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Modulation of Oral Heat and Cold Pain by Irritant Chemicals

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Abstract

Common food irritants elicit oral heat or cool sensations via actions at thermosensitive transient receptor potential (TRP) channels. We used a half-tongue, 2-alternative forced-choice procedure coupled with bilateral pain intensity ratings to investigate irritant effects on heat and cold pain. The method was validated in a bilateral thermal difference detection task. Capsaicin, mustard oil, and cinnamaldehyde enhanced lingual heat pain elicited by a 49 °C stimulus. Mustard oil and cinnamaldehyde weakly enhanced lingual cold pain (9.5 °C), whereas capsaicin had no effect. Menthol significantly enhanced cold pain and weakly reduced heat pain. To address if capsaicin's effect was due to summation of perceptually similar thermal and chemical sensations, one-half of the tongue was desensitized by application of capsaicin. Upon reapplication, capsaicin elicited little or no irritant sensation yet still significantly enhanced heat pain on the capsaicin-treated side, ruling out summation. In a third experiment, capsaicin significantly enhanced pain ratings to graded heat stimuli (47 °C to 50 °C) resulting in an upward shift of the stimulus–response function. Menthol may induce cold hyperalgesia via enhanced thermal gating of TRPM8 in peripheral fibers. Capsaicin, mustard oil, and cinnanamdelyde may induce heat hyperalgesia via enhanced thermal gating of TRPV1 that is coexpressed with TRPA1 in peripheral nociceptors.

Key words: capsaicin, cold pain, heat pain, menthol, oral irritation, TRP channel

Introduction

Irritant chemicals in spices, as well as temperature, play important roles in the overall perception and palatability of foods and beverages. In recent years, 6 thermosensitive transient receptor potential (TRP) ion channels have been identified, several of which also respond to irritant chemicals (Clapham et al. 2001; Jordt et al. 2003; Patapoutian et al. 2003; Tominaga and Caterina 2004; Reid 2005; Wang and Woolf 2005; Dhaka et al. 2006). Thus, TRPV1 responds to capsaicin, the pungent chemical in chili peppers, as well as noxious heating at and above the pain threshold of 42 °C (Caterina et al. 1997). TRPM8 responds to the cooling agent menthol as well as temperatures below ~ 28 °C (McKemy et al. 2002; Peier et al. 2002). TRPA1 was originally reported to respond to intense cooling below ~ 18 °C (Bandell et al. 2004), as well as mustard oil, cinnamaldehyde, bradykinin, and other pungent chemicals (Bandell et al. 2004; Jordt et al. 2004; Bautista et al. 2006). Although the temporal dynamics of oral irritation induced by capsaicin (Green 1989; Dessirier et al. 1997; Prescott 1999), menthol (Cliff and Green 1996; Dessirier et al. 2001), mustard oil (Simons et al. 2003), and cinnamaldehyde (Prescott and Swain-Campbell 2000) have been investigated in human psychophysical studies, less is known regarding the effects of these irritants on thermal pain, particularly in the oral cavity. Capsaicin transiently enhanced cutaneous heat pain (LaMotte et al. 1991; Simone and Ochoa 1991; LaMotte et al. 1992; Torebjork et al. 1992), and in the oral cavity, the burning sensation of capsaicin was enhanced by temperatures increasing into the noxious range (Green 1986). Mustard oil and cinnamaldehyde induced heat hyperalgesia in skin (Koltzenburg et al. 1994; Schmelz et al. 1996, Olausson 1998). Menthol enhanced oral cold sensation evoked by cooling down to 10 °C (Green 1985) and resulted in cold hyperalgesia on the skin (Wasner et al. 2004; Namer et al. 2005; Hatem et al. 2006). Based on the paucity of data regarding irritant chemical modulation of thermally evoked oral pain, the aim of our first set of experiments was to investigate the effects of pretreatment of the tongue with 4 different irritants on heat and cold pain. We investigated capsaicin, menthol, mustard oil, and cinnamaldeyde because these chemicals have been studied most in relation to their effects

on thermosensitive TRP channels. We employed a sensitive half-tongue, 2-alternative forced-choice (2-AFC) method coupled with bilateral ratings of the intensity of thermally evoked pain, which our laboratory has used in the past to investigate temporal dynamics of oral chemical irritation and cross-interactions between irritants (e.g., Simons et al. 2003). Because capsaicin, cinnamaldehyde, and mustard oil enhanced heat pain, we conducted a second experiment to rule out the possibility that this effect was due to summation of the perceptually similar heat sensations evoked by the thermode and chemical stimulus. Finally, in a third experiment, we investigated the effect of capsaicin on heat pain over a broader range of temperatures. These results have appeared in abstract format (Albin et al. 2006).

Materials and methods

Subjects

A total of 188 subjects was recruited using Experimetrix, an online university psychology experiment Web site. One hundred and thirty-six subjects (37 M, 99 F; ages 19–55) participated in Experiment 1, 36 subjects (27 F, 9 M; ages 18–33) in Experiment 2, and 16 (10 F, 6 M; ages 20–55) in Experiment 3 (4 also participated in Experiments 1 and/or 2). Subjects were all nonsmoking university students and staff who did not consume spicy food for 2 days prior to testing. They were also asked to refrain from eating, drinking, chewing gum, brushing their teeth, and using mouthwash for 1 h before the experiment as verified at the beginning of each session. The experimental protocol was approved by University of California, Davis, Human Subjects Internal Review Board.

Chemical stimuli

A stock solution of 6% L-menthol (from dry crystals; Givaudan Flavors Corp., Cincinnati, OH) was prepared in ethanol and 20% Tween-80. This stock solution was diluted daily—to avoid recrystallization—with deionized (DI) water to make a 0.3% (19 mM) menthol solution (Table 1). Forty microliters was pipetted onto a filter paper at the time of application and applied unilaterally with 40 μl of vehicle containing matching concentrations of Tween-80 (1%) and ethanol (3.8%) in DI water.

A 0.001% (10 ppm, 33 μM) capsaicin solution was prepared by diluting 1% capsaicin in 70% ethanol stock solution (Sigma Chemical Co., St Louis, MO) with DI water. Filter papers were prepared daily by pipetting 40 μl of the dilute solution and drying them ahead of time to prevent confounding effects of ethanol. A predried capsaicin disk and an untreated disk were rehydrated with 40 μl of DI water immediately before application.

A 1% (103 mM) mustard oil solution was prepared by diluting 98% mustard oil (allyl isothiocyanate; Fluka, St Louis, MO) in mineral oil. Thirty microliters was pipetted onto a 1.5-cm (176.7-mm^2) filter paper disk (Whatmann, Maidstone, UK) immediately before application. Thirty microliters of mineral oil (vehicle) was pipetted onto a separate filter paper.

A 0.2% (16 mM) cinnamaldehyde solution was prepared by diluting a 98% stock solution with DI water. Forty microliters of cinnamaldehyde solution and 40 μl of DI water (vehicle) were pipetted onto separate filter papers immediately before application.

Table 1 Irritant concentration, volume, and application times

	v/v (%)	Concentration	Volume (μl)	Time (s)
Menthol	0.3	19 mM	40	30
Capsaicin	0.001	32.7 μM	40	30
Mustard oil	1	103 mM	30	15
Cinnamaldehyde	0.2	15.9 mM	40	30

Thermal stimulation

A square Peltier thermode (4.60 × 4.60 cm; NTE-2, Physitemp Instruments, Clifton, NJ) was used for thermal stimulation in 3 different experiments (see below). In Experiment 1, it was either preheated to 49 °C ± 0.2 °C or precooled to 9.5 °C ± 0.2 °C before each session. The higher temperature was selected because it provided a tongue–thermode interface temperature above the benchmark heat pain threshold of ~45 °C (Hardy et al. 1952). The 9.5 °C temperature was selected because it resulted in a thermode–tongue interface temperature of ~13 °C or less (see below) which is in the range of cold pain, although it is noted that the cold pain threshold is highly variable across subjects (Chen et al. 1996; Beise et al. 1998). The temperature of the thermode was feedback controlled and maintained within ±0.2 °C. In Experiment 2, only the 49 °C ± 0.2 °C stimulus was used, and in Experiment 3, 4 different temperatures were tested (47, 48, 49, and 50 ± 0.2 °C). In the latter experiment, a thermocouple (IT-21; 0.08 s time constant; Physitemp Instruments) was placed in the center of the thermode to record the tongue–thermode interface temperature. The thermometer output was also routed to a computer through a Powerlab interface (ML820; ADInstruments, Colorado Springs, CO) to allow continual recording of temperature using Chart software (ADInstruments). A fresh piece of violet-colored plastic wrap (Reynolds Wrap; Alcoa Consumer Products, Richmond, VA) was placed over the thermode as a sanitary barrier for each subject.

Thermal difference detection

At the beginning of each session in Experiment 1 (see below), subjects performed a thermal difference detection task. After rinsing with DI water, subjects pressed their tongue against a preheated or precooled thermode with a 0.5 °C temperature differential between the 2 halves of the thermode. The temperature difference was accomplished by placing a thin strip of polyethylene plastic underneath the sanitary

barrier to cover exactly one-half of the thermode (2.30 cm wide × 4.60 cm long). This insulation barrier created a temperature difference of 0.54 °C +/− 0.05 °C (standard error of the mean) between the 2 sides of the thermode, as measured using thermocouples (IT-210.08 s time constant; Physitemp Instruments) placed in the center of each half of the thermode. The thermocouple was connected to a thermometer (BAT-12, Physitemp Instruments) whose output was routed to a computer through a Powerlab interface to record the temperature using Chart software. The resulting comparison temperatures were 48.5 °C versus 49.0 °C for heat and 10.0 °C versus 9.5 °C for cold. Subjects were told that the thermode consisted of 2 adjacent metal blocks, which could be separately controlled. During the heat session, subjects were asked to choose which side of their tongue was more painful in a 2-AFC procedure. They were also asked to rate the heat pain intensity on each side of their tongue using a 0–10 rating scale, where 0 = "no heat pain" and 10 = "most intense heat pain imaginable" (Price et al. 1983). In the cold condition, subjects chose on which side cold pain was stronger (2-AFC) and rated the cold pain intensity from 0–10, where 0 = "no cold pain" and 10 = "most intense cold pain imaginable." The insulated side was randomized and counterbalanced across all subjects.

Experiment 1: irritant modulation of heat or cold pain

After the thermal difference detection task, the plastic insulator was removed without the knowledge of the subjects. Subjects were then treated with 1 of 4 chemical irritants (mustard oil, capsaicin, cinnamaldehyde, or menthol). Each irritant was tested in a separate session with the hot thermode and again with the cold thermode such that there was a total of 8 test sessions with a minimum of 2 days in between successive sessions.

The procedure is illustrated in Figure 1. The irritant was applied by filter paper (1.5 cm diameter, 176.7 mm^2; Whatmann) onto one side of the dorsal anterior tongue and a second identical filter paper soaked with vehicle simultaneously applied onto the opposite side using forceps. While the filter papers were on the tongue, subjects were asked to close their mouth and avoid touching the sides or roof of the oral cavity. This minimized any cooling effects of inhalation or spreading irritation to other parts of the mouth. Capsaicin, menthol, and cinnamaldehyde filter papers were left on for 30 s, whereas mustard oil was left on for 15 s due to faster acting irritation (Table 1). After filter papers were removed, subjects immediately pressed their tongue against the thermode, which in the first session was uniformly preheated to 49 °C. They then performed the 2-AFC and ratings procedures. Subjects repeated this 2-AFC and bilateral ratings procedure at 1.5, 5, and 10 min after original irritant application. The same procedure (including thermal detection at the beginning of the session) was repeated with a cold (9.5 °C) stimulus in a second session at least 2 days later.

The thermode–tongue interface was recorded in pilot studies for Experiments 1 and 2 and was routinely recorded in Experiment 3. With the thermode preset at 9.5 °C, the interface temperature reached a stable level within 7–15 s after the subject pressed the tongue to the thermode, with the interface temperature being 2.5 °C to 3.5 °C higher (i.e., ~13 °C or less). Similarly, when preheated at 49 °C, the interface temperature stabilized at a level 2.5 °C to 3.5 °C lower after 7–15 s. The interindividual differences in the interface temperatures achieved were presumably due to differences in thermal conductivity of the tongue.

Experiment 2: capsaicin desensitization

Capsaicin desensitization of the tongue was accomplished as follows. First, capsaicin (10 ppm, 33 μM) was applied to one side of the tongue using a large cotton swab (Puritan 6-inch cotton-tipped applicators, Harwood Products Co., Guilford, ME). Vehicle (DI water) was applied to the other side also by a large cotton swab. The 2 cotton swabs were dipped into the capsaicin or vehicle solution and then each was rolled over one side of the anterior dorsal surface of the tongue simultaneously. Subjects periodically (once per minute) rated the irritation experienced on the capsaicin-treated side using the 0–10 numeric rating scale.

When irritation levels reached 0 (usually within 10 min), a rehydrated capsaicin filter paper disk was applied by forceps to the side previously receiving capsaicin, and a vehicle-treated disk was simultaneously applied to the opposite side in the same manner. This second application of capsaicin was analogous to the single irritant application in Experiment 1.

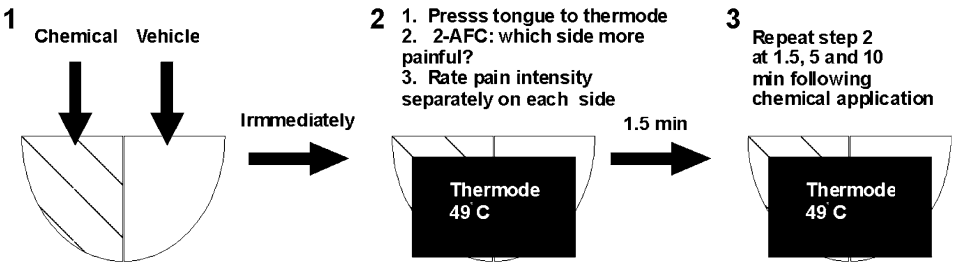

Figure 1 Schematic of experimental procedure.

Thus, an equivalent amount of capsaicin was delivered unilaterally before heat testing but was not accompanied by irritation. The filter papers were left on for 30 s and then removed. Thermal testing was as in Experiment 1, except that subjects provided a rating of the intensity of irritant sensation (using the same 0–10 rating scale) immediately prior to each heat stimulus. A rating of 0 or 1 was taken as evidence for capsaicin self-desensitization. Subjects then pressed the tongue against the preheated thermode, chose which side was more painful in the 2-AFC, and provided bilateral pain intensity ratings immediately following the second application of capsaicin, and again 1.5, 5, and 10 min later, as in Experiment 1.

Experiment 3: capsaicin enhancement of heat pain across the noxious range

Subjects participated in 2 training sessions and 3 test sessions. Each session lasted 45–55 min. In the first training session, subjects were familiarized with the 4 stimulus temperatures used (47, 48, 49, or 50 ± 0.2 °C) and then practiced rating heat pain intensity elicited by these different temperatures. For each stimulus rating, subjects placed their tongue on the thermode for 7–15 s while the thermode–tongue interface temperature was recorded. A thermocouple (IT-210.08 s time constant; Physitemp Instruments) was placed in the center of the thermode to record the tongue–thermode interface temperature. The thermometer output was also routed to a computer through a Powerlab interface (ML820; ADInstruments) to allow continual recording of temperature using Chart software (ADInstruments). The temperature increased over the first several seconds and reached a stable plateau level within 7–15 s, at which time the heat pain rating was obtained using the 0–10 numeric rating scale. Subjects were allowed to use decimals if desired. They were provided feedback as to whether the ratings were consistent with the rank order of the stimulus temperatures. In the second training session, subjects practiced half-tongue ratings of heat pain after unilateral application of capsaicin and were provided similar feedback. Small cotton swabs (Qosmetix, Edgewood, NY: mini round tips swab with white handle; 3 inch, 2.7 mm head) were dipped in capsaicin and vehicle. Each swab was used to simultaneously paint opposing sides (right and left) of the anterior dorsal tongue surface of the tongue.

In the 3 test sessions, each subject was tested under one of the following conditions: 1) vehicle application to the whole anterior tongue, 2) capsaicin (10 ppm, 30 μM) application to the whole anterior tongue, or 3) application of capsaicin and vehicle separately to opposite halves of the tongue (half-tongue procedure). At least 2 days rest was given between a capsaicin session and the next test session.

In each session in which capsaicin was applied to the whole or half tongue, we followed a procedure similar to that of Experiment 2 to ensure that the capsaicin-treated area of the tongue was desensitized. This was accomplished using the following reapplication scheme, which is illustrated for the half-tongue procedure in Figure 2. Capsaicin was applied to either one-half of the dorsal tongue (half-tongue condition) or to both sides (whole-tongue condition), using large cotton swabs at the beginning of each session. In the whole-tongue vehicle condition, DI water was applied to both sides of the tongue in the same manner. Subjects were asked to rate irritation each minute on a 0–10 numeric scale. Once irritation levels reached less than 1, subjects were given a temperature warm-up to refamiliarize them with the range of thermode temperatures. For their warm-up, subjects firmly pressed the dorsal anterior tongue against the thermode as the thermode temperature was increased from 43 °C to 50 °C and then back down over 15 s. After the initial desensitizing and warm-up period, the test period began. In the whole-tongue vehicle–only condition (no capsaicin), the initial desensitization period was skipped and testing began at the warm-up step.

During the test period, capsaicin was reapplied such that heat sensitization was present but capsaicin irritation was not. To accomplish this, capsaicin was reapplied using small cotton swabs. After a 4-min rest period, subjects rated their irritation (to ensure irritation levels had dropped to less than 1) (Figure 2). In this desensitized state, as in Experiment 2, subjects rated pain evoked by a heat stimulus at one of the temperatures (Figure 2) and then rated pain elicited by a second stimulus at a different temperature, 1.5 min later. This 1.5-min interstimulus interval was chosen because it allows enough time for nociceptors and pain sensation to recover from fatigue (Beitel and Dubner 1976; Price et al. 1977).

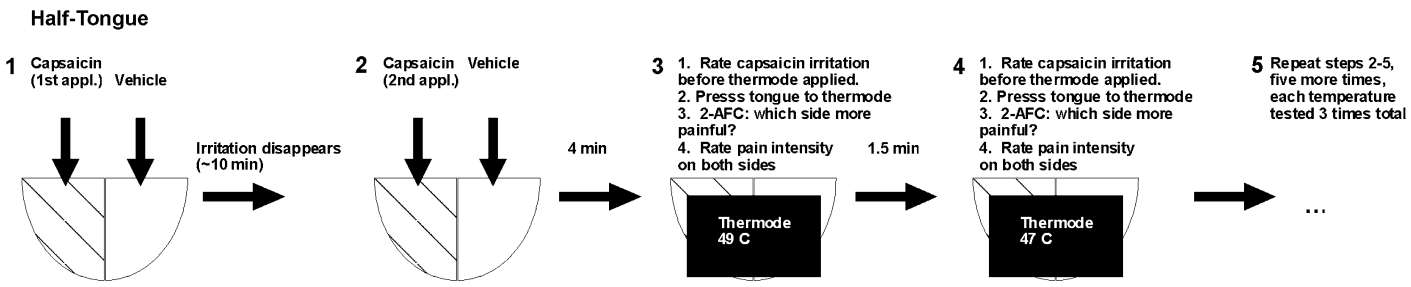

Figure 2 Flow diagram showing capsaicin application and thermal testing procedure for half-tongue condition.

Capsaicin was then reapplied, followed by a 4-min rest and a pair of heat stimuli just as before (Figure 2). This pattern was followed for a total of 6 rounds. Therefore, a total of 12 test stimuli were delivered in each session (6 blocks in which 2 different temperatures were delivered in each block). Each individual temperature was rated 3 times per session, and temperatures were randomized and counterbalanced for early, middle, and late presentation within a session.

Data analysis

Experiments 1 and 2

For 2-AFC data in Experiments 1 and 2, the total number of subjects choosing the irritant-treated side as more painful was counted and analyzed for significance from binomial tables. Thus, if a significant majority of subjects chose the irritant-treated side as more painful, this was interpreted as sensitization of heat or cold pain. A minimum of 30 subjects was used for each 2-AFC procedure as the binomial distribution approaches a normal distribution with this number of observations. If the 2-AFC data resulted in a significant difference, d' was calculated (Ennis 1993) to estimate the magnitude of the difference in heat pain perception between sides in each condition. Intensity ratings of heat pain for the irritant-treated and vehicle-treated sides were compared with repeated-measures analysis of variance (ANOVA) (SPSS 14.0 software, Chicago, IL) followed, where appropriate, by post hoc Bonferroni tests.

Experiment 3

Ratings data were subjected to a regression analysis such that the slope of the temperature–response function was calculated for every replication performed by each subject. These data were then subjected to a repeated-measures ANOVA with subject, replication, and treatment serving as main effects. The ANOVA procedure was conducted separately for the half-tongue and whole-tongue conditions.

Results

Thermal difference detection

In the first experiment, 90% of subjects could discriminate an actual 0.5 °C temperature difference in both hot and cold sessions (Figure 3). A significant number of subjects (111 out of 124 subjects, $P < 0.001$) chose the hotter side correctly (Figure 3, left-hand open bar). The mean intensity ratings were also significantly greater for the hotter side (Figure 3, left-hand black and gray bars, $P < 0.001$). A significant number of subjects (111 out of 123 subjects, $P < 0.001$) also chose the colder side correctly (Figure 3, right-hand open bar), and mean intensity ratings were also significantly different (Figure 3 right-hand black and gray bars; $P < 0.001$). The d' values were not significantly different for hot (1.77) and cold (1.83) thermal difference detection tasks.

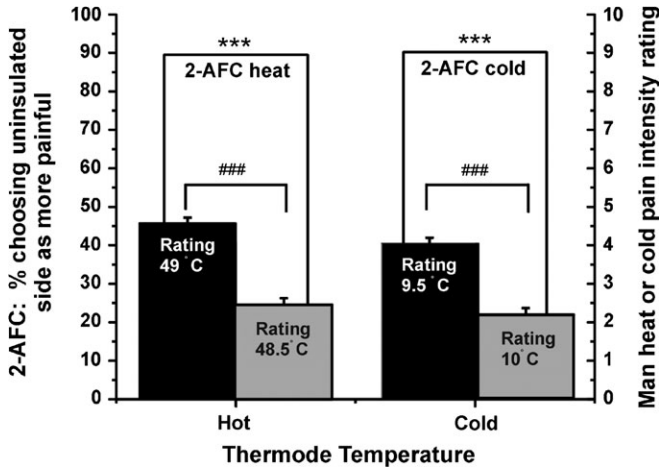

Figure 3 Detection of thermal difference. Open left-hand bar: percentage of subjects choosing the uninsulated (hotter) side of the tongue as more painful in 2-AFC (left-side *y* axis). Open right-hand bar: percent choosing uninsulated (colder) side as more painful in 2-AFC. Lower pairs of bars show mean intensity ratings of heat pain (left side) and cold pain (right side) for higher (black bars) and lower temperatures (gray bars) (right-side *y* axis). $***P < 0.001$, binomial (2-AFC). $^{###}P < 0.001$, paired *t*-test (ratings). Error bars here and in all subsequent figures: standard error of the mean.

Experiment 1: menthol

Pretreatment with menthol slightly but significantly reduced heat pain (Figure 4A) and significantly increased the perceived intensity of cold pain (Figure 4B). Although a minority of subjects (12 out of 32) chose the vehicle-treated side as having greater heat pain (i.e., the majority chose the menthol-treated side as less painful) in the 2-AFC (Figure 4A, open bars), this did not reach statistical significance. However, heat pain intensity ratings were significantly lower on the menthol-treated side (ANOVA, $F_{1,96} = 11.5$, $P < 0.001$) across all time points (Figure 4A, open squares) even though the mean rating difference between the 2 sides (0.39) was small. There was no treatment × time interaction indicating that the difference between the 2 sides did not change over time. For cold pain, the menthol-pretreated side was chosen as more painful by a significant majority (Figure 4B, open bars, 22 out of 32 subjects) at 1.5 and 5 min, although not immediately, suggesting that menthol's effect takes time to develop. The magnitude of cold pain enhancement was equivalent at 1.5 and 5 min ($d' = 0.69$ for both time points). In agreement with the 2-AFC results, cold pain intensity ratings were significantly higher on the menthol-treated side ($F_{1,93} = 29.6$, $P < 0.001$). However, the treatment × time interaction term was not significant, indicating that the difference in pain between sides was similar across all time points (Figure 4B, open squares).

Experiment 1: capsaicin

Capsaicin enhanced the perceived intensity of heat but not cold pain. A significant proportion of subjects chose the

capsaicin-pretreated side as more painful initially and at 1.5 min (Figure 5A, open bars, 23 out of 30 and 27 out of 30 for initial and 1.5 min time points). The magnitude of capsaicin enhancement of heat pain was greatest at 1.5 min ($d' = 1.81$). Heat pain ratings were significantly higher on the capsaicin-pretreated side ($F_{1,87} = 51.66$, $P < 0.001$) of the tongue and the significant ($F_{3,87} = 10.51$, $P < 0.001$) treatment × time interaction term suggested that the difference in pain ratings was higher at 1.5 and 5 min. Capsaicin did not affect cold pain at any time point (Figure 5B).

Experiment 1: mustard oil

Mustard oil enhanced the perceived intensity of heat pain. A significant proportion of subjects (23 out of 30) chose the mustard oil–treated side as more painful initially and at 1.5 min

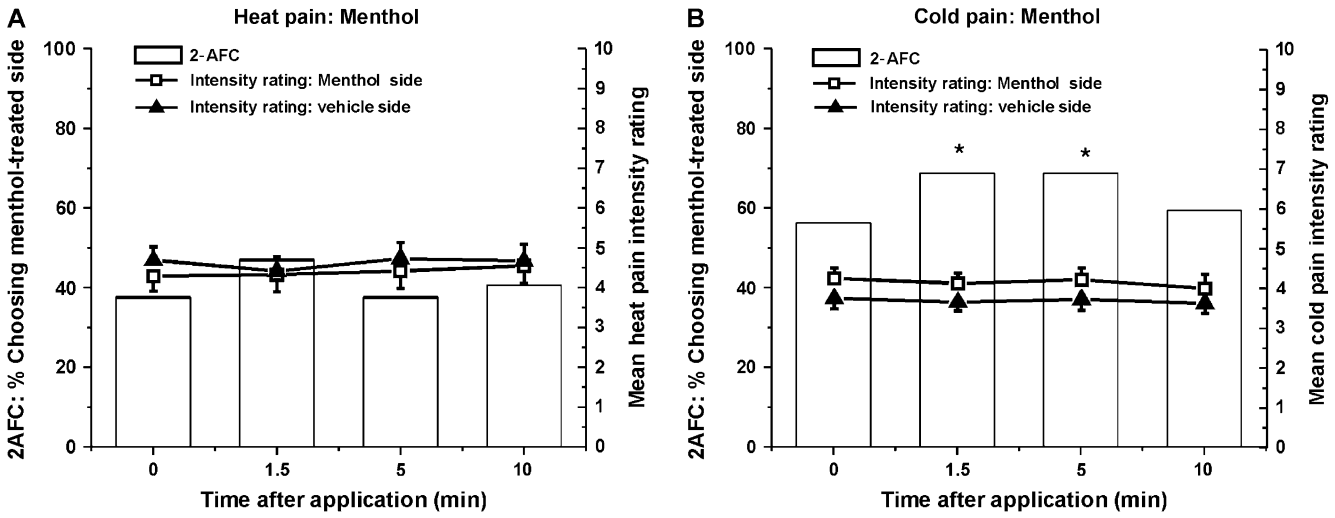

Figure 4 Menthol enhancement of cold pain and weak reduction of heat pain. **(A)** Heat pain. 2-AFC (open bars): percentage of subjects choosing menthol-treated side of the tongue as hotter (left axis) versus time after menthol application. Mean heat pain intensity ratings (line graphs) for the menthol-treated (open squares) and vehicle-treated side (black triangles) versus time after application of menthol. Error bars: standard error of the mean. Menthol did not affect heat pain in the 2-AFC, but weakly reduced heat pain intensity ratings (ANOVA, $F_{1,96} = 11.5$, $P < 0.001$) across all time points. **(B)** Cold pain (format as in A). Menthol enhanced cold pain at 1.5 and 5 min. $*P < 0.05$ for 2-AFC (binomial test). Cold pain intensity ratings were significantly higher on the menthol-treated side ($F_{1,93} = 29.6$, $P < 0.001$).

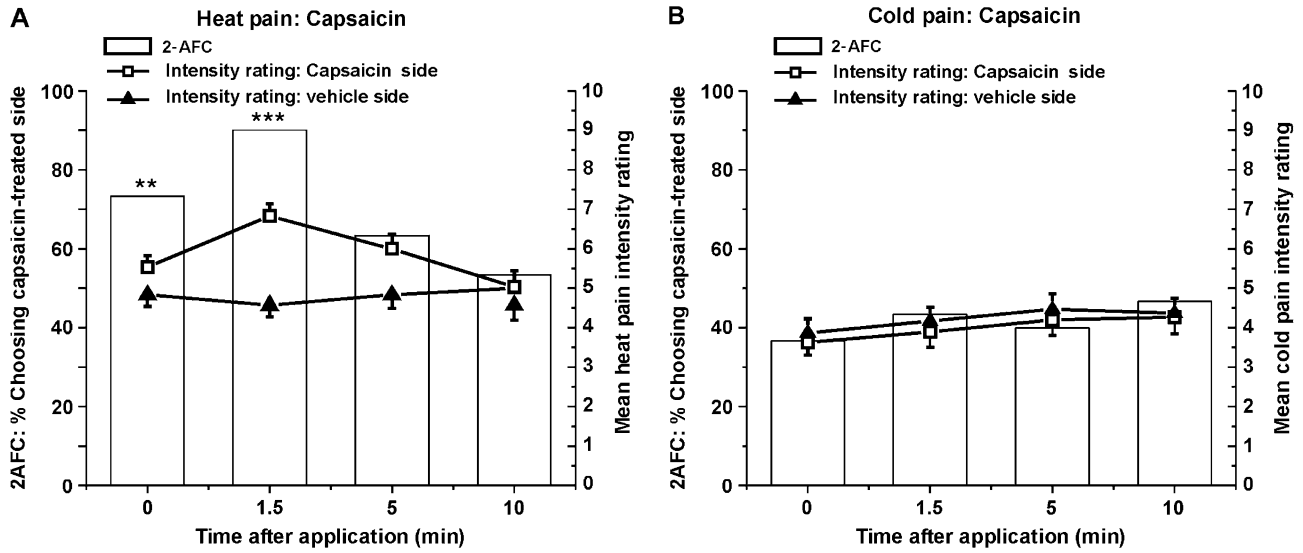

Figure 5 Capsaicin enhancement of heat but not cold pain. **(A)** Heat pain (format as in Figure 3A). $**P < 0.01$, $***P < 0.001$ for 2-AFC (binomial test). Heat pain ratings were significantly higher on the capsaicin-pretreated side ($F_{1,87} = 51.66$, $P < 0.001$) of the tongue. **(B)** Cold pain (format as in Figure 3B). Capsaicin had no effect on cold pain.

(Figure 6A, open bars). Across all time points, mustard oil evoked significantly higher pain ratings compared with vehicle (Figure 6A, open squares; ANOVA, $F_{1,90} = 19.3$, $P < 0.001$).

Mustard oil initially enhanced cold pain in that a significant proportion of subjects (21 out of 30) chose the mustard oil–treated side as more painful immediately after application (Figure 6B, open bars). Although there was no significant effect of mustard oil ($F_{1,87} = 1.65$, $P = 0.198$) on cold pain ratings, there was a significant time × treatment interaction ($F_{3,87} = 6.7$, $P < 0.001$) indicating that mustard oil cold pain ratings decreased across time, whereas vehicle cold pain ratings stayed relatively constant.

Experiment 1: cinnamaldehyde

Cinnamaldehyde enhanced heat pain (Figure 7A). A significant proportion of subjects (22 out of 32) chose the cinnamaldehyde side as more painful initially and at 1.5 min (Figure 7A, open bars), although there was no significant effect of cinnamaldehyde on heat pain ratings ($F_{1,186} = 2.9$, $P = 0.093$). A significant treatment × time interaction term ($F_{3,186} = 4.54$, $P = 0.004$) indicated that the pain ratings on the cinnamaldehyde-treated side tended to decrease across time, whereas those on the vehicle-treated side tended to slightly increase (Figure 7A).

Cinnamaldehyde significantly enhanced cold pain ratings overall (Figure 7B, ANOVA, $F_{1,183} = 5.15$, $P = 0.024$), although the difference in mean ratings between the 2 sides was small (0.29) and was not confirmed by the 2-AFC data (Figure 7B, open bars).

Experiment 2: capsaicin desensitization

This paradigm allowed us to test if heat pain elicited by the thermode was enhanced by capsaicin pretreatment in the absence of its burning sensation. Twenty-nine of the 32 subjects exhibited desensitization of capsaicin irritation prior to at least 1 trial with heat. Data from 4 subjects were excluded from analysis because they did not rate the irritation elicited by the second application of capsaicin as 0 or 1 at any time throughout the 10-min duration of the experiment. The number of subjects reporting capsaicin irritation as 1 or 0 increased progressively for each time point of heat testing (Figure 8, numbers in parentheses). At each time point, a significant majority chose the capsaicin-treated side as more painful (Figure 8, open bars). Mean heat pain intensity ratings were significantly higher on the capsaicin-treated side across all time points (Figure 8, open squares; ANOVA, $F_{1,51} = 150.3$, $P < 0.001$). The significant treatment × time interaction term ($F_{3,51} = 6.9$, $P < 0.001$) indicated that the propensity of capsaicin to enhance heat pain decreased over time. The enhancement of heat pain in the desensitized condition was greater and of longer duration compared with Experiment 1. For the initial and 1.5-min time points, d' was infinite as 100% of subjects chose the capsaicin-treated side, in comparison with Experiment 1 for which the corresponding d' values were 0.87 and 1.81. The d' values for the 5- and 10-min time points were 1.76 and 1.54, compared with an absence of effect at these time points in Experiment 1.

Experiment 3: capsaicin enhancement of heat pain across the noxious range

Half-tongue condition

All subjects reported ratings of <1 for capsaicin irritant intensity prior to the heat stimulus under both half-tongue and whole-tongue conditions. Although subjects differed ($F_{15,30} = 26.6$, $P < 0.001$) in the intensity of perceived pain,

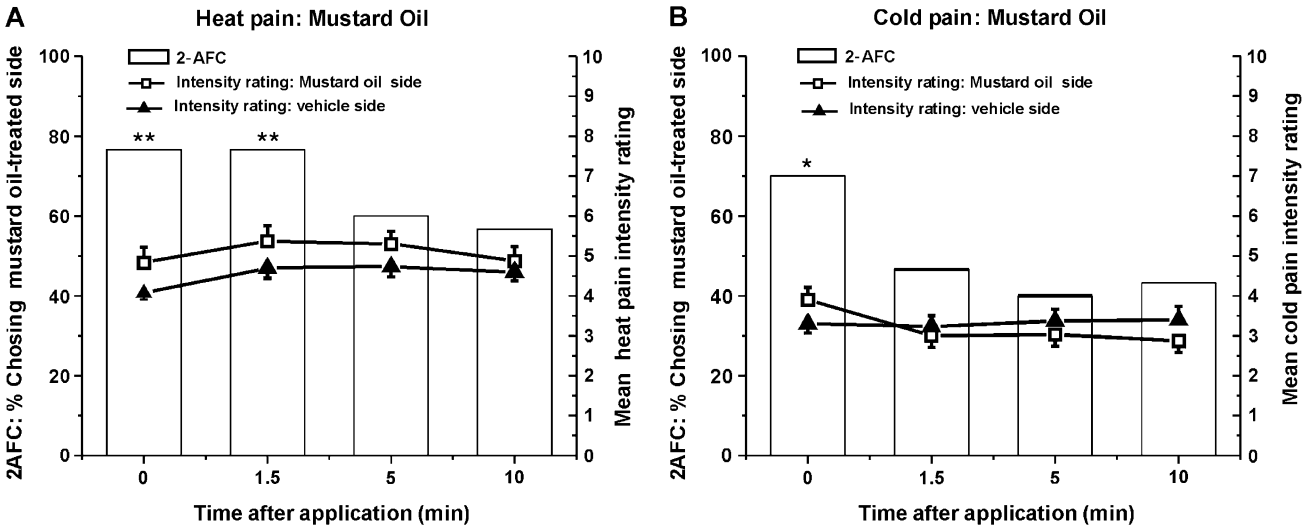

Figure 6 Mustard oil enhancement of heat and cold pain. **(A)** Heat pain (format as in Figure 3A). $^{**}P < 0.01$, binomial (2-AFC). Across all time points, mustard oil evoked significantly higher pain ratings compared with vehicle (open squares; ANOVA, $F_{1,90} = 19.3$, $P < 0.001$). **(B)** Cold pain (format as in Figure 3B). Mustard oil transiently enhanced cold pain in the 2-AFC.

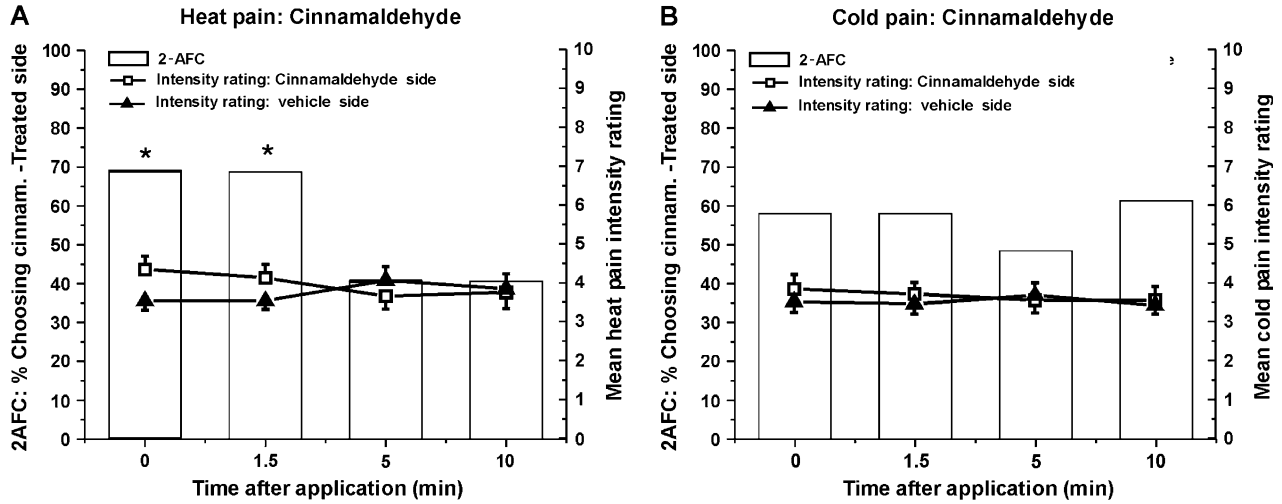

Figure 7 Cinnamaldehyde enhancement of heat and cold pain. **(A)** Heat pain (format as in Figure 3A). *$P < 0.05$, binomial (2-AFC). **(B)** Cold pain (format as in Figure 3B). Cinnamaldehyde slightly enhanced cold pain ratings overall (ANOVA, $F_{1,183} = 5.15$, $P = 0.024$) although this was not significant in the 2-AFC.

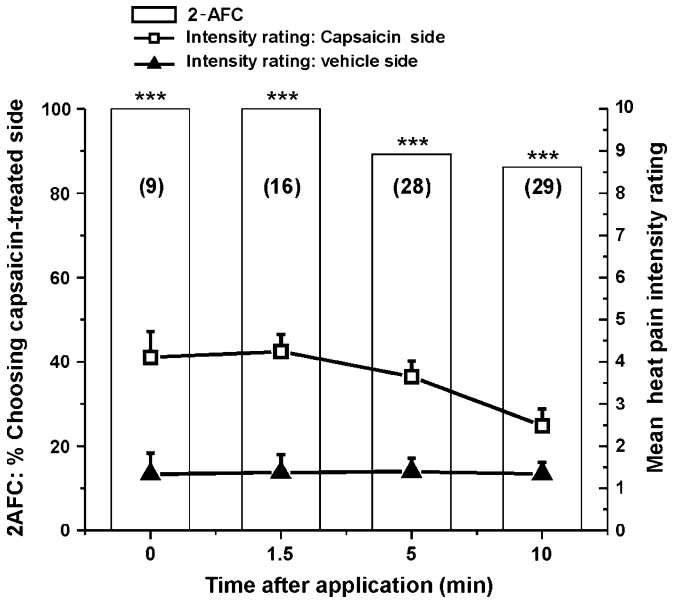

Figure 8 Capsaicin enhancement of heat pain in desensitized tongue. 2-AFC (open bars): percentage of subjects choosing capsaicin-treated side of the tongue as hotter (left axis) versus time after second capsaicin application. Tongue was pretreated with capsaicin prior to second capsaicin application to induce self-desensitization. Numbers in parentheses: number of subjects rating second capsaicin application as 0 or 1. Line graphs show mean heat pain intensity ratings for the capsaicin-treated (open squares) and vehicle-treated side (black triangles) versus time after application of capsaicin. ***$P < 0.001$, binomial (2-AFC). Mean heat pain intensity ratings were significantly higher on the capsaicin-treated side at all time points (open squares; ANOVA, $F_{1,51} = 150.3$, $P < 0.001$).

the slopes of the stimulus–response curves were remarkably stable across replications ($F_{2,30} = 1.5$, $P = 0.233$). The mean slope of the temperature–response function on the capsaicin-treated side of the tongue (1.7 ± 0.2) was significantly ($F_{1,30} = 128.3$, $P < 0.001$) steeper than that obtained on the vehicle-treated side (1.1 ± 0.2) (Figure 9A). Even when the 47 °C data point (essentially 0) was removed on the vehicle-treated side, the slope differences remained significant indicating that capsaicin pretreatment resulted in a leftward shift and increased slope of the stimulus–response function relative to the vehicle-treated side (Figure 9A). Interestingly, the slope on the capsaicin-treated side was not different from the average slope obtained under the whole-tongue condition (see below), suggesting that the slope on the vehicle-treated side was reduced under the half-tongue condition.

Whole-tongue condition

As in the half-tongue condition, there was a significant effect of subject ($F_{15,30} = 7.3$, $P < 0.001$) but not replication ($F_{2,30} = 0.07$, $P = 0.929$), indicating that although differences existed among individual subjects' ratings, the slopes of the temperature–response functions were stable across replications ($F_{2,30} = 0.07$, $P = 0.929$). However, in contrast to the half-tongue condition, capsaicin applied to the whole tongue did not significantly affect ($F_{1,30} = 2.1$, $P = 0.160$) the slope of the temperature–response curve (1.6 ± 0.2), which was not different from that obtained under the whole-tongue vehicle condition (1.7 ± 0.2) (Figure 9B).

Discussion

Detection of a thermal difference

Subjects reliably (90%) detected a thermal difference of 0.5 °C across the tongue, consistent with previous studies. Handwerker et al. (1982) reported detection rates of ~77% for a 0.4 °C difference and ~90% for a 0.8 °C difference superimposed on a 46 °C background temperature on forearm skin. Another study reported that 75% of subjects detected

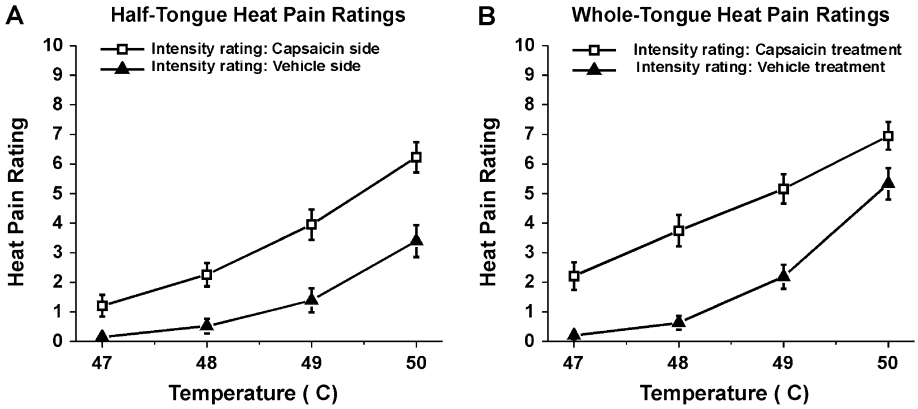

Figure 9 Heat pain stimulus–response functions. **(A)** Half-tongue condition. Graph plots mean heat pain ratings versus stimulus temperature (n = 16 subjects). Open squares: capsaicin-treated side; filled triangles: vehicle-treated side. Error bars: standard error of the mean. **(B)** Whole-tongue condition. Graph as in (A) for ratings when capsaicin (open squares) or vehicle (filled triangles) was applied to both sides of the tongue.

an ~0.5 °C difference on a 38 °C background temperature and an ~0.1 °C difference on a 47 °C background (Robinson et al. 1983). A difference detection of 0.74 °C (on a 32 °C background) was reported for the tongue tip (Grushka et al. 1987).

The ability of a significant proportion of subjects (90%) to detect a small temperature difference (0.5 °C) between the 2 sides validated the 2-AFC paradigm. As most of the significant effects were detected by less than 90% of subjects, we estimate that the thermal sensitization we observed is equivalent to a temperature difference of 0.5 °C or less. The half-tongue 2-AFC paradigm has been validated previously for distinguishing sweet intensities using the sweet blocker sylvestre gymneste (Simons et al. 2002), carbonation tingling using a carbonic anhydrase blocker (Dessirier et al. 2000), and nicotine irritation using the nicotinic receptor blocker mecamylamine (Dessirier et al. 1998).

Menthol

Menthol significantly enhanced cold pain, consistent with an older psychophysical study showing that menthol enhanced perceived oral cold sensations at temperatures down to 10 °C (Green 1985) as well as more recent studies reporting cold hyperalgesia following cutaneous application of 30–40% menthol (Wasner et al. 2004; Namer et al. 2005; Hatem et al. 2006). Menthol also weakly reduced heat pain, consistent with common experience that cooling the oral cavity reduces the burning sensation associated with heat or spicy food. However, other recent studies reported that menthol had minimal or no effect on cutaneous heat pain (Wasner et al. 2004; Namer et al. 2005; Hatem et al. 2006) or ratings of nociceptive oral sensations elicited by heating up to 45 °C (Green 2005). Earlier studies reported menthol to either enhance or reduce sensations of innocuous warmth (Green 1985, 1986, 1992).

We recently reported the presence of lamina I neurons in trigeminal subnucleus caudalis (Vc) that respond to noxious heating and innocuous and noxious cooling of the tongue (Zanotto et al. 2006). These Vc neurons also responded to menthol in a desensitizing temporal pattern consistent with its oral irritant sensation (Cliff and Green 1994; Dessirier et al. 2001). Menthol strongly enhanced the responses of these Vc neurons to cooling but not noxious heating of the tongue (Zanotto et al. 2006), similar to our present data showing menthol enhancement of cold pain with only a small effect on heat pain. The properties of these Vc neurons are consistent with a role in neural circuits that mediate the nociceptive sensations elicited by lingual menthol and cooling. The divergent effects of menthol on cold versus heat pain argue in favor of a peripheral site of action and against central sensitization. Otherwise, one would expect that both heat and cold pain would be enhanced because these qualities may be signaled by a common population of spinal cord neurons responding to both noxious heat and noxious cold (Khasabov et al. 2001).

Menthol activates the cold-transducing ion channel TRPM8 and enhances cold-evoked currents (McKemy et al. 2002; Peier et al. 2002). It has been known for some time that menthol enhances responses of cold receptors (Hensel and Zottermann 1951; Schafer et al. 1986) that presumably express TRPM8. Noxious cold stimuli excite unmyelinated and thinly myelinated polymodal nociceptors that also respond to noxious heat (Bessou and Perl 1969; LaMotte and Thalhammer 1982; Campero et al. 1996; Simone and Kajander 1996, 1997), and it is currently uncertain if polymodal nociceptors express TRPM8. Dorsal root ganglion (DRG) neurons expressing TRPM8 were activated by noxious cold stimulation (Mizushima et al. 2006). About 50% of cold- and menthol-sensitive DRG and trigeminal ganglion cells additionally responded to capsaicin (McKemy et al. 2002; Reid et al. 2002; Viana et al. 2002; Xing et al. 2006), suggesting that they may be nociceptors coexpressing TRPM8 and TRPV1. However, recent studies using in situ hybridization (Kobayashi et al. 2005) and immunohistochemistry (Abe

et al. 2005) reported low incidences (1.5–4.6%) of coexpression of TRPM8 and TRPV1 (or their mRNAs) in DRG or trigeminal ganglion neurons. Thus, cold pain that was presently observed to be enhanced by menthol might be mediated via activation of TRPM8-expressing polymodal nociceptors or innocuous cold receptors, assuming that the latter have access to central neurons involved in signaling pain. Both possibilities are supported by reports that neurons in lamina I of spinal cord or Vc that respond to noxious heat and pinch also respond to cooling at variable thresholds both in the innocuous and noxious ranges (Craig et al. 2001; Zanotto et al. 2006; Zhang et al. 2006).

If menthol enhances cold pain via TRPM8 ion channels expressed in cold receptors or polymodal nociceptors, the mechanism is obscure. Speculatively, action of menthol at its binding site on TRPM8 may enhance thermal gating of the ion channel by an as yet poorly understood interaction.

Innocuous cooling of the skin can elicit nociceptive sensations of stinging, burning, or pricking (Green and Pope 2003). Innocuous cooling of skin pretreated with 10% menthol enhanced such nociceptive sensations when the cooling probe statically contacted the skin, whereas dynamic contact of the cool thermode against menthol-pretreated skin for 5 s reduced the nociceptive sensations, a phenomenon termed contact suppression (Green and Schoen 2007). In the present experiment, the tongue was cooled by dynamic contact, which would be expected to reduce nociceptive sensations on the menthol-pretreated side. Despite the likely presence of contact suppression, subjects nonetheless rated the menthol-treated side as having stronger cold pain. This implies that menthol enhancement of cold pain exceeded the effect of contact suppression and/or that contact suppression observed in the innocuous cool range (20 °C to 28 °C) is weaker or absent at noxious cold temperatures.

Mustard oil and cinnamaldehyde

Mustard oil and cinnamaldehyde significantly enhanced heat pain, consistent with previous studies reporting that these agents produce heat hyperalgesia (Koltzenburg et al. 1994; Schmelz et al. 1996; Olausson 1998; Namer et al. 2005). Both irritants also weakly enhanced cold pain, possibly consistent with central sensitization which mustard oil is well known to induce (Woolf et al. 1994). It was recently reported that cutaneous application of 10% cinnamaldehyde resulted in heat hyperalgesia and cold ''hypoalgesia'' (Namer et al. 2005). The difference between this latter finding and our finding that cinnamaldehyde weakly enhanced cold pain may be attributed to the different concentrations as well as site (forearm vs. tongue) and method of application. One speculative mechanism of mustard oil and cinnamaldehyde sensitization of heat pain is via a TRPA1-mediated enhancement of thermal gating of TRPV1, which is invariably coexpressed with TRPA1 in sensory neurons (Peier et al. 2002; Story et al. 2003; Kobayashi et al. 2005).

Both mustard oil and cinnamaldehyde act at TRPA1 ion channels which have been implicated in transducing noxious cold (Bandell et al. 2004; Jordt et al. 2004; Kwan et al. 2006). However, neither agent evoked cold sensations but instead elicited sensations of burning pain (Cliff and Heymann 1992; Prescott and Swain-Campbell 2000; Namer et al. 2005). The transient and weak enhancement of cold pain by mustard oil and cinnamaldehyde might be consistent with an action via TRPA1. However, a role for TRPA1 in transducing noxious cold has been challenged (Jordt et al. 2004; Bautista et al. 2006) and remains unresolved.

Capsaicin

Capsaicin significantly enhanced heat pain but not cold pain. This finding is consistent with prior psychophysical studies showing that intradermal capsaicin enhanced heat pain intensity within a small region around the injection site for up to 2 h (LaMotte et al. 1991, 1992; Torebjork et al. 1992). Topical cutaneous application of capsaicin resulted in an initial heat pain sensitization followed by long-term desensitization, with no effect on cold pain (Simone and Ochoa 1991); this is consistent with our results. In the oral cavity, capsaicin enhanced warmth and reduced innocuous cold; the burning sensation elicited by capsaicin (2 ppm) was enhanced in a temperature-dependent manner from 37 °C to 45 °C (Green 1986). In a more recent study, pretreatment of the tongue with capsaicin (300 or 900 μM) significantly reduced the intensity of cold- and heat-evoked nociceptive sensations tested 15 min later (Green 2005). This is not inconsistent with our results, which showed enhancement of heat pain during the initial 5-min period post-capsaicin that abated within 10 min (Figure 5). TRPV1 responds to temperatures above the pain threshold (Caterina et al. 1997), and the present results might be explained by a capsaicin enhancement of thermal gating of TRPV1 expressed in polymodal nociceptors mediating thermal pain sensation. Moreover, in Experiment 2, subjects were desensitized to capsaicin irritation yet still exhibited significant heat pain sensitization. This implies that the first application of capsaicin rendered TRPV1 insensitive to further activation by capsaicin at the presumed TM3–TM4 binding site on the intracellular side of the membrane (Jordt and Julius 2002) while rendering TRPV1 more sensitive to activation by heat presumably at a separate site.

The results of Experiment 2 showed that heat pain was enhanced by capsaicin applied to the predesensitized tongue so that it elicited minimal or no burning sensation. This argues against one potential explanation for the capsaicin enhancement of heat pain observed in Experiment 1, whereby subjects summed the perceptually similar heat sensations elicited by capsaicin and the 49 °C thermode to formulate their overall intensity rating. It is noteworthy that capsaicin enhancement of heat pain was greater in Experiment 2 compared with Experiment 1 because the desensitized subjects reported a

larger difference in intensity ratings between capsaicin- versus vehicle-treated sides, d' values were greater, and enhancement of heat pain persisted longer. For this reason, and also to assess capsaicin enhancement of heat pain over a wider range of stimulus intensities, we undertook Experiment 3. The results were generally consistent with Experiment 2 in that under the half-tongue application condition, heat pain ratings on the vehicle-treated side were lower compared with the condition of whole-tongue vehicle application (Figure 9A,B, respectively, filled triangles). Although we are uncertain as to the cause of this difference, we offer a few speculative possibilities. One is that the half-tongue condition provides a more accurate comparison of the difference in sensations experienced simultaneously on each side compared with whole-tongue ratings with or without capsaicin as assessed on separate days. Another possibility is that the use of the same scale in Experiments 2 and 3 to rate the intensity of both capsaicin irritation and heat pain may have led subjects to provide lower ratings for the latter. This is because the lower bound of the irritation scale was "no sensation," whereas that for pain was "no pain." Subjects with relatively little training in the use of such scales may have had difficulty in shifting context within the same session using scales with quite different end points. This would be avoided by the use of a single scale for both sensory qualities.

In Experiments 2 and 3, we assumed that capsaicin desensitization would eliminate irritancy from capsaicin, but we did not make any a priori assumptions about the effect on heat pain. The results show clearly that capsaicin pretreatment enhanced, rather than lowered, heat pain, via potential mechanisms discussed earlier. Under the whole-tongue condition of Experiment 3, capsaicin pretreatment resulted in a parallel leftward shift of the stimulus–response function with enhanced ratings of suprathreshold stimuli and a lower extrapolated heat pain threshold, consistent with thermal hyperalgesia. This result indicates that while the intensity of heat pain is increased, discriminability of small temperature changes in the noxious range is not altered by capsaicin.

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