). Twenty-three percent (19/82) responded to the 37°C warm stimulus. Warmth-responsive units exhibited a low level of spontaneous activity (0.29 impulses/sec) that was numerically higher than that of warmth-insensitive units (0.16 impulses/sec). Fifty-two percent (16/30) and 25% (12/48) exhibited increased firing following intradermal injection of histamine or 5-HT, respectively. All unit recording sites were located in the superficial dorsal horn at a mean depth of 125.3 +/− 11.0 μm (SE) below the surface of the lumbar spinal cord. For most units the location was confirmed by post hoc histological identification of lesion sites (Figs. 4, 5, 7, and 8, and insets).

In 5-HT-responsive units, mean warmth-evoked responses were significantly greater following intradermal injection of 5-HT compared with preinjection. The example of Fig. 3 shows an increased response to the warm stimulus following intradermal injection of 5-HT, which elicited a prolonged increase in firing of the dorsal horn neuron.

Figure 4*A* shows mean peak warming-evoked responses vs. time for the 5-HT-sensitive units. Mean ongoing activity is also presented to show significantly increased firing during the first 5 min post-5-HT. Prior to intradermal injection of 5-HT, only 7 of 12 units responded to the warming stimulus, while all 12 units responded at 5 min post-5-HT (Table 2). The mean warming-evoked response was significantly greater than the preinjection response (Fig. 4, *A–D*) out to 15 min post-5-HT ($P < 0.05$; paired *t*-test). Responses elicited by the same warming stimulus were not affected following intradermal injection of 5-HT in 5-HT-insensitive units (Fig. 5, *A–D*). Prior to intradermal injection of 5-HT, only 5 of 36 units responded to the warming stimulus in 5-HT-insensitive units (Table 2).

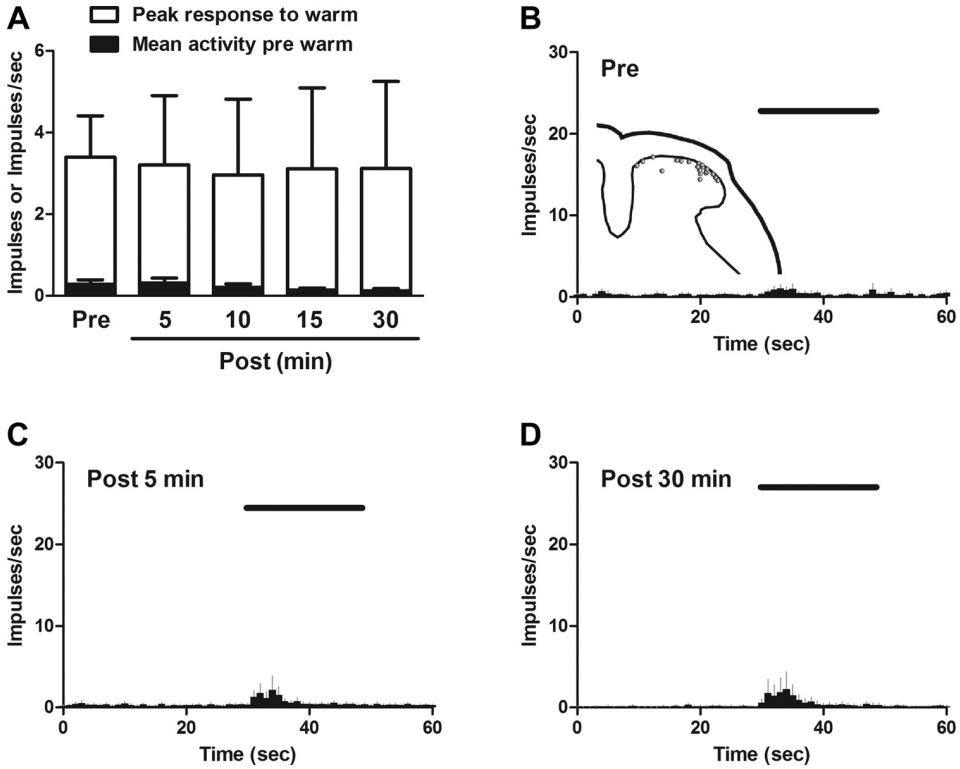

Fig. 5. Warming-evoked responses in 5-HT-insensitive dorsal horn neurons. *A*: 5-HT-insensitive units ($n = 36$). Bar graphs indicate mean peak responses to warming stimulus (open column) and mean unit firing rate over a 30-s period just prior to the warming stimulus (closed column) vs. time following intradermal injection of 5-HT (at time 0). Pre-5-HT responses are shown at the Pre time point. Error bars: SE. *B*: peristimulus-time histogram (bins: 1 sec) of mean responses to warming stimulus before 5-HT. Inset: histologically recovered recording sites (dots) compiled on representative lumbar section. *C*: as in *B* for 5 min after 5-HT. *D*: as in *B* for 30 min after 5-HT.

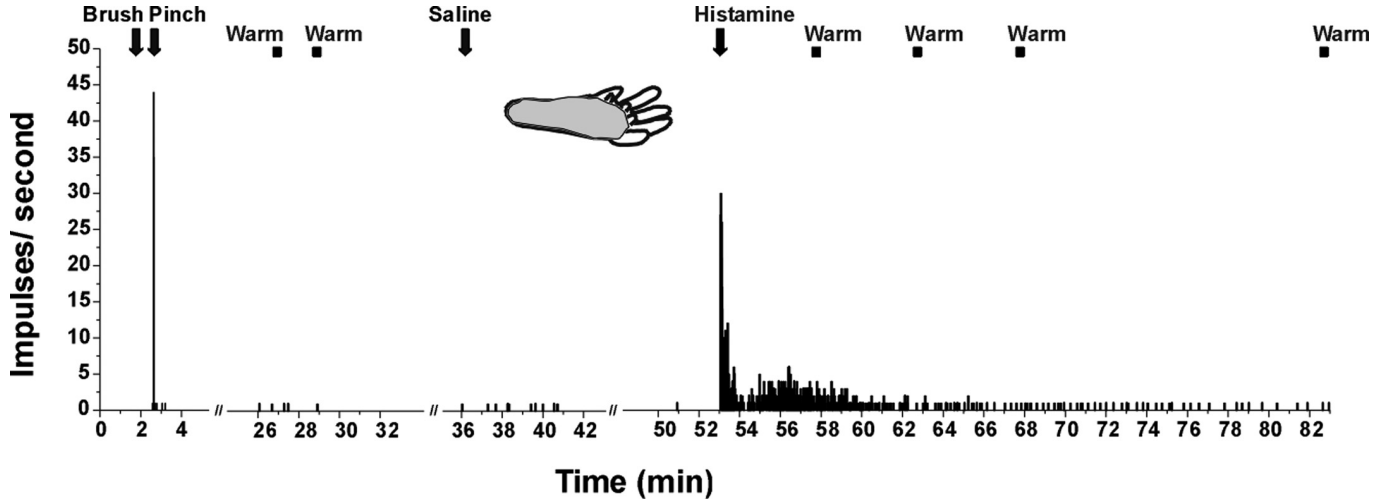

Fig. 6. Individual example showing warming-evoked response of dorsal horn neuron before and after histamine. Peristimulus-time histogram (bins: 1 sec) showing, from left to right, cotton brush and pinch stimuli, warming stimulus, intradermal injection of saline, intradermal histamine, and warming stimuli. Bars show time of warming stimuli. Top middle inset: mechanosensitive receptive field (gray) on hindpaw.

The example of Fig. 6 shows weak responses to the warming stimulus prior to histamine. Intradermal injection of histamine elicited a prolonged increase in firing of the dorsal horn neuron. In this typical example, following histamine there was no enhancement of the response to warming. Figure 7*A* shows mean peak warming-evoked responses vs. time for the histamine-sensitive units. In histamine-responsive units, the mean firing rate was significantly increased 5 min posthistamine (Fig. 7*A*). Intradermal injection of histamine had no significant effect on firing evoked by the warming stimulus (Fig. 7, *A–D*). Prior to intradermal injection of histamine, only 4 of 15 units responded to the warming stimulus in histamine-sensitive units (Table 2). Responses elicited by the warming stimulus were not affected following intradermal injection of histamine in histamine-insensitive units (Fig. 8, *A–D*). Prior to intradermal injection of histamine, only 11 of 23

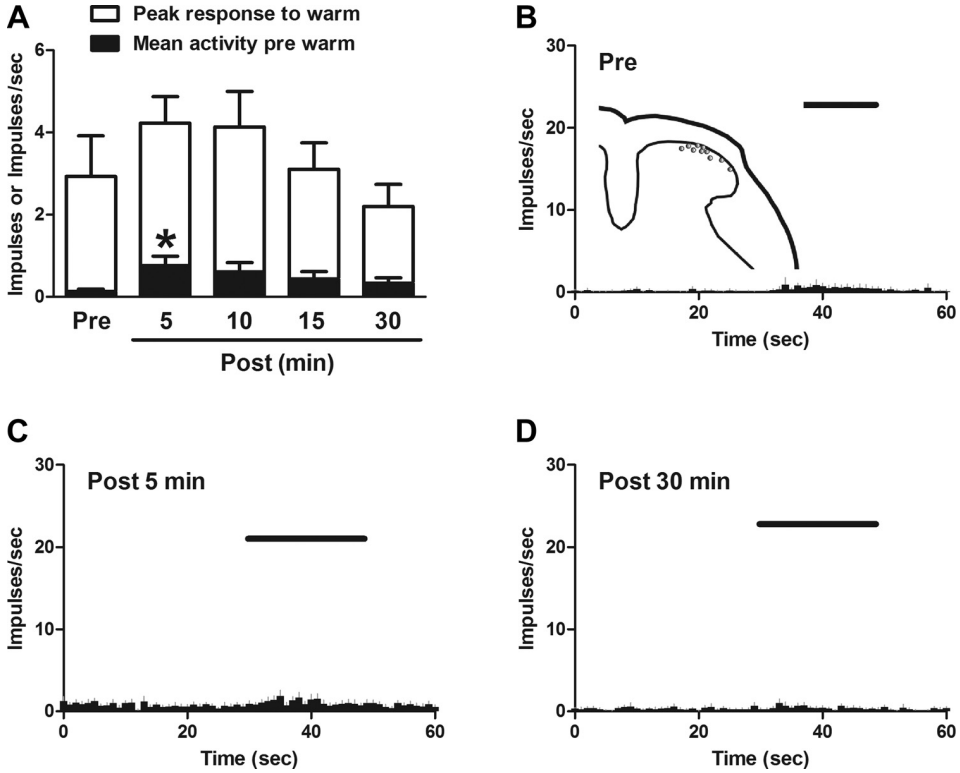

Fig. 7. Warming-evoked responses in histamine-sensitive dorsal horn neurons. *A*: histamine-sensitive units ($n = 15$). Bar graphs indicate mean peak responses to warming stimulus (open column) and mean unit firing rate over a 30-s period just prior to the warming stimulus (closed column) vs. time following intradermal injection of histamine (at time 0). Prehistamine responses are shown at the Pre time point. Error bars: SE. *Significant difference compared with corresponding responses before histamine ($P < 0.05$; paired *t*-test). *B*: peristimulus-time histogram (bins: 1 sec) of mean responses to warming stimulus before histamine. Inset: histologically recovered recording sites (dots) compiled on representative lumbar section. *C*: as in *B* for 5 min after histamine. *D*: as in *B* for 30 min after histamine.

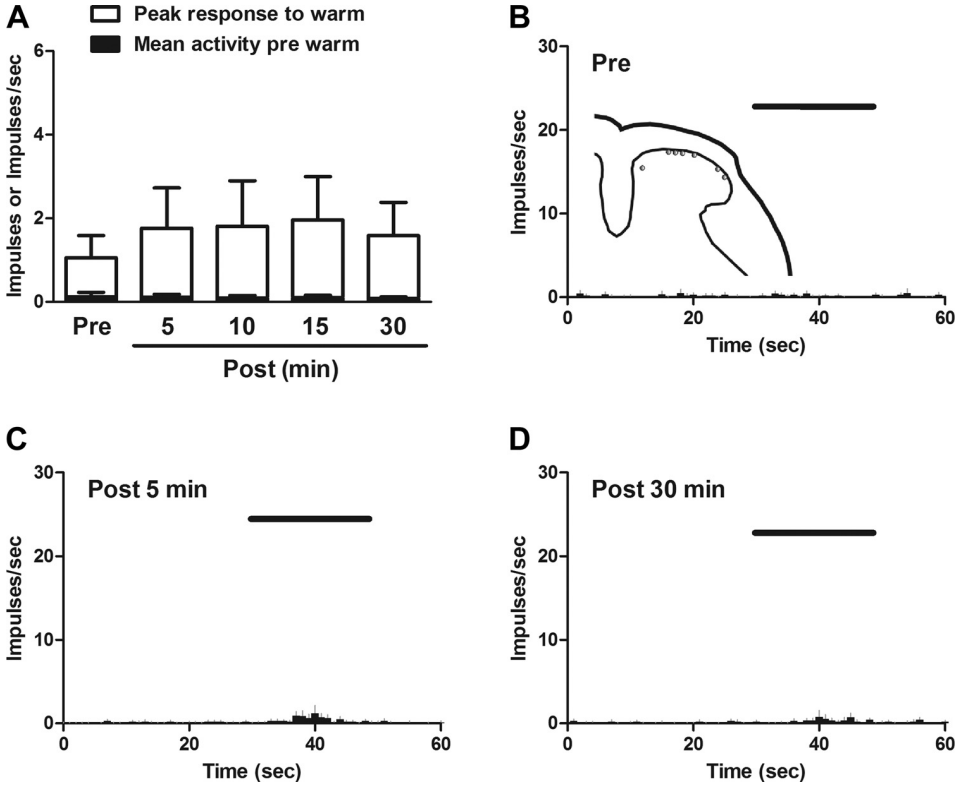

Fig. 8. Warming-evoked responses in histamine-insensitive dorsal horn neurons. *A*. histamine-insensitive units ($n = 14$). Bar graphs indicate mean peak responses to warming stimulus (open column) and mean unit firing rate over a 30-s period just prior to the warming stimulus (closed column) vs. time following intradermal injection of histamine (at time 0). Prehistamine responses are shown at the Pre time point. Error bars: SE. *B*: peristimulus-time histogram (bins: 1 sec) of mean responses to warming stimulus before histamine. Inset: histologically recovered recording sites (dots) compiled on representative lumbar section. *C*: as in *B* for 5 min after histamine. *D*: as in *B* for 30 min after histamine.

units responded to the warming stimulus in histamine-insensitive units (Table 2).

DISCUSSION

We presently observed that scratching behavior elicited by 5-HT, but not histamine, SLIGRL, or BAM8-22, was significantly increased during exposure to a warm environment that elevated skin temperature to >36°C. Similarly, DRG cell responses to 5-HT, but not histamine, were significantly greater at a bath temperature of 35°C. We additionally investigated the interaction of skin temperature and pruritogens at the level of spinal dorsal horn neurons and observed that only those neurons that were directly activated by intradermal 5-HT exhibited enhanced firing during innocuous warming (37°C) of skin in the receptive field. Of these, some did not respond to the warming stimulus prior to 5-HT, but after intradermal injection of 5-HT they exhibited increases in firing during warming that were superimposed on the 5-HT-evoked increase in neuronal activity. Other neurons responded to the warming stimulus prior to intradermal injection of 5-HT, but after intradermal 5-HT exhibited a higher firing rate during warming. Neurons that did not respond to intradermal 5-HT, and both histamine-responsive and histamine-unresponsive neurons, did not exhibit any increase in firing during skin warming compared with that observed prior to intradermal injection of the pruritogen. These results are consistent with the possibility that innocuous warming enhances the 5-HT-evoked responses of pruriceptors, such that 5-HT-evoked responses of second-order dorsal horn neurons and resultant scratching behavior are enhanced when the skin is warmed.

Since the ongoing responses to 5-HT were clearly enhanced, one possibility is that warming could enhance the biochemical reactions elicited by 5-HT and/or its receptors. However, to our knowledge there is no evidence supporting this idea. Another possibility is that the warmth-sensitive TRP ion channel TRPV4 (Chung et al. 2003) might be involved in 5-HT-evoked itch. We recently reported that 5-HT-evoked scratching behavior and responses of DRG cells depend at least partly on TRPV4 (Akiyama et al. 2016). It is currently not known which 5-HT receptor subtype might interact with TRPV4 in pruriceptive nerve endings. Five-HT receptor subtypes 5-HT2 (Nojima and Carstens 2003; Yamaguchi et al. 1999), 5-HT3 (Ostadhadi et al. 2015), 5-HT7 linked to TRPA1 (Morita et al. 2015), and recently 5-HT1F (Stantcheva et al. 2016) have been implicated in itch. Both 5-HT and histamine sensitized DRG neuronal responses to the selective TRPV4 channel agonist 4a-phorbol 12, 13-didecanoate (Cenac et al. 2010). This is consistent with an interaction of 5-HT and TRPV4 to increase pruriceptor activity. It was very recently reported that mice lacking TRPV4 in the epidermis exhibited an ~30% reduction in histamine-evoked scratching (Chen et al. 2016). However, we presently did not observe any effect of warming on histamine-evoked scratching or responses of DRG or spinal dorsal horn neurons. We speculate that TRPV4 may play a greater role in warmth enhancement of itch elicited by 5-HT than histamine. The mechanism is unknown, but might speculatively involve acti-

vation of TRPV4 by innocuous warming to elicit a depolarization that sums with 5-HT-evoked depolarization in pruriceptive primary afferents that coexpress TRPV4 and 5-HT receptors.

It is also conceivable that 5-HT sensitizes TRPV4 expressed in warm fibers that converge onto 5-HT-sensitive dorsal horn neurons, enhancing their response to innocuous warming as a signal of increased itch. The mechanism underlying this speculative interaction is unknown. We believe that central sensitization of 5-HT-responsive dorsal horn neurons is an unlikely explanation for the enhancement of warming-evoked responses, since a majority of 5-HT-sensitive dorsal horn neurons also likely responds to intradermal histamine (Akiyama et al. 2010), but only 5-HT-responsive (and not histamine-responsive) neurons exhibited enhanced responses to warming.

Five-HT-responsive superficial dorsal horn neurons plausibly signal itch, since 5-HT elicits itch-related scratching behavior in rodents (Akiyama et al. 2012, 2009c; Mishra and Hoon 2013; Sun et al. 2009; Yamaguchi et al. 1999) and itch sensation in humans (Hosogi et al. 2006; Rausl et al. 2013; Weisshaar et al. 2004, 1997). Five-HT-responsive dorsal horn neurons additionally respond to other pruritogens (Akiyama et al. 2009a), and such pruriceptive neurons respond to intrathecal bombesin, which is thought to target itch-signaling neurons (Akiyama et al. 2014b). In the present study, 25% of superficial dorsal horn neurons responded to intradermal injection of 5-HT. This is consistent with previous reports that intradermal injection of 5-HT activated 27% of trigeminothalamic tract neurons (Moser and Giesler 2014), which are implicated in signaling itch sensation (Davidson et al. 2014), as well as 27% of C-type dorsal root ganglion neurons (Hachisuka et al. 2010). The warmth-induced increase in responses of 5-HT-sensitive superficial dorsal horn neurons may thus be encoded as enhanced itch.

Although we found that warming the skin to 37°C enhanced 5-HT-evoked firing of dorsal horn neurons, recently Lipshetz and Giesler (2016) found that warming the skin to 40°C had almost no effect on responses of dorsal horn trigeminothalamic neurons to 5-HT injections in rats. This discrepancy may be due to a species difference (mouse vs. rats) and/or size of warming devices (1.27-cm-diameter vs. 3- by 3-mm contact surface).

In the present study, we found that 23% of warming-responsive superficial dorsal horn neurons exhibited spontaneous activity, consistent with previous reports (Andrew and Craig 2001; Khasabov et al. 2001). They were classified as HT or WDR. Previous studies reported that warming-responsive spinal neurons are classified as HT, WDR, or mechano-insensitive (Andrew and Craig 2001; Khasabov et al. 2001; Matthews et al. 2006). They respond to noxious heat stimuli (>45°C) (Andrew and Craig 2001; Courtney et al. 1972; Khasabov et al. 2001; Matthews et al. 2006), while warming-sensitive primary afferent C-fibers do not respond to noxious heat stimuli (>45°C) (Duclaux and Kenshalo 1980; LaMotte and Campbell 1978), suggesting that warming-sensitive spinal neurons receive inputs from multiple fibers including innocuous warm receptors and noxious heat-sensitive nociceptors.

Intradermal injection of histamine did not affect warming-evoked responses of histamine-responsive or histamine-insensitive dorsal horn neurons. In contrast, a previous study from our laboratory reported that identical intradermal injection of histamine resulted in enhanced responses to innocuous mechanical stimuli of histamine-responsive (but not histamine-insensitive) dorsal horn neurons (Akiyama et al. 2014a). Since warm fibers do not respond to mechanical stimuli (LaMotte and Campbell, 1978), these results suggest that histamine differentially sensitizes mechanoreceptive, but not thermoreceptive (warm), input to spinal itch-signaling neurons. In contrast, the present data indicate that 5-HT, but not histamine, enhances warmth sensitivity of pruriceptive dorsal horn neurons, suggesting differential mechanisms for the evocation of itch by innocuous touch (alloknesis) as opposed to innocuous skin warming. These findings may have clinical implications. Alloknesis, which accompanies many types of chronic itch, might benefit from antihistamine treatment, whereas warmth-evoked itch in conditions such as atopic dermatitis might be better treated by interfering with 5-HT receptors or TRPV4.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

T.A. conceived and designed research; T.A., M.N., A.D., M.I., and M.I.C. performed experiments; T.A., M.N., A.D., and M.I. analyzed data; T.A., M.N., A.D., M.I., and E.C. interpreted results of experiments; T.A. prepared figures; T.A. drafted manuscript; T.A. and E.C. edited and revised manuscript; T.A. and E.C. approved final version of manuscript.

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