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Neurons in Superficial Trigeminal Subnucleus Caudalis Responsive to Oral Cooling, Menthol, and Other Irritant Stimuli

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Zanutto KL, Merrill AW, Carstens MI, Carstens E. Neurons in superficial trigeminal subnucleus caudalis responsive to oral cooling, menthol and other irritant stimuli. *J Neurophysiol* 97: 966–978, 2007. First published December 6, 2006; doi:10.1152/jn.00996.2006. The recent discoveries of cold-sensitive transient receptor potential (TRP) channels prompted us to investigate the responses of neurons in trigeminal subnucleus caudalis (Vc) to intraoral cooling and agonists of TRPM8 and TRPA1. Single units responsive to lingual cooling were recorded in superficial laminae of Vc in thiopental-anesthetized rats. All units responded to noxious heat and 88% responded to menthol. Responses increased with menthol concentration from 0.1 to 1% (6.4–64 mM) and plateaued at 10% (640 mM). Noxious cold-evoked responses were significantly enhanced after menthol in a concentration-dependent manner. Constant-flow application of 1% menthol elicited a phasic discharge that adapted over 2–8 min and significantly enhanced subsequent cold-evoked but not heat-evoked responses; vehicle (10% ethanol) was ineffective. Reapplication of menthol 15 min later elicited a significantly reduced response (self-desensitization). Vc units were similarly excited phasically by 1% menthol dissolved in 40% ethanol. The 40% ethanol briefly excited Vc units during the first minute and reduced subsequent responses to noxious heat and cold while exhibiting neither self-desensitization nor cross-desensitization to menthol. Menthol cross-desensitized Vc responses to 40% ethanol. Most menthol-responsive units also responded to the TRPA1 agonists cinnamaldehyde and mustard oil, and the TRPV1 agonist capsaicin. Units in superficial Vc receive convergent input from primary afferents that express TRPM8, TRPA1, and/or TRPV1 channels, either directly or indirectly via intersubnuclear pathways. The convergent nature of these units suggests a general role in signaling noxious stimuli.

INTRODUCTION

Menthol is a popular additive to many foods and alcoholic beverages and is used as well in oral hygiene, tobacco, and other consumer products because it imparts a cool fresh sensation. Menthol is known to excite peripheral cold receptors (Hensel and Zotterman 1951; Schafer et al. 1986) by interacting with the cold-sensitive transient receptor potential (TRP) channel TRPM8 (McKemy et al. 2002; Peier et al. 2002). In addition to its cooling action, menthol imparts an irritant sensation at higher concentrations (Cliff and Green 1994, 1996; Dessirier et al. 2001; Green and McAuliffe 2000). Recent evidence indicates that TRPM8 may be expressed in two distinct populations of cold-sensitive dorsal root ganglion (DRG) neurons, one sensitive to menthol but not capsaicin and the other sensitive to menthol, capsaicin, ATP, and acidic stimuli; the former were suggested to be cold receptors and the latter nociceptors (Xing et al. 2006). Trigeminal nociceptors

and thermoreceptors innervating the oral cavity project to trigeminal subnucleus caudalis (Vc) where they excite second-order neurons, including neuronal populations in superficial laminae that respond to cooling or warming (Dickenson et al. 1979; Dostrovsky and Hellon 1978; Hutchison et al. 1997) or to noxious heat and irritant chemicals (Carstens et al. 1998; Meng et al. 1997) with some also responding to cold (Carstens et al. 1998; McHaffie et al. 1994). The latter nociceptive neurons appear similar to polymodal or HPC type spinothalamic tract neurons in lamina I of lumbar or cervical spinal cord that respond in a graded manner to cooling as well as noxious heating (Craig et al. 2001; Zhang et al. 2006). Because very little is known about central trigeminal neurons that may convey the cooling and irritant qualities of intraoral menthol, we presently investigated if menthol excites cold-sensitive neurons in superficial laminae of Vc. We hypothesized that menthol would excite cold- and noxious heat-responsive Vc neurons and would enhance responses to subsequent cooling consistent with menthol sensitization of cold receptors (Schafer et al. 1986) via TRPM8 (McKemy et al. 2002). Because many mentholated products such as mouthwash or liquors also contain the solvent ethanol, we additionally investigated interactions between menthol and ethanol on Vc neuronal activity. We further tested if cold- and menthol-sensitive Vc neurons additionally respond to other irritant chemicals known to act at different thermosensitive TRP channels, including cinnamaldehyde and mustard oil that act at TRPA1 (Bandell et al. 2004; Jordt et al. 2004) and capsaicin and ethanol that act at TRPV1 (Caterina et al. 1997; Trevisani et al. 2002). An abstract of portions of this work has appeared (Carstens et al. 2005).

METHODS

Surgery

These experiments were conducted under a protocol approved by the University of California, Davis Institutional Animal Care and Use Committee. Forty-eight male Sprague-Dawley rats, 405–611 g, were anesthetized with thiopental (85 mg/kg ip). After induction, a catheter was placed in either the external jugular vein or the lateral tail vein, and anesthesia was maintained by constant intravenous infusion of thiopental at a rate sufficient to maintain areflexia as described previously (Carstens et al. 1998; Dessirier et al. 2000). The electrocardiogram (EKG) was recorded continually, and core body temperature was monitored and maintained by external heat. A laminectomy exposed the upper cervical spinal cord and lower brain stem to allow access to Vc (for details, see Carstens et al. 1998; Dessirier et al. 2000).

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Recording and stimulation

Extracellular single-unit recordings were made using an insulated tungsten microelectrode (Frederick Haer, Bowdoin, ME) driven into the caudal medulla by hydraulic microdrive (Kopf Instruments, Tujunga, CA) to a depth of ~80–150 μm . Action potentials were amplified by conventional means and fed through a Powerlab interface (AD Instruments, Grand Junction, CO) to a computer for continuous display on one channel with the EKG and tongue temperature simultaneously displayed on two other channels, using Chart 5.0 software (ADInstruments). Action potential data were simultaneously routed to a second computer that displayed each digitized action potential as well as spike trains on-line; data were saved to the hard drive for off-line spike sorting using custom software (Forster and Handwerker 1990).

Thermal stimulation

Units were isolated that responded to ice water applied to the anterior tongue. In some experiments, ice water (~3°C) and water heated to 53°C delivered in a ~0.25-ml bolus to the tongue served as noxious cold and hot stimuli, respectively. The tongue surface temperature was monitored with a thermocouple (IT-21; 0.08-s time constant; Physitemp Instruments, Clifton, NJ). The ice water produced a rapid drop in tongue surface temperature that recovered within ~30 s (see example in Fig. 3A, *bottom*), and typically elicited a response of similar duration (see Fig. 3A, *2nd and 3rd traces from top*). In most experiments, a feedback-controlled Peltier thermode (Physitemp NTE-2A, 13 mm diam) was placed against the anterior tongue to allow computer-controlled delivery of noxious heat (53°C) or cold (to -10°C) stimuli from an adapting temperature of 34°C. The temperature at the tongue-thermode interface was continually monitored by a separate thermocouple (IT-21; Physitemp) connected to a BAT-12 thermometer (Physitemp) the output of which was routed through the Powerlab interface and simultaneously displayed on the computer screen along with the action potential data and EKG using Chart 5.0. In most experiments using the thermode, a single ramp decrease in temperature from 36 to -10°C was delivered, followed later by a ramp increase from 36 to 58°C. In the cooling mode, the Peltier thermode produced a fairly slow decline in temperature (see examples in Fig. 3, *B and C, bottom*) that allowed an estimation of the response threshold, taken as the tongue-thermode interface temperature at which the firing rate increased by two- to threefold (see examples in Fig. 3, *B and C, 2nd traces from top*). In the heating mode, the temperature increased at a rate of ~10°C/s, and thresholds were determined in the same manner. In several experiments, graded cooling and heating stimuli were delivered. For graded cooling, the tongue temperature was successively lowered in 10°C increments from 35 to 25, 15, 5, and -5°C, with a 30-s stimulus duration at each temperature and a 5-s period to change temperature. For graded heating, the thermode was programmed to increase from an adapting temperature of 36–42, 48, and 54°C for 10 s at each temperature with a 90-s duration between successive heat stimuli. Although the highest temperatures are noxious, they were brief and did not appear to produce tissue damage or result in increased background firing (see Figs. 7–10). The 90-s interstimulus interval was based on a previous report that responses of primate facial nociceptors to repeated noxious heat stimuli ($\leq 55^\circ\text{C}$) were stable at interstimulus intervals >90 s while exhibiting fatigue (desensitization) at shorter interstimulus intervals (Beitel and Dubner 1976). The intra-epithelial temperature near the nociceptive endings was lower (for heating) or higher (for cooling) than the thermode-tongue interface temperature; we did not presently attempt to measure to the thermal gradient using an intra-epithelial thermistor to avoid tissue injury.

Chemical stimulation

The following chemicals were used: L-menthol (0.1–10%, 6.4–640 mM, Givaudan, Cincinnati OH) dissolved in 40% ethanol, or in 10%

ethanol/1% polyoxyethylene-sorbitan monooleate (Tween-80; Sigma-Aldrich Chemical, St. Louis MO); ethanol 10 or 40% (2.17 and 8.68 M) in distilled water; cinnamaldehyde [1 or 10% (76, 760 mM) in mineral oil, Sigma], mustard oil [10% (1 M) in mineral oil, Sigma] and capsaicin [0.01 or 0.1% (0.3, 3 mM); from a stock solution of 1% in 80% ethanol; Sigma]. The 1% menthol concentration was selected because this or slightly lower (0.3%) concentrations elicit irritation in humans (Cliff and Green 1994, 1996; Dessirier et al. 2001), and many commercial products contain menthol in concentrations $\leq 9\%$ or higher. The chemicals were delivered either by syringe as a bolus of 0.1 ml for 1 min followed by rinse with isotonic saline or by electronic syringe pump set to deliver the fluid at a constant rate of ~0.5 ml/min for a period of 1 or 10 min, followed by rinse with isotonic saline. A strip of Parafilm was placed under the tongue to cover surrounding skin surfaces and prevent them from being contacted by the chemical stimuli.

Treatment groups

After initially characterizing thermal and mechanical responsiveness of the unit, units were formally tested with noxious cold and heat stimuli, followed 2 min later by the first chemical, which was either menthol or ethanol. Subsequent stimuli were delivered according to six different protocols as described in the following text. One unit was tested per animal except for two cases in which two units distinguishable by amplitude and waveform were recorded simultaneously.

Group 1: concentration-dependent response to menthol and enhancement of cold-evoked responses

For each of seven units in this group, responses to the series of graded stepwise decreases in temperature, followed by graded noxious heat stimuli, were recorded first (see Fig. 4A). Menthol at 0.1% (6.4 mM) was then applied by constant flow for 60 s to the tongue, followed 90 s later by repetition of the cooling sequence. A rest period of ≥ 40 min was imposed before testing the next-higher menthol concentration, 0.5% (32 mM) followed by cooling. This pattern was repeated twice more with 1 and 10% menthol (64 and 640 mM, respectively), allowing ≥ 60 min between menthol applications to generate complete menthol dose-response curves for each unit and to determine effects of each menthol application on responses to graded cooling (see Fig. 5).

Group 2: constant-flow superfusion of 1% menthol in 10% ethanol

Menthol was applied by constant flow for a longer (10 min) period to determine if responses adapt over time. For nine Vc units, noxious cold and heat stimuli were delivered by Peltier thermode at 2-min intervals, followed 2 min later by constant-flow superfusion of 1% menthol in 10% ethanol vehicle for 10 min (see Fig. 7). Two minutes after the end of menthol, the noxious cold and heat stimuli were delivered as before. To test if the initial menthol stimulus affected subsequent responses to menthol or thermal stimuli, menthol was reapplied in the same manner for 10 min, beginning 15 min after the end of the first menthol application, followed by noxious cold and heat as before. Four of the units in this group were initially tested with 10% ethanol (see following text) and were included since the ethanol vehicle had no effect.

To test for menthol cross-desensitization of responses to ethanol, 40% ethanol was delivered by constant-flow superfusion for 10 min, beginning 15 min after the second menthol application, followed by noxious cold and heat as before (see Fig. 7C). Responses to 40% ethanol postmenthol were compared with responses elicited by ethanol when it was delivered as the first stimulus (see following text and Fig. 10). Ethanol application was repeated in the identical manner 15 min later.

Group 3: constant-flow superfusion of 10% ethanol

This group served as a vehicle control. Noxious cold and heat stimuli, followed by 10% ethanol, were applied in the identical manner as described for the preceding group. Because 10% ethanol did not significantly affect Vc neuronal firing rate, four of these units were subsequently used in group 2 and one in group 5.

Group 4: constant-flow superfusion of menthol in 40% ethanol

The rationale for this group was to provide controlled delivery of menthol dissolved directly in a higher ethanol concentration to more closely mimic common menthol- and alcohol-containing products such as mouthwash or peppermint liquor. For eight Vc units, the tongue was initially stimulated with cold water followed by hot water. For six units, the tongue was first cooled and then heated 1 min later, by computer-controlled Peltier thermode. For all 14 units, the thermal stimulus was followed 2 min later by constant-flow application of menthol for 10 min (see Fig. 9). One minute after the end of menthol application, noxious cold and then heat stimuli were tested as before. Menthol was then reapplied in the same manner for 10 min, beginning 15 min after the end of the first menthol application followed 1 min later by the noxious cold and heat stimulus sequence. This was done to determine if subsequent responses to menthol and thermal stimuli were conditioned by the initial 10-min period of menthol application.

Group 5: constant-flow superfusion of 40% ethanol

Ethanol at concentrations more than ~15% excites superficial Vc units (Carstens et al. 1998). We therefore determined the neuronal response to 40% ethanol alone when delivered first in seven Vc units (1 unit previously received 10% ethanol that was ineffective). This also allowed us to assess the relative contribution of ethanol to the responses elicited by menthol in 40% ethanol (group 4). A noxious cold stimulus was delivered by Peltier thermode, followed 1 min later by noxious heat, followed 2 min later by constant-flow superfusion with 40% ethanol for 10 min. Cold and heat stimuli were reapplied as before starting 1 min after the end of ethanol application (see Fig. 10). Ethanol was reapplied in the same manner 15 min after the end of the first ethanol application, followed by noxious cold and heat stimuli in the same sequence as before. This was done to determine if subsequent responses to ethanol and thermal stimuli were affected by prior ethanol. Next, to test for possible ethanol cross-desensitization of responses to menthol, a solution of 1% menthol in 10% ethanol was delivered by constant-flow superfusion for 10 min, starting 15 min after the end of the second 40% ethanol application followed by noxious cold and heat stimuli in the same sequence as before. The response to menthol postethanol was compared with Vc responses to menthol when it was delivered as the first stimulus (group 4). The same menthol solution was then reapplied for 10 min, beginning 15 min after the end of the previous menthol application, to test for self-desensitization. This was followed by noxious cold and heat as before.

Group 6: bolus application of 1% menthol in 40% ethanol

The rationale for this group was to deliver menthol dissolved directly in ethanol and applied in a manner to mimic the natural ingestion of common alcohol- and menthol-containing products. For 12 Vc units, cold water was applied. In some experiments, noxious heat (53°C water) was delivered 2 min later. However, because heat was not tested for all units in the group, only responses to cold water were analyzed. Menthol (1% in 40% ethanol) was then applied as a bolus 2.5 min after the cold stimulus, left on for 60 s, and then rinsed with isotonic saline. This was followed 2 min later by reapplication of cold water to determine if menthol affected the thermal response. Menthol and cold stimuli were similarly reapplied 15 min after the first

menthol rinse, to determine if the subsequent responses to menthol and cooling were conditioned by prior menthol (see Fig. 11).

Responses to other irritants

After testing in groups 1–6 was completed, we then applied a panel of additional irritant chemicals in the following order: cinnamaldehyde (1 or 10%), mustard oil (10%), capsaicin (0.01 or 0.1%), and mustard oil 10%; responses to noxious cold and heat stimuli were recorded after each chemical stimulus application (see Figs. 12 and 13).

Histology

At the completion of each study, an electrolytic lesion was made at the recording site. The brain stem was then removed and postfixed in 10% buffered formalin for at least one week before cutting in 50- μ m frozen sections using a microtome. Sections were counterstained with Neutral Red and lesions identified under the light microscope.

Data analysis

Responses were quantified as the number of action potentials during the period of stimulus application, subtracting the spontaneous activity recorded during an equivalent time period prior to stimulus application (i.e., 1 min of spontaneous activity preceding cooling by Peltier thermode was subtracted from 1 min of activity following the onset of the cold stimulus; 30 s of spontaneous activity was subtracted from the 30-s period of activity after application of cold water). Paired *t*-test were used to evaluate the significance of the change in firing rate before and after bolus menthol application. Comparisons of responses elicited by different chemical stimuli were also made using paired or unpaired *t*-test. One-way ANOVA with post hoc least significant difference (LSD) test was done to determine significant differences in firing rate (assessed in 1-min bins) before and during application of irritant chemicals. The responses to heating and cooling the tongue before and after chemical stimulation were also evaluated using either paired or unpaired *t*-test. $P < 0.05$ was considered significant for all tests.

RESULTS

General properties and responses to thermal stimuli and menthol

Data were obtained from 50 units. Unit recording sites were histologically localized to the dorsomedial aspect of superficial Vc (Fig. 1).

Units were selected based on responses to cold stimulation of the tongue. All units included in the present analysis additionally responded robustly to noxious heating of the tongue as well as tongue pressure-pinch and irritant chemical stimuli. A typical example of such a unit's responses to multiple thermal and chemical stimuli is shown in Fig. 2. This unit responded to noxious cold, heat, and menthol and additionally to cinnamaldehyde, mustard oil, and capsaicin. Figure 3 shows individual examples of three different Vc unit responses to cooling, with cold water (Fig. 3A, 2nd trace) or by Peltier thermode (Fig. 3, B and C, 2nd traces). The mean cold threshold was $24.1 \pm 9.8^\circ\text{C}$ (mean \pm SD), range: 3–34°C ($n = 14$). Responses to noxious heating by Peltier thermode began at a mean threshold of $39.4 \pm 2.4^\circ\text{C}$, range: 35.1–45°C, $n = 23$. We rarely encountered cold-sensitive units that did not respond to noxious heat; two of these (not included in the present sample) responded to menthol but not capsaicin and may have been cold-specific neurons (Craig et al. 2001).

When subsequently tested with 1% menthol, 42/48 (88%) responded; examples of the initial response to menthol are shown

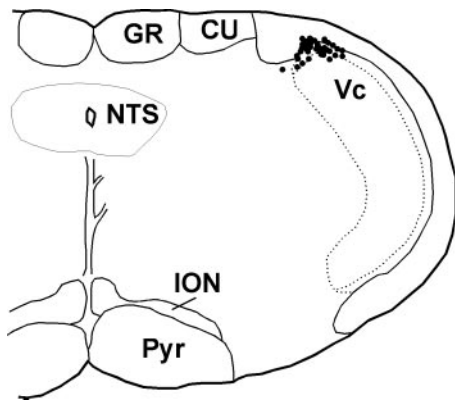


FIG. 1. Histologically localized recording sites (dots, $n = 38$) compiled on representative section through medulla. CU, cuneate n.; ION, GR, gracile n.; inferior olivary n.; NTS, n. of solitary tract; Pyr, medullary pyramid; Vc, trigeminal subnucleus caudalis.

in Fig. 3, A–C (*top traces*). Note that after menthol, units' responses to the cold stimulus were enhanced and prolonged (Fig. 3, A–C, *3rd traces from top*). Seven of 20 cells tested initially responded to cooling in the innocuous range (Fig. 3B, *2nd trace*) and exhibited a large and prolonged increase in firing in the noxious cold range postmenthol (Fig. 3B, *3rd trace*). Eight units responded weakly or with no detectable increase in firing during cooling with the thermode prior to menthol (although they all responded to ice water) but exhibited a robust response postmenthol as illustrated in Fig. 3C (*2nd and 3rd traces*), suggesting that menthol sensitized a "subliminal fringe" of cold nociceptors (or cold receptors). The remaining five cells exhibited enhanced responses to cooling postmenthol with no change in threshold. Overall the mean threshold was not significantly different postmenthol ($21 \pm 2.4^\circ\text{C}$).

In seven cases, we recorded Vc unit responses to graded noxious cooling and heating. Figure 4A shows an individual example of a unit that responded weakly to graded cooling of the tongue; this unit responded robustly to graded noxious heat stimuli. After application of 1% menthol to the tongue, this unit's responses to graded cooling were markedly enhanced (Fig. 4B) as described in greater detail in the following text. The properties of these Vc units are thus consistent with polymodal or HPC categories of lamina I spinothalamic tract neurons in cats and rats (Craig et al. 2001; Zhang et al. 2006).

Group 1: concentration-dependent response to menthol and enhancement of cold-evoked responses

In seven experiments, we recorded Vc responses to increasing concentrations of menthol, delivered at long (>60 min)

interstimulus intervals to reduce or avoid tachyphylaxis. Figure 5 shows averaged responses to 0.1% (6.4 mM; Fig. 5A), 0.5% (32 mM; Fig. 5B), 1% (64 mM; Fig. 5C), and 10% menthol (640 mM; Fig. 5D). Figure 6 plots mean responses versus menthol concentration to show a significant concentration dependency with saturation to show a significant concentration dependency with saturation >1%. Tachyphylaxis was observed to repeated application of menthol at a 15-min interstimulus interval (see following text), and we do not know if tachyphylaxis to 1% menthol persists beyond 60 min, so we cannot rule out the possibility that the response to 10% menthol was reduced due to prior stimulation with 1% menthol.

Cold-evoked responses were enhanced in a concentration-dependent manner by menthol. The right halves of the averaged PSTHs in Fig. 5 show responses to stepwise graded cooling. After 0.5, 1, and 10% menthol, there were significant increases in firing to the -5°C (Fig. 5B), 15, 5, and -5°C (Fig. 5C), and 25, 15, 5, and -5°C stimuli (Fig. 5D), respectively, compared with prementhol responses.

Group 2: constant-flow superfusion of 1% menthol in 10% ethanol

In a separate group of nine Vc units, menthol was applied by constant flow for a longer (10 min) period to determine if responses adapt over time. For five of the nine units tested, menthol was the first chemical stimulus applied, whereas four of the units were initially tested with 10% ethanol vehicle, which had no effect (see following text). Figure 7A shows that menthol significantly increased the mean firing rate during the first 3 min of application ($P < 0.05$, $n = 9$). The response to noxious cold applied by thermode postmenthol was significantly greater than prementhol ($P < 0.001$) with five of eight units exhibiting enhanced cold responses. The response to noxious heat was not significantly different. Reapplication of menthol 15 min later elicited no significant change in firing (Fig. 7B). The total response during the second 10-min period of menthol application was significantly lower compared with the first menthol application ($P < 0.001$). The cold response after the second menthol application was significantly greater than that before and after the first menthol application ($P < 0.001$ for both). The response to heat was significantly greater than that before and after the first menthol application ($P < 0.05$ and $P < 0.0001$, respectively).

To test if ethanol responses were affected by prior menthol, 40% ethanol was applied 15 min after the second menthol application (Fig. 7C). Ethanol did not significantly affect the mean firing rate when compared with the 1 min immediately prior to application. Because Vc units responded significantly to 40% ethanol when not preceded by menthol (Fig. 10A; see

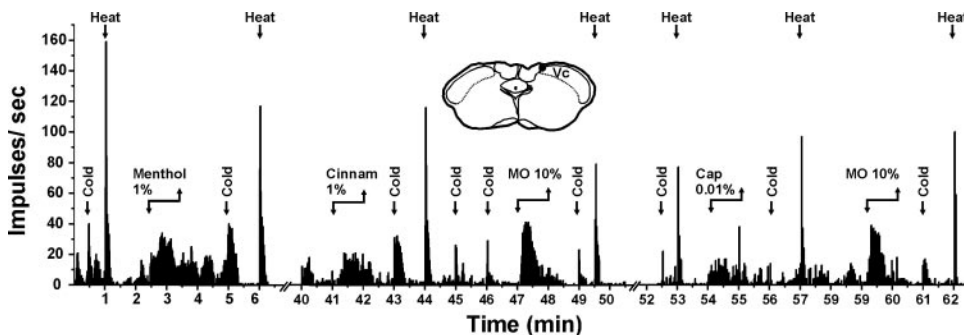


FIG. 2. Representative individual example of cold- and menthol-responsive Vc unit that additionally responded to noxious heat and other irritant chemicals. Shown are peristimulus time histograms (PSTHs; bin width: 1 s) of activity. Arrows, time of application of noxious cold (0 – 3°C water) or heat (53°C water) stimuli; bars with downward and upward arrows, start and end of chemical application. Cinnam, cinnamaldehyde; MO, mustard oil; Cap, capsaicin. *Inset*: recording site (dot).

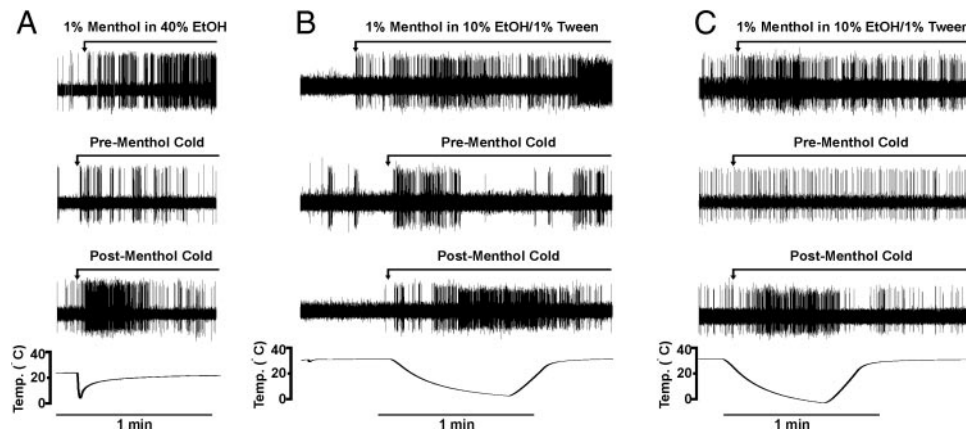


FIG. 3. Examples of recordings of Vc unit responses to menthol and cold stimuli. *A*: traces show spike records of Vc unit's responses to 1% menthol (in 40% ethanol; *top*), noxious cooling by lingual application of ice water prior to menthol (*2nd trace*), and noxious cooling postmenthol (*3rd trace*). Pre- and postmenthol traces are aligned consecutively to allow direct comparison; menthol was actually applied between pre- and postmenthol cold stimuli. *Bottom*: recording of tongue surface temperature, which dropped abruptly with application of ice water. *B*: format as *A* for a different Vc unit's responses to menthol (*top*) and cooling delivered by feedback-controlled Peltier thermode, pre (middle)- and postmenthol (*3rd trace*). *Bottom*: temperature recorded at interface between thermode and tongue surface. *C*: format as in *B* for a different Vc unit.

following text), these results are consistent with menthol cross-desensitizing responses to ethanol. The cold response postethanol was significantly lower compared with the cold responses before and after the first and second menthol applications ($P < 0.001$ for all). The heat response was not significantly different from that prior to menthol. Reapplication of 40% ethanol 15 min later resulted in a significant increase in firing during the first min compared with baseline ($P < 0.01$) but not when compared with the first ethanol application (Fig. 7D). The firing rate during minutes 2, 8, and 10 of the second ethanol application was significantly higher than during the first ($P < 0.05$). The cold response was not significantly different from the prementhol response but was significantly higher than that after the first application of ethanol and significantly lower than that after the second application of menthol ($P < 0.01$ for both). The response to heat was not significantly different compared with those before menthol or after the first ethanol application.

Group 3: constant-flow superfusion of 10% ethanol

Ethanol 10% (vehicle control) was applied first in five experiments. It did not significantly affect the firing rate (Fig.

8). The cold response after the ethanol application [29.8 ± 33.7 (SE) spike/min] was significantly lower than the preethanol response (61.6 ± 34.8 spike/min, paired *t*-test, $P < 0.05$); however, the heat response did not change significantly.

Group 4: constant-flow superfusion of menthol in 40% ethanol

Because many mentholated products contain alcohol, we investigated responses of Vc units to constant-flow application of menthol dissolved in a higher (40%) ethanol concentration. In eight experiments, noxious cold and hot water stimuli were used, whereas in the remaining six, the cold and heat stimuli were delivered by feedback-controlled Peltier thermode. Figure 3, *B* and *C* (*2nd* and *3rd traces*), shows a typical example of two Vc units' responses to cold stimuli delivered by Peltier thermode pre- and postmenthol as well as their initial responses to menthol applied by constant flow to the tongue (Fig. 3, *B* and *C*, *top traces*). There was a build-up in firing during the initial 1.5 min of menthol application, as well as a prolonged cold response postmenthol (Fig. 3, *B* and *C*, *bottom traces*).

Figure 9A shows averaged responses of Vc units to menthol (in 40% ethanol) and thermal stimuli. The mean increase in

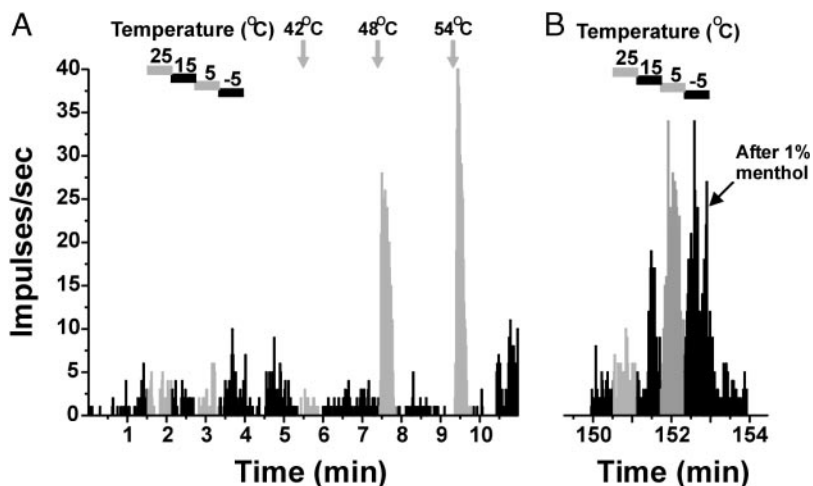


FIG. 4. Individual example of Vc unit's responses to graded noxious cooling and heating. *A*: peristimulus time histogram (PSTH) shows unit's responses to stepwise decreases (alternating gray and black bars above corresponding segments of PSTH; *left*) and graded increases (gray arrows above corresponding segments of PSTH) in tongue temperature. *B*: responses of same unit in *A* to graded stepwise decreases in tongue temperature recorded after application of 1% menthol, ~ 2.5 h later.

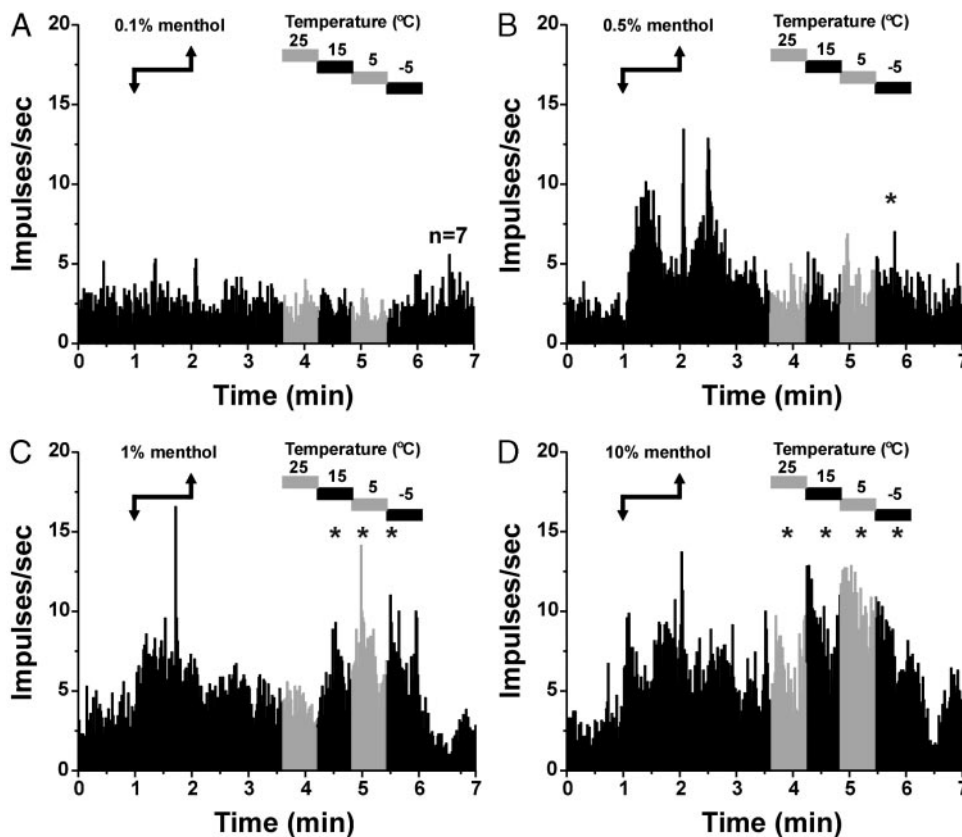


FIG. 5. Concentration-related increases in response to menthol and enhancement of cold-evoked responses (group 1). *A*: averaged PSTH of responses of 7 Vc units to lingual application of 0.1% (6.4 mM) menthol, followed 1.5 min later by graded step-wise cooling (indicated by alternating gray and black bars and corresponding segments of PSTH). Error bars omitted for clarity. *B*: PSTH (format as in *A*) of response to 0.5% (32 mM) menthol. *, significantly greater than prementhol response to -5°C . *C*: 1% (64 mM) menthol. *, significantly greater than prementhol responses to 15, 5, and -5°C . *D*: 10% (640 mM) menthol. *, significantly greater than corresponding response prementhol.

firing rate (impulse/min) was significant for each of the first 3 min after the onset of the menthol superfusion (Fig. 9*A*, left, $P < 0.002$, ANOVA, $n = 14$) followed by a decline toward baseline firing levels. The mean response to cooling with the thermode was significantly greater postmenthol compared with the prementhol cold response ($P < 0.001$, paired t -test, $n = 6$) with five of the six cells showing an enhanced response. Similarly, the mean response of the eight units to the cold water stimulus was significantly ($P < 0.001$, paired t -test, data not shown) greater postmenthol [268 ± 69.4 (SE) spike/min] compared with prementhol (50.1 ± 21.7 spike/min) with 7/8 exhibiting enhanced responses to cold postmenthol. After a 15-min break, menthol was reapplied and elicited a response that, however, was significantly smaller than the first response (paired t -test, $P < 0.01$) indicative of self-desensitization (Fig.

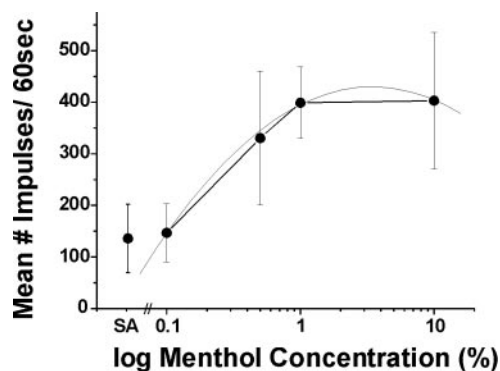


FIG. 6. Semi-log plot of mean responses versus menthol concentration. SA, spontaneous activity prior to menthol application. Error bars: SE. Curve shows 2nd-order polynomial curve fit; the R^2 value was statistically significant ($P < 0.05$).

9*B*). After the second menthol application, the mean response to noxious cold applied by thermode was significantly greater than prementhol ($P < 0.001$, paired t -test) although it was lower compared with the first postmenthol cold response. The mean response to cold water after the second menthol application was also significantly greater than prementhol ($P < 0.001$, paired t -test) and not significantly different from the first postmenthol cold response. Responses to noxious heat delivered either by thermode (Fig. 9, *A* and *B*) or hot water (not shown) were not significantly changed after the first or second application of menthol compared with prementhol responses.

Group 5: constant-flow superfusion of 40% ethanol

The vehicle used in group 4, 40% ethanol, can also activate Vc units (Carstens et al. 1998). This was verified presently in vehicle control experiments (Fig. 10). When ethanol 40% was applied as the first chemical in the experiment, it significantly ($P < 0.01$, $n = 7$) increased firing relative to preethanol baseline during the initial 1 min (Fig. 10*A*). Responses to noxious cold and heat (applied by Peltier thermode) postethanol treatment were both significantly lower compared with preethanol responses when baseline activity was subtracted ($P < 0.01$ for both). When 40% ethanol was reapplied after a 15-min break, it again significantly ($P < 0.001$, $n = 5$) excited Vc units during the initial 1 min (Fig. 10*B*). The cold response was not significantly different from the preethanol level (but was higher compared with the cold response after the 1st ethanol treatment, $P < 0.001$, unpaired t -test), and the heat response was significantly lower compared with that prior to the first ethanol treatment ($P < 0.001$, unpaired t -test).

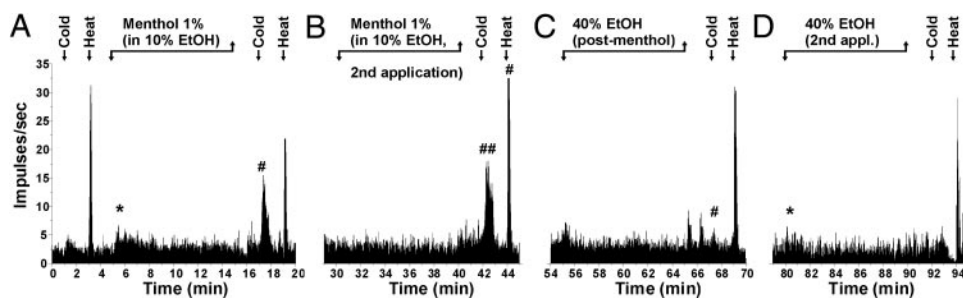


FIG. 7. Vc responses to menthol and thermal stimuli and effects on responses to ethanol (group 2). *A*: averaged PSTH of responses of 9 Vc units to noxious cold and heat applied by thermode before and after constant-flow application of 1% menthol. *, significantly different from prementhol baseline during initial 3 min ($P < 0.05$). #, significantly different from prementhol ($P < 0.001$). *B*: reapplication of menthol 15 min later. ##, significantly different from responses before and after 1st menthol application ($P < 0.001$). #, significantly different from responses before and after first menthol application ($P < 0.05$). *C*: response to 40% ethanol 15 min later. #, cold response significantly lower compared with pre- and postmenthol ($P < 0.001$ for all). *D*: response to reapplication of 40% ethanol 15 min later. *, significantly different from preethanol baseline ($P < 0.05$).

We then tested whether responses to menthol (in 10% ethanol) were affected by prior application of 40% ethanol. Figure 10C shows that, 15 min after the second application of 40% ethanol, application of menthol still significantly ($P < 0.05$) increased firing. The total response during the 10-min menthol application was significantly higher compared with the first ethanol application (unpaired t -test, $P < 0.001$). This indicates that 40% ethanol did not cross-desensitize or otherwise depress responses to menthol. Moreover, the average response to cold postmenthol was significantly greater compared with cold responses recorded before and after the first and second ethanol applications ($P < 0.001$ for both, unpaired t -test). Individually, four of the five units tested in this group exhibited increased responses to cold postmenthol. The response to heat postmenthol was significantly lower compared with preethanol ($P < 0.05$, unpaired t -test). The second menthol application resulted in no significant increase in firing (Fig. 10D); firing at 6 and 8 min after onset of menthol was significantly lower compared with baseline ($P < 0.05$). The mean firing rate during the second menthol application was significantly lower compared with the first menthol application for all 10 min (paired t -test, $P < 0.05$). This is consistent with menthol self-desensitization. Additionally, the total response over the 10-min period was significantly lower than during the second ethanol treatment ($P < 0.001$, unpaired t -test). The response to cold was not significantly different from that after

the first menthol application and was significantly greater than pre- and postethanol. The response to heat was not significantly different from that preethanol and was significantly greater than that after the first menthol application ($P < 0.001$) and second ethanol application ($P < 0.01$).

Group 6: bolus application of 1% menthol in 40% ethanol

This group was included to mimic more natural ingestion of a menthol- and alcohol-containing product. Figure 11 shows averaged PSTHs of responses of 12 Vc units to ice water ($\sim 3^\circ\text{C}$) and menthol. The first menthol application significantly elevated mean firing (prementhol: 201.9 ± 56.5 spike/60 s, mean \pm SE; postmenthol: 590.1 ± 179.4) and resulted in a significant ($P < 0.001$, paired t -test) enhancement of the mean cold-evoked response (19.9 ± 18.5 spike/30 s prementhol vs. 85.1 ± 69.7 postmenthol). Of the 12 cells so tested, 6 exhibited a marked and significantly (t -test, $P < 0.05$) larger cold response postmenthol, whereas 4 units exhibited a numerically (but not significantly) greater cold response postmenthol with no change in the remaining 2 units. Reapplication of menthol 15 min later elicited a smaller but still significant increase in firing (prementhol: 156.3 spike/30 s \pm 50.5; postmenthol: 275.4 ± 71.2); however, this response was significantly lower compared with the initial menthol response ($P < 0.001$, paired t -test), indicating self-desensitization. After the second menthol, the mean cold-evoked response was further enhanced (387.8 spike/30 s \pm 106.3) and was significantly larger than after the first menthol application ($P < 0.001$, paired t -test).

Responses to other irritants

In 38 units, additional chemicals were tested after applications of menthol and ethanol. The order of chemical presentation was always cinnamaldehyde (1 or 10%), mustard oil (10%), capsaicin (0.01 or 0.1%), and finally re-application of 10% mustard oil. Of 23 units tested with 1% cinnamaldehyde, 14 (61%) exhibited increased firing. The averaged response of all units to 1% cinnamaldehyde is shown in Fig. 12A. Eight of 10 other units responded to 10% cinnamaldehyde and their averaged response is shown in Fig. 12B. At both concentrations of cinnamaldehyde, the firing increased slowly during the application period; the mean response to 10% but not 1% cinnamaldehyde was significant ($P < 0.001$, paired t -test). The presence of a response in those units sensitive to cinnamaldehyde indicates that the lingual afferents projecting to Vc were

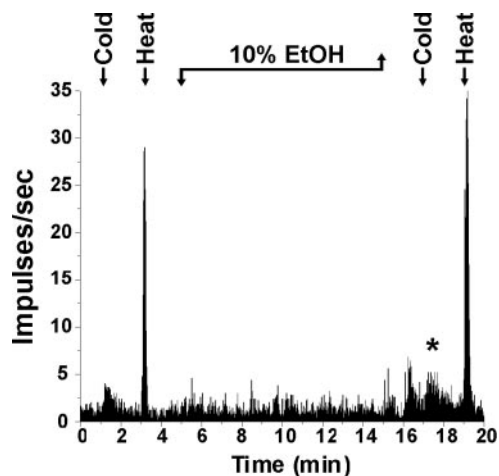


FIG. 8. Control application of 10% ethanol vehicle (group 3). *, significantly different from cold response preethanol.

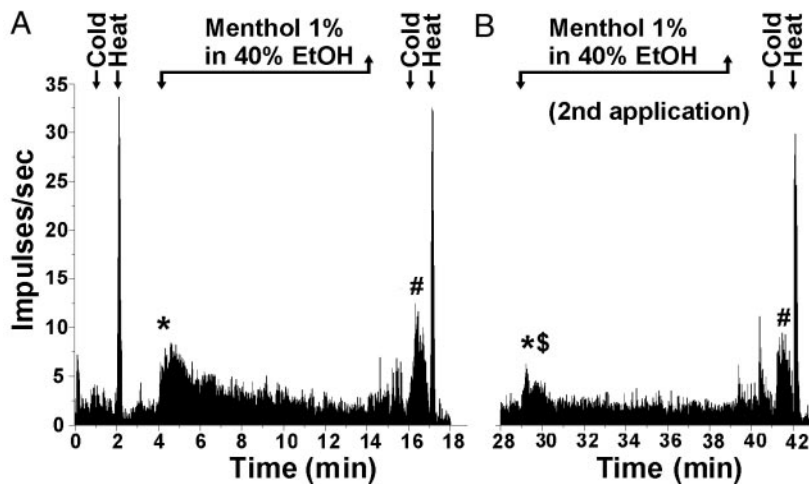


FIG. 9. Vc responses to thermal stimuli and constant-flow application of menthol in 40% ethanol (group 4). *A*: averaged PSTH of Vc units' responses to thermal and menthol stimuli. The PSTH for thermal stimuli delivered by Peltier thermode (in 6 units) is continuous with the averaged response to menthol (in 40% ethanol) for 14 units (8 of which received thermal stimuli by hot and cold water; data not shown). *, significantly different from baseline during initial 3 min after onset of menthol. #, significantly different from prementhol cold response. *B*: mean response to 2nd application of menthol 15 min later ($n = 14$), followed by responses to cold and heat delivered by thermode ($n = 6$). *, significantly different from baseline during 1st 2 min. \$: significantly different compared with first menthol application. #, significantly different from prementhol cold response.

not cross-desensitized by prior application of menthol or ethanol. The absence of responses observed in a minority of units might be a true negative response or might be attributed to desensitization by prior stimuli.

Mustard oil was applied after cinnamaldehyde and elicited responses in 33/37 (89%) of the units. The averaged response of these units, shown in Fig. 12*C*, was statistically significant ($P < 0.001$, paired t -test). The presence of a robust response indicates that lingual afferents projecting to the Vc units were not cross-desensitized by the preceding menthol, ethanol, or cinnamaldehyde stimuli. The response peaked within 15–20 s and then declined despite the continued presence of mustard oil consistent with our previous report (Simons et al. 2004). Most (28/37) of these units were retested with a second mustard oil application after intervening stimulation with capsaicin. The averaged response, shown in Fig. 12*D*, was reduced and the preceding spontaneous firing level was greater, compared with the first mustard oil response ($P < 0.001$, unpaired t -test). The reduced response is consistent with cross-desensitization by capsaicin and/or self-desensitization.

After mustard oil, 0.01% capsaicin was tested in 32 units of which 24 (75%) responded; the averaged response shown in Fig. 12*E* was statistically significant ($P < 0.001$). Ten of 14 units (71%) responded to a higher (0.1%) capsaicin concentration; in 5 cases, the higher dose was applied after application of the lower capsaicin concentration which did not elicit a response, whereas in 6 units, the 0.1% concentration was tested

post 0.01% capsaicin and postmustard oil. The remaining three were tested after mustard oil but without the intervening lower concentration of capsaicin. Figure 12*F* shows the averaged responses to all 14 units tested with 0.1% capsaicin, which was also statistically significant ($P < 0.001$). The data suggest a concentration-related increase in firing, although this is confounded by the likelihood of self-desensitization for the units tested with both concentrations. Nevertheless, the positive response of a majority of the units is consistent with their receiving input from TRPV1-expressing afferent fibers. The absence of a response in a minority of units may reflect desensitization due to prior chemicals.

Responses to noxious heat and cold stimuli were compared prior to and after the application of each chemical. For each chemical tested, thermal responses were variably affected with no statistically significant overall trend. However, these comparisons are confounded by the numerous chemicals applied, and the modulation of thermal responses needs to be tested for each chemical in a separate experiment.

Eight Vc units were subjected to the identical battery of thermal and chemical stimuli, and their averaged response is shown in Fig. 13. The population responded significantly to each of the irritant chemicals tested ($P < 0.001$ for each, paired t -test). When mustard oil was reapplied after an intervening trial with capsaicin, the second response to mustard oil was significantly lower compared with the first ($P < 0.001$, paired t -test; spontaneous activity subtracted). Although there is a

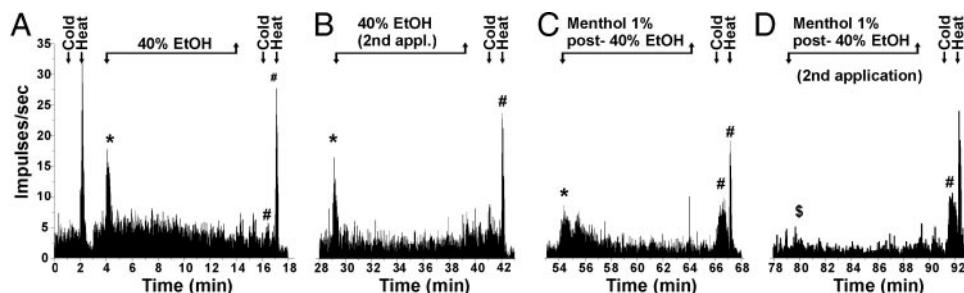


FIG. 10. Vc response to 40% ethanol and lack of cross-desensitization of menthol-evoked response (group 5). *A*: averaged PSTH of response of 7 Vc units to cold and heat applied by thermode followed by constant-flow application of 40% ethanol to the tongue. *, significantly different from preethanol baseline during 1st min ($P < 0.01$). #, significantly different from preethanol cold and heat responses ($P < 0.001$ for both). *B*: 2nd application of 40% ethanol 15 min later ($n = 5$ of the 7 units shown in *A*). *, significantly different from preethanol baseline, #, significantly different from preethanol heat response ($P < 0.001$). *C*: response to 1% menthol (same units as in *B*). *, significantly different from prementhol baseline for first 3 min. #, significantly different from corresponding responses preethanol ($P < 0.001$ and $P < 0.05$ for cold and heat, respectively). *D*: response to 2nd application of 1% menthol 15 min later. \$, significantly different from response to 1st application of menthol ($P < 0.05$). #, significantly different from pre- and postethanol responses to cold ($P < 0.001$).

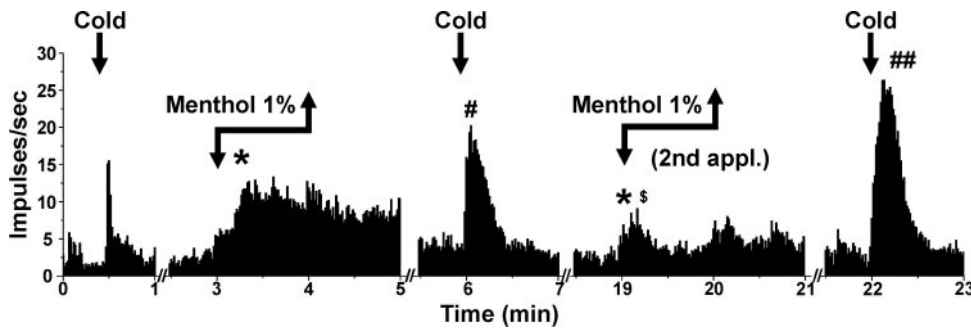


FIG. 11. Vc unit responses to noxious cold and bolus application of menthol to the tongue (group 6). Averaged PSTHs (bin width: 1 s; $n = 12$ units) show responses to cold (arrow) and 2 applications of menthol (bars with downward and upward arrows) given 15 min apart. Error bars omitted for clarity. *, significantly different from prementhol firing rate ($P < 0.01$). #, significantly different from prementhol cold response. ##, significantly different from response after 1st menthol application ($P < 0.001$). \$, significantly different from first menthol response ($P < 0.001$).

visible trend for responses to noxious heat to rise over time, there was also a progressive increase in spontaneous activity. With baseline activity subtracted, none of the heat-evoked responses recorded after irritant chemical stimuli were significantly different from the response prior to cinnamaldehyde. The same was generally true for responses to noxious cold, except that cold-evoked responses were significantly lower ($P < 0.05$) after capsaicin and the second mustard oil application, compared with precinnamaldehyde. These latter cold

responses were recorded on an elevated background firing rate caused by the prior irritant stimuli.

DISCUSSION

The present study isolated cold-responsive units in superficial laminae of dorsomedial Vc, all of which additionally responded to noxious heating and most (88%) to menthol. A majority also responded to cinnamaldehyde, mustard oil, cap-

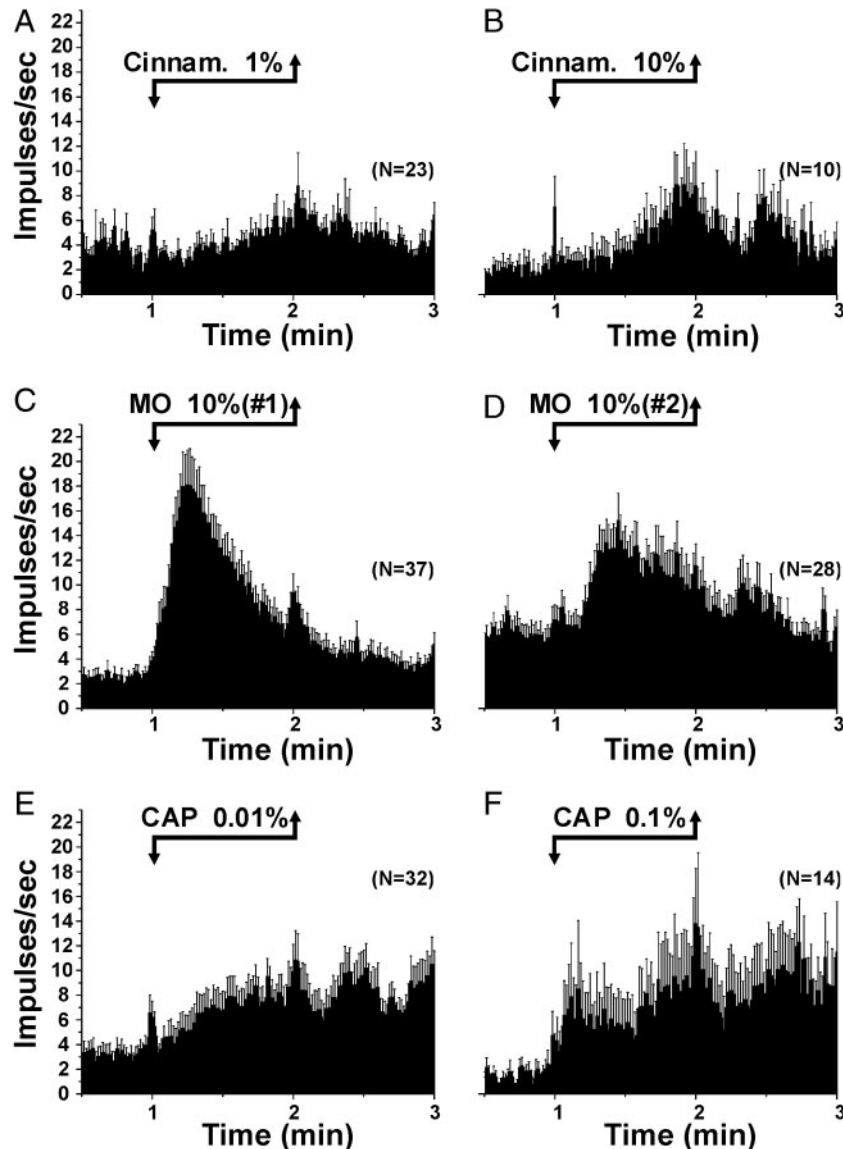


FIG. 12. Responses of cold- and menthol-sensitive Vc units to other irritant chemicals. *A*: averaged PSTH of responses of 23 units to 1% cinnamaldehyde given after cells had been tested with menthol and ethanol. Error bars: SE. *B*: PSTH as in *A* for mean response of 10 units to 10% cinnamaldehyde. *C*: mean response to 10% mustard oil, given after cinnamaldehyde. *D*: mean response of 28 of the 37 units in *C* to a 2nd application of 10% mustard oil, given after an intervening capsaicin stimulus. *E*: mean response to 0.01% capsaicin, given after cinnamaldehyde and mustard oil. *F*: mean response to 0.1% capsaicin. Five of these units were previously stimulated with 0.01% capsaicin.

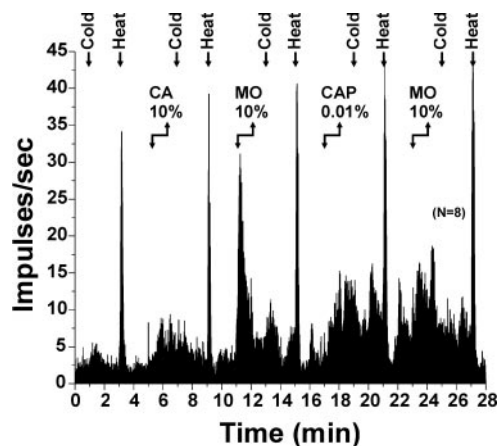


FIG. 13. Mean response of 8 units receiving an identical battery of stimuli. Downward arrows indicate onset of noxious cold or heat stimuli delivered by Peltier thermode contacting the anterior dorsal tongue surface. Upward and downward connected arrows indicate on- and offset of application of each indicated chemical (CA, cinnamaldehyde; MO, mustard oil, CAP, capsaicin). Mean responses to each irritant chemical were statistically significant ($P < 0.001$ for each, paired t -test).

saicin, and 40% ethanol. The broadly tuned chemical responsiveness of these units confirms earlier studies (Amano et al. 1986; Carstens et al. 1998; Dessirier et al. 2000) and implies that they receive input from lingual primary afferents expressing TRPM8, TRPV1, and/or TRPA1. The input may be direct via convergence from primary afferents expressing one or a combination of these TRP channels and/or indirectly via inter-subnuclear connections (Hirata et al. 2003; Jacquin et al. 1990) from second-order neurons in more rostral subnuclei such as oralis that are known to receive nociceptive input (e.g., Dallel et al. 1999).

Responses to heating, cooling, and menthol

All of the present cold-responsive Vc units additionally responded vigorously to noxious heating of the tongue as well as pressure-pinch and irritant chemical stimuli. Mean thresholds for cold- and heat-evoked responses are consistent with properties of lamina I spinothalamic tract neurons in cats and rats (Craig et al. 2001; Zhang et al. 2006) and WDR units in superficial and deeper laminae of Vc (Carstens et al. 1998; Hutchison et al. 1997; Malick et al. 2000; McHaffie et al. 1994; Meng et al. 1997; Simons et al. 2004). Our observation that some superficial Vc units had cold thresholds in the noxious range is consistent with previous studies reporting that noxious orofacial cooling evokes c-fos expression in superficial Vc (Strassman et al. 1993), and noxious cooling of hindlimb skin elicits c-fos expression in superficial and deeper laminae of the lumbar spinal cord (Abbadie et al. 1994; Doyle and Hunt 1999) and excites spinothalamic (Craig et al. 2001) and spinoparabrachial (Bester et al. 2000) projection neurons in lamina I.

Only two cold-sensitive units were presently encountered that did not respond to noxious heat, and both responded to menthol but not capsaicin. We assume that these were cold-specific units (Craig et al. 2001), and they were not included in the present analysis. The remainder of the cold-sensitive Vc units responded vigorously to noxious heat as well as pressure-pinch and irritant chemicals. When tested, Vc units exhibited increasing responses to graded increases in the intensity of

noxious heat, with some also exhibiting graded noxious cold-evoked responses that became more apparent after menthol (Figs. 4 and 5). These properties are consistent with those of "polymodal" or HPC classes of lamina I spinothalamic projection neurons in rat (Zhang et al. 2006) and cat (Craig et al. 2001). Although a small fraction of cold-specific units exhibited a "paradoxical" response to noxious heat, it was usually weak and had a higher threshold (Craig et al. 2001; Zhang et al. 2006) compared with the present Vc units. We therefore conclude that it is unlikely that any of the presently reported Vc units were cold-specific neurons with "paradoxical" responses to noxious heat.

Many of the present Vc units responded to cooling in the innocuous range (threshold $\sim 24^{\circ}\text{C}$) as well as to menthol, implying input from primary afferents expressing TRPM8. 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). Co-expression of TRPM8 and TRPV1 could account for the responses of unmyelinated and thinly myelinated nociceptors to noxious heat and cold (Bessou and Perl 1969; Campero et al. 1996; LaMotte and Thalhammer 1982; Simone and Kajander 1996, 1997). Responsiveness of Vc neurons to noxious heat and cold is consistent with input from such nociceptive afferents. 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 co-expression of TRPM8 and TRPV1 (or their mRNAs) in DRG or trigeminal ganglion neurons. This suggests that the major source of cold input to Vc neurons may be capsaicin-insensitive innocuous cold receptors (Xing et al. 2006) rather than nociceptors. An additional potential source of input is from cold-sensitive fibers that express neither TRPM8 nor TRPA1 (Munns et al. 2006). Cold receptors signal innocuous cooling via connections with a select population of cooling-specific spinothalamic tract neurons in lamina I (Han et al. 1998). If innocuous cold receptors also excite pain-signaling Vc neurons, then skin cooling should be expected to elicit sensations of both innocuous cool and pain. Indeed relatively small decreases in skin temperature in the innocuous range can elicit nociceptive sensations (stinging, burning, prickle) as well as cold (Green and Pope 2003). Similarly, although Vc unit responses to noxious heat and capsaicin are explained by input from primary afferents expressing TRPV1, the heat threshold ($\sim 39^{\circ}\text{C}$) is in the innocuous range. It is conceivable that innocuous warm receptors, modulated by warm-sensitive TRPV3 and TRPV4 channels expressed in skin keratinocytes (Chung et al. 2004; Moqrich et al. 2005), converge onto Vc neurons to impart innocuous heat sensitivity. Activation of nociceptive Vc neurons by innocuous warm receptor input might explain the recent observation that innocuous warming can elicit pain sensation in some subjects (Green et al. 2006).

Innocuous cold receptors typically exhibit phasic responses to changes in temperature followed by a tonic response at the new temperature level (Schaefer et al. 1988). However, phasic responses during step temperature changes were not consistently observed for the present Vc units (Fig. 5). Possible reasons for a lack of consistent phasic response to cold might be that the rate of temperature change was too slow ($< 2^{\circ}\text{C/s}$) and/or that temporal dispersion of input from cold receptors blurred the phasic responses arriving at the Vc neuron. Fur-

thermore, some of the Vc units had cold thresholds in the noxious range, possibly reflecting input from nociceptors responsive to noxious cold which did not exhibit marked phasic responses (Kajander and Simone 1997, 1998).

There was a significant, concentration-dependent enhancement of cold-evoked Vc responses postmenthol that was not accompanied by a change in threshold as might have been predicted by the moderate (2.5°C) reduction in cold thresholds of trigeminal ganglion cells by menthol (McKemy et al. 2002). Human psychophysical studies report cold (but not heat) hyperalgesia after cutaneous application of 30–40% menthol (Hatem et al. 2006; Namer et al. 2005; Wasner et al. 2004). If the present Vc neurons signal pain sensation, then menthol enhancement of their cold-evoked responses represents a possible mechanism for cold hyperalgesia. Several Vc units exhibited enhanced responses to cooling in the noxious range postmenthol, suggesting that menthol sensitized cold nociceptors either via enhancement of TRPM8 or, speculatively, TRPA1. However, the latter possibility is mitigated by a recent report that menthol significantly reduced the activation of TRPA1 by noxious cold or cinnamaldehyde (Macpherson et al. 2006). That menthol significantly enhanced cold- but not heat-evoked responses of Vc units argues against central sensitization.

Vc unit responses desensitized during constant-flow application of menthol, consistent with a progressive reduction in psychophysical ratings of irritation elicited by repeated intraoral application of menthol (Cliff and Green 1994, 1996; Dessirier et al. 2001). After a 15-min rest period, reapplication of menthol evoked significantly weaker responses in Vc units indicative of self-desensitization, consistent with psychophysical studies (Cliff and Green 1994, 1996; Dessirier et al. 2001). Desensitization of successive neural and perceptual responses to repeated menthol might be mediated by desensitization of TRPM8 expressed in cold receptors and/or nociceptors via a recently described phospholipase C-mediated decrease in phosphatidylinositol 4,5 bisphosphate that is thought to regulate TRPM8 ion channel activity (Rohacs et al. 2005). Another possibility is that after the initial excitation, the decline in firing was due to a local anesthetic effect of menthol (especially at higher concentrations) or the 40% ethanol vehicle. If this were the case, however, the anesthetic effect was short-lived since responses to cooling were enhanced, not reduced, within 2–4 min after cessation of menthol.

Response to ethanol and cross-interactions with menthol and thermal stimuli

Ethanol 40%, but not 10%, excited Vc units consistent with our previous study (Carstens et al. 1998). Excitation by ethanol was brief and firing quickly returned to baseline, consistent with a psychophysical study showing a progressive decline in irritant ratings elicited by repeated application of 47.5% ethanol (Prescott and Swain-Campbell 2000). Reapplication of 40% ethanol elicited a significant Vc response indicating absence of tachyphylaxis, also consistent with previous electrophysiological (Carstens et al. 1998) and psychophysical studies (Prescott and Swain-Campbell 2000). Furthermore, menthol elicited a significant Vc response when applied after 40% ethanol, indicating absence of cross-desensitization. In contrast, menthol appeared to cross-desensitize Vc unit responses to 40% ethanol (Fig. 7C).

Ethanol activates and sensitizes nociceptors via TRPV1 (Trevisani et al. 2002). However, 10% ethanol did not significantly enhance Vc responses to noxious heat (Fig. 8) and 40% ethanol significantly depressed responses to noxious heat and cold (Fig. 10). The latter might reflect a local anesthetic action that, however, subsided within 15 min because reapplication of ethanol elicited a significant response. Moreover, application of menthol in 40% ethanol elicited a significant response (Figs. 9A and 11), arguing against a local anesthetic effect. Ethanol inhibits TRPM8 (Weil et al. 2005), which might account for the significant reduction in Vc cold-evoked responses after both 10 and 40% ethanol.

Responses to mustard oil, cinnamaldehyde and capsaicin

Most Vc units additionally responded to the TRPA1 agonists mustard oil and cinnamaldehyde as well as the TRPV1 agonist capsaicin. While neurons expressing TRPA1 invariably co-express TRPV1, TRPA1 and TRPM8 are rarely co-expressed (Kobayashi et al. 2005; Peier et al. 2002; Story et al. 2003). This implies that Vc units receive convergent input from separate populations of primary afferents, one expressing TRPM8 (with or without TRPV1), and the other co-expressing TRPA1 and TRPV1.

TRPA1 has been implicated in transducing noxious cold (Bandell et al. 2004; Story et al. 2003) and mechanical stimuli (Nagata et al. 2005). However, the former has been challenged (Jordt et al. 2004), and knockout mice lacking TRPA1 exhibit neither auditory nor cold pain deficits (Bautista et al. 2006). Moreover, cutaneous cinnamaldehyde induces burning pain but not cold sensation (Namer et al. 2005). In the oral cavity, mustard oil elicits a desensitizing pattern of burning irritation (Simons et al. 2003) and Vc unit firing (Fig. 12C) (Simons et al. 2004).

In psychophysical studies, mustard oil and capsaicin exhibit self-desensitization and mutual cross-desensitization (Simons et al. 2003). Vc units still responded to capsaicin after mustard oil, indicating that cross-desensitization was not absolute. Vc unit responses to mustard oil were significantly reduced after capsaicin (Fig. 13) consistent with partial cross-desensitization. Interestingly, cooling reduced ongoing activity postcapsaicin (Fig. 13), an effect that apparently outweighed excitatory effects of cooling. Suppression of postcapsaicin ongoing activity by cooling may relate to the well-known ability of a cold drink to quickly reduce the oral burn experienced when eating spicy food.

Mustard oil also sensitizes Vc responses to noxious heat (Simons et al. 2004). However, we did not presently observe significant sensitization of heat-evoked responses after mustard oil, cinnamaldehyde, or capsaicin possibly because the Vc units had already reached a maximal response level due to the multiple stimuli delivered before these latter chemicals were tested.

Chemesthesis

The present results showing a broad range of chemical and thermal inputs to superficial Vc neurons is consistent with the notion of chemesthesis (Green 1996; Green et al. 1990). Presumably, these Vc neurons signal chemesthetic sensations (irritation or pain) in the oral mucosa, regardless of the triggering stimulus. For example, both noxious hot and innocuous

cold stimuli excited Vc units. Although such convergence represents a loss of quality coding, the chemesthetic responses of these Vc units is signaled in parallel with more specialized pathways devoted to individual sensory qualities such as warmth, cool, and touch that are well localized and discriminated within the oral cavity. Activation of the broadly tuned Vc neurons may provide a general pain signal, the thermal quality of which is identified by co-activation of cold or warm receptors. The properties of the present Vc neurons make them good candidates to convey the chemesthetic qualities of spicy foods, tingly drinks, and other consumables such as oral hygiene or tobacco products that contain alcohol or other irritants (e.g., nicotine). It is therefore interesting that many individuals develop preferences for products that contain innately aversive irritant chemicals that activate trigeminal pain-signaling neurons (Prescott and Stevenson 1995; Rozin and Schiller 1980; Rozin et al. 1981).

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