

# Novel Menthol-Derived Cooling Compounds Activate Primary and Second-Order Trigeminal Sensory Neurons and Modulate Lingual Thermosensitivity

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## Abstract

We presently investigated 2 novel menthol derivatives GIV1 and GIV2, which exhibit strong cooling effects. In previous human psychophysical studies, GIV1 delivered in a toothpaste medium elicited a cooling sensation that was longer lasting compared with GIV2 and menthol carboxamide (WS-3). In the current study, we investigated the molecular and cellular effects of these cooling agents. In calcium flux studies of TRPM8 expressed in HEK cells, both GIV1 and GIV2 were approximately 40- to 200-fold more potent than menthol and WS-3. GIV1 and GIV2 also activated TRPA1 but at levels that were 400 times greater than those required for TRPM8 activation. In calcium imaging studies, subpopulations of cultured rat trigeminal ganglion and dorsal root ganglion cells responded to GIV1 and/or GIV2; the majority of these were also activated by menthol and some were additionally activated by the TRPA1 agonist cinnamaldehyde and/or the TRPV1 agonist capsaicin. We also made *in vivo* single-unit recordings from cold-sensitive neurons in rat trigeminal subnucleus caudalis (Vc). GIV1 and GIV2 directly excited some Vc neurons, GIV1 significantly enhanced their responses to cooling, and both GIV1 and GIV2 reduced responses to noxious heat. These novel cooling compounds provide additional molecular tools to investigate the neural processes of cold sensation.

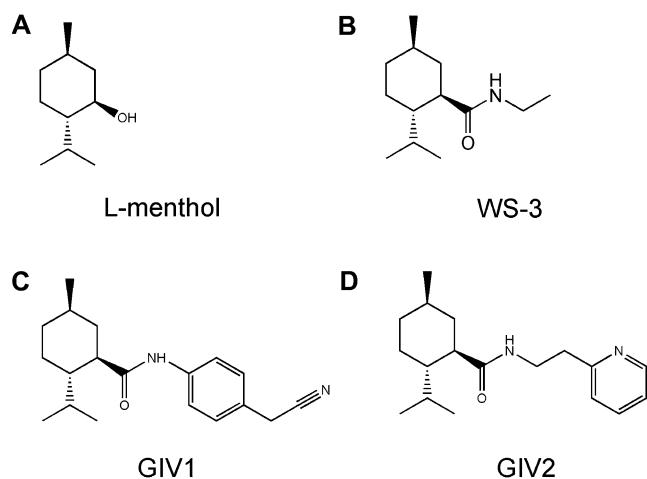
**Key words:** calcium imaging, capsaicin, cinnamaldehyde, cold, trigeminal, TRPM8

## Introduction

Menthol from mint oils is used in many pharmaceutical and over the counter oral hygiene products because of its cooling and analgesic properties (Eccles 1994). The cooling sensation elicited by menthol has been attributed to its action at the transient receptor potential (TRP) channel, TRPM8, which is also activated by temperatures less than 25 °C as well as by other chemicals that elicit cooling sensations (McKemy et al. 2002; Peier et al. 2002; Reid et al. 2002). Menthol enhances the cooling-evoked responses of peripheral cold fibers (Swandulla et al. 1985, 1986; Schäfer et al. 1986; Reid and Flonta 2001) in a calcium-dependent manner (Schäfer et al. 1986; Reid and Flonta 2001) via an enhancement of thermal gating of TRPM8 (McKemy et al. 2002). Menthol also enhances cold-evoked responses of neurons in superficial laminae of trigeminal subnucleus caudalis (Vc) (Zanotto et al. 2007). In addition, menthol exhibits nasal pungency and elicits irritation of the oral mucosa at high concentrations (Cliff and Green 1994; Gwartney and Heymann 1995; Dessirier et al. 2001).

Menthol is a monocyclic terpene (Figure 1A) and shares a common chemical structure with many other TRP channel agonists (Behrendt et al. 2004; Vogt-Eisele et al. 2007). L-menthol evokes a stronger cooling sensation than D-menthol (Green 1985; Eccles et al. 1988), indicating that tertiary structure is important for binding to its target receptor. Many synthetic derivatives of menthol have been used in personal hygiene products. The most commercialized have been compounds generated by Wilkinson Sword Ltd. in the 1970s, including WS-3 (Figure 1B), WS-23, and WS-5 (Watson et al. 1978). Many of these compounds are more efficacious than L-menthol in eliciting cooling sensation, and some are nearly odorless. These represent important characteristics for cooling compounds in personal hygiene products because nasal and oral irritant properties limit the concentrations of menthol that can be used.

There have been many recent advances in the generation of cooling compounds (Erman 2007; Furrer et al. 2008). In the



**Figure 1** Structure of TRPM8 agonists L-menthol and WS-3 versus novel cooling compounds GIV1 and GIV2. **A.** L-menthol, **B.** WS-3, **C.** GIV1, **D.** GIV2.

present study, we examined the effectiveness of 2 novel cooling compounds, GIV1 and GIV2 (Figure 1C,D). In a toothpaste medium, GIV1 and GIV2 were much more effective at producing a cooling sensation compared with WS-3 (Furrer et al. 2008; Compound 2j and 2k, respectively). In the present study, we investigated the physiological basis of the cooling effects of these compounds. GIV1 and GIV2 were found to be much more potent than L-menthol in activating TRPM8 transfected in HEK cells and activated overlapping populations of menthol-sensitive rat trigeminal ganglion (TG) and dorsal root ganglion (DRG) cells. Using in vivo electrophysiology, GIV1 and GIV2 were shown to directly activate some cold-sensitive Vc neurons in rats, with GIV1 enhancing responses to cooling and both agents reducing responses to noxious heat. These menthane carboxyamides therefore represent powerful cooling agents as well as useful molecular tools to help elucidate the mechanisms underlying thermal sensations.

## Materials and methods

### Chemicals

For fluorimetric imaging plate reader (FLIPR) studies, all agonists including L-menthol, cinnamaldehyde, and menthol carboxamide (WS-3, Givaudan Flavors) and the novel cooling compounds, N-(4-cyanomethylphenyl)-p-menthanecarboxamide (GIV1) and N-(2-(Pyridin-2-yl)ethyl)-3-p-menthanecarboxamide (GIV2) (Givaudan) were prepared at a stock concentration of 10 mg/mL in dimethyl sulfoxide (DMSO), from which concentration curves were prepared in assay buffer on the day of the experiment. For calcium imaging studies, GIV1 and GIV2 were first dissolved in PG at a concentration of 30 mM and further diluted to working concentrations in Ringers solution. L-menthol, cinnamaldehyde, and capsaicin were dissolved

in ethanol (EtOH) and further diluted to working concentrations in Ringers solution (0.015% EtOH).

For in vivo electrophysiology, GIV1 and GIV2 were dissolved in either 70% DMSO or propylene glycol (PG), respectively. One percent L-menthol (Givaudan) was dissolved in 10% ethanol + 1% Tween-80 (Sigma), 10% cinnamaldehyde (Sigma) was dissolved in 1.0% Tween-80, and 0.01% capsaicin (Sigma) was dissolved in 10% ethanol.

### TRPM8 and TRPA1 calcium imaging studies

HEK293 cell lines stably expressing either hTRPM8 or hTRPA1 were generated for these studies, and receptor activation was monitored by calcium imaging in a FLIPR. For generation of stable cell lines, constructs containing either TRPM8 or TRPA1 were linearized and transfected into the HEK293 host cells. The TRPM8 cDNA clone was generated by reverse transcriptase-polymerase chain reaction using mRNA isolated from human DRG, which were purchased from ABS Bio Inc. The resulting hTRPM8 cDNA was fully sequenced for accuracy, and the protein encoded by this construct is identical to the human TRPM8 sequence associated with GenBank accession ID NP 076985. The cDNA clone for human TRPA1 was obtained directly from Origene, Inc, and the protein encoded by this cDNA is identical to the human TRPA1 sequence associated with GenBank accession ID NM 007332. The hTRPM8 cDNA was prepared in pcDNA3.1 (Invitrogen) allowing for constitutive expression of the transgene, whereas we used pcDNA4-TO (Invitrogen) to allow for tetracycline-regulated expression of hTRPA1. After 48 h post-transfection, the cells were treated with 200 µg/mL Zeocin (Invivogen) to select for stable transfectants. After 2–4 weeks, zeocin-resistant colonies were selected, expanded, and clones expressing hTRPM8 were identified based on a robust response to 100 µM L-menthol, whereas TRPA1 clones were selected based on their response to 250 µM trans-cinnamaldehyde. TRPA1 expression was induced for 4 h by the addition of 0.5 µg/mL tetracycline 4 h prior to initiation of calcium flux studies for receptor activity.

For FLIPR assays of TRP channel activation, cells were pre-plated on Day 0 at a density of 9500 cells per well in DMEM + 10% FBS in black, clear bottom 96-well plates that had been precoated with 0.001% poly (ethyleneimine) (molecular weight = ~60 000, Acros Organics). On day 2, agonists were evaluated via calcium imaging using Fluo-4. Briefly, growth medium was discarded, and the cells were incubated in the dark for 1 h at 37 °C in 50 µL loading buffer consisting of 1.5 µM Fluo-4 AM (Invitrogen) and 2.5 µM probenecid (Sigma-Aldrich) in DMEM (no FBS). After incubation, the plates were washed 5× with 100 µL of assay buffer (in mM: 130 NaCl, 5 KCl, 10 HEPES, 2 CaCl<sub>2</sub>, and 10 glucose, pH 7.4.) and further incubated in the dark at room temperature for 30 min. The cells were then washed 5× with 100 µL assay buffer and then calcium responses to serial dilution curves of

agonists were measured in a FLIPR<sup>TETRA</sup> (Molecular Devices). Data were collected with 2–4 replicates from  $n = 3$  experiments and processed with GraphPad Prism version 5.0 (GraphPad Software).

#### TG and DRG cell culture and calcium imaging

Under a protocol approved by the UC Davis Institutional Animal Care and Use Committee, juvenile (~3 weeks, ~100 g) male Sprague–Dawley rats were euthanized under isoflurane anesthesia and trigeminal, and lumbosacral ganglia were extracted and placed into Petri dishes containing Hanks Buffered Salt Solution (Gibco, Invitrogen Life Sciences). Ganglia were minced with fine spring scissors and incubated in 40  $\mu$ L papain (no. 3126, Worthington Biochemical Company) with 1 mg L-cysteine (Sigma) in 1.5-mL Hanks solution for 5 min in a 37 °C rocking water bath. Cells were centrifuged at 200  $\times g$  for 2 min and media suctioned away. The ganglia were then incubated in 2 mg/mL collagenase type II (CLS2, Worthington) in Hanks Solution for 5 min in a 37 °C rocking water bath. Cells were centrifuged again at 200  $\times g$  for 1 min and gently triturated with completed media consisting of Earle's minimal essential media (Gibco) and 10% donor horse serum (Quad Five) with 1% 100  $\times$  MEM vitamin solution and penicillin–streptomycin (Gibco) through polished glass pipettes. The cells were plated in 40- $\mu$ L aliquots on 25-mm round glass coverslips (Bellco) coated with 1 mg/mL poly-D-lysine (Sigma) for 1 h. Cells were given 2 mL of completed media 1 h postplating in a 37 °C water-jacketed CO<sub>2</sub> injected incubator, and fresh media was given after 24 h.

Cells were incubated 1 h in 1 mM Fura 2AM (F1221, Invitrogen Life Sciences) dissolved in DMSO (Sigma) to a final concentration of 10  $\mu$ M in 5 mM glucose-supplemented Ringers solution (140 mM NaCl, 4 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, and pH adjusted to 7.4 with 4.54 mL of 1 M NaOH) containing 0.1% Pluronic (F127, Invitrogen). Cells were rinsed with Ringer's solution and allowed to rest for 10 min before being placed on a custom-made aluminum perfusion block designed to fit a thermal stage (HCC100A, Dagan Corporation) and viewed through a Nikon inverted microscope (Eclipse TS100). Fluorescence images obtained at 340/380-nm wavelengths were viewed through a CoolSnap camera attached to a Lambda LS lamp and a Lambda 10-3 optical filter changer (Sutter Instrument Company). Ratiometric measurements were made using Simple PCI (Compix Inc.) with an intermittent pause of 3 s between measurements. Solutions were administered to one end of the perfusion block by a gravity fed, solenoid-operated perfusion system (ValveLink 8.2, AutoMate Scientific) and removed via a vacuum line at the other end.

Chemical stimuli were 250  $\mu$ M L-menthol, 200  $\mu$ M CA, or 1  $\mu$ M capsaicin (dissolved in 0.015% EtOH in Ringers) and 100  $\mu$ M GIV1 and GIV2 (dissolved in 0.25% PG). In pilot

experiments, we determined that the 100  $\mu$ M concentration of the GIV compounds elicited responses comparable to those elicited by 250  $\mu$ M L-menthol and 200  $\mu$ M CA. Each chemical was applied for 30 s, except capsaicin which was applied for 10 s. Vehicles were applied separately as controls and had no effect (data not shown). Ratiometric measurements for GIV1 or GIV2 application were obtained during a 30-s application period, followed subsequently by application of menthol and CA in a randomized order to avoid order effects. Capsaicin was always applied next-to-last to avoid desensitization, followed lastly by Ringers containing a high K<sup>+</sup> concentration (144 mM) to identify neurons. A positive response to a chemical was defined as at least a 20% change in corrected ratio response (= response postapplication – baseline previous to application). The incidence of responses of GIV1- and GIV2-sensitive TG and DRG cells to menthol, CA, and capsaicin were compared using Fisher's Exact tests.

#### Vc electrophysiology

Methods were the same as described previously (Zanotto et al. 2007, 2008). Briefly, male Sprague–Dawley rats (381–556 g) were anesthetized with thiopental (induction: 85 mg/kg intraperitoneal; maintenance 10 mg/kg/h intravenously). The caudal medulla was exposed surgically to allow extracellular single-unit recordings from second-order neurons in the dorsomedial aspect of Vc using tungsten microelectrodes. Unit activity was amplified, digitized, and displayed using a Powerlab interface (AD Instruments) and custom software (Forster and Handwerker 1990). Recorded action potentials were sorted by spike size and waveform, and responses quantified as number of action potentials per second.

Ice-cold water was used as a search stimulus to identify cool-sensitive units. In the first set of experiments, we recorded unit responses to sequential topical (lingual) application of ice water (0–3 °C), followed 1 min later by hot water (53 °C), followed 2 min later by one of the chemicals (i.e., GIV1, GIV2, menthol, or CA), followed 2 min later by the cold- and hot-water stimuli. The entire sequence was repeated using the same cold- and hot-water stimuli but a different chemical, at least 20 min after the previous trial, in order to reduce potential carryover effects. However, subsequent analysis revealed no evidence for carryover effects of various chemicals (i.e., no evidence for cross-sensitization or cross-desensitization). Responses to chemical stimuli were determined by comparing activity 60 s prior to chemical stimulation with activity during the 60-s period following chemical application by paired *t*-test. Chemical modulation of thermal responses was analyzed by comparing mean cold- or heat-evoked responses before with those recorded following chemical stimulus application by paired *t*-test. For these comparisons, baseline activity was subtracted from the thermally evoked responses.

In a second set of experiments, cold, heat, and chemical stimuli were applied, with 2 differences. First, the thermal stimuli were delivered using a feedback-controlled Peltier thermode (2TE-2A Physitemp) that contacted the dorsal anterior tongue surface. The thermode warmed or cooled the tongue at a rate of  $\sim 1^{\circ}\text{C/s}$ . Temperature at the thermode-tongue interface was recorded online using a fast thermocouple (IT-23, Physitemp) connected to a digital thermometer (BAT-12; Physitemp). Use of controlled thermal stimuli allowed us to determine thresholds for cold- and heat-evoked responses, which were calculated as the temperature at which the neuronal firing rate changed by 2 or more SD relative to the preceding baseline firing rate. The second difference was timing. The cold stimulus was applied first, followed 2 min later by the heat stimulus, followed 2 min later by the chemical. We then recorded activity for 20 min before re-applying the cold and hot stimuli. As in the first set of experiments, a 20-min rest period intervened between successive application of chemicals to avoid carryover effects and chemicals were applied in a randomized order. At the conclusion of recording experiments, an electrolytic lesion was made at the recording site to allow histological verification as previously described (Zanotto et al. 2007).

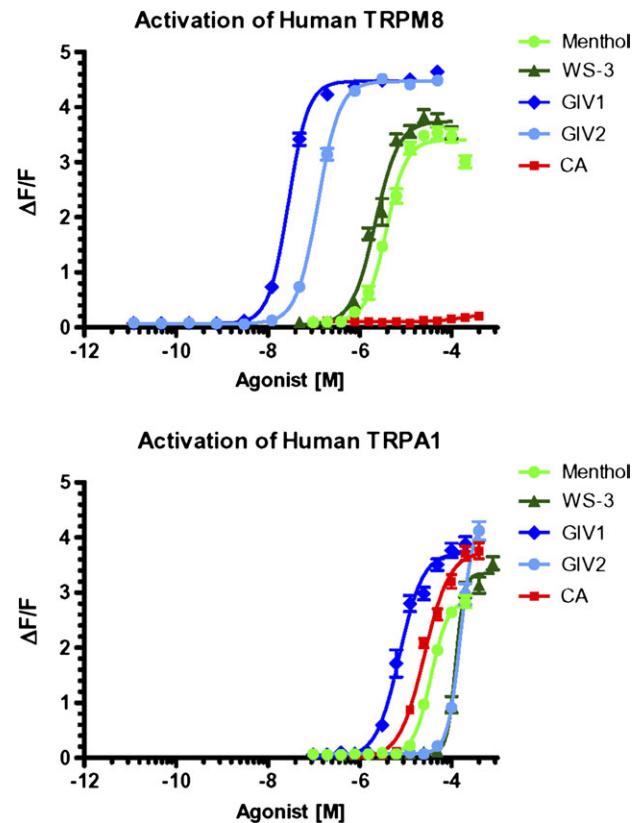
## Results

### Calcium flux studies

HEK cells stably expressing either hTRPM8 or hTRPA1 were exposed to either the TRPM8 agonists L-menthol or WS-3, TRPA1 agonist cinnamaldehyde (CA) (Bandell et al. 2004, 2005), or novel menthane compounds GIV1 or GIV2. The GIV1 and GIV2 EC<sub>50</sub> activation concentrations for hTRPM8 were 28.1 nM and 128 nM, respectively (Figure 2, upper panel). Comparing to the EC<sub>50</sub> value for menthol, 3.8  $\mu\text{M}$ , GIV1 and GIV2 are almost 140-fold and 31-fold, respectively, more efficacious at activating to TRPM8. We found that GIV1 was a more potent agonist for TRPA1 than the CA reference, with an EC<sub>50</sub> of 7.3  $\mu\text{M}$  versus 25.8  $\mu\text{M}$  for CA (Figure 2, lower panel). Interestingly, we found that the remaining menthane, WS-3 (120.5  $\mu\text{M}$ ), menthol (36.6  $\mu\text{M}$ ), and GIV2 (159.1  $\mu\text{M}$ ), were much weaker agonists of TRPA1 than GIV1. Comparisons of potencies obtained in TRPM8 and TRPA1 expressing cells suggest that although GIV1 and GIV2 activate both TRP channels, they are 260- to 1300-fold more selective for TRPM8 than TRPA1.

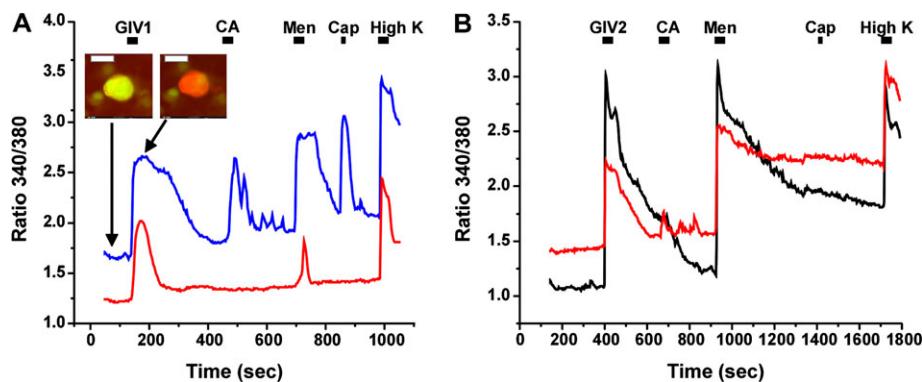
### Calcium imaging of TG and DRG cells

Fifteen percent of TG and 11% of DRG cells responded to GIV1, whereas 24% of TG and 10% of DRG cells responded to GIV2. Examples of 2 DRG cells' responses to GIV1 are shown in Figure 3A. Both additionally responded to menthol, whereas the cell indicated by the blue trace also responded to CA and capsaicin. Examples of 2 TG cells' responses to GIV2 are shown in Figure 3B. Both additionally responded to menthol but not capsaicin or CA.



**Figure 2** Novel menthane preferentially activate hTRPM8. HEK293 cells stably expressing hTRPM8 (upper graph) or hTRPA1 (lower graph) were stimulated with agonists, and calcium mobilization was quantified using a FLIPR-Tetra. The calcium mobilization signals are presented in  $\Delta F/F$ , where  $\Delta F/F = (F - F_0)/F_0$ , in which peak fluorescence responses ( $F$ ) after compound addition have been corrected for and normalized to background fluorescence ( $F_0$ ). The dose-response curves were plotted using GraphPad Prism 5 (GraphPad Software) and fitted using a 4-parameter logistic equation. Data were collected from 4 replicates of a  $n = 3$  experiments. The EC<sub>50</sub> values determined from these data were: Menthol: hTRPM8 =  $3.7 \pm 0.1 \mu\text{M}$ , hTRPA1 =  $36.7 \pm 1.8 \mu\text{M}$ ; WS-3: hTRPM8 =  $2.2 \pm 0.1 \mu\text{M}$ , hTRPA1 =  $120.6 \pm 12.1 \mu\text{M}$ ; GIV1: hTRPM8 =  $28 \pm 2.2 \text{ nM}$ , hTRPA1 =  $7.3 \pm 1.1 \mu\text{M}$ ; GIV2: hTRPM8 =  $123.1 \pm 4.4 \text{ nM}$ , hTRPA1 =  $159.1 \pm 7.5 \mu\text{M}$ ; CA: hTRPA1 =  $25.9 \pm 0.4 \mu\text{M}$ .

The percentages of GIV1- and GIV2-sensitive TG and DRG neurons that responded to additional chemical stimuli are shown in the pie charts of Figure 4A–D. A significant proportion of GIV1-sensitive TG and DRG cells responded to menthol (79.4% and 41.2%, respectively; Figure 4A,B, green and blue wedges;  $P < 0.05$ , Fisher's Exact test). Similarly, a significant percentage of GIV2-sensitive TG and DRG cells responded to menthol (77.4% and 70.6%, respectively;  $P < 0.05$ ) (Figure 4C,D). A significant proportion of GIV2-sensitive TG cells also responded to CA (41.9%, Figure 4C;  $P < 0.05$ ). Data for GIV1- and GIV2-insensitive TG and DRG cells are presented in Figure 4E–H, where it may be seen that much lower percentages of cells responded to menthol (7–12.1%), whereas larger percentages responded to capsaicin. Overall, 23.1% of all TG



**Figure 3** Examples of DRG cell responses to GIV compounds. **A.** Graph plots 340/380-nm ratios versus time for 2 DRG cells responding to GIV1 (100  $\mu$ M). Both cells also responded to menthol (250  $\mu$ M) and high K<sup>+</sup>. The cell indicated by the blue trace also responded to CA (200  $\mu$ M) and capsaicin (1  $\mu$ M), whereas the cell indicated by the red trace did not. Insets are photomicrographs of the cell indicated by the blue trace, prior to and after application of GIV1 at the time points indicated by the arrows. Calibration bar = 20  $\mu$ m. **B.** Graph as in A for 2 TG cells responsive to GIV2 (100  $\mu$ M), menthol (250  $\mu$ M), and high K<sup>+</sup>. Neither cell responded to capsaicin.

responded to menthol, 18.2% to CA, and 54.4% to capsaicin. Sixteen percent of all DRG cells responded to menthol, 10.9% to CA, and 23.7% to capsaicin, consistent with previous studies (Winter et al. 1988; Caterina et al. 2000; Nealen et al. 2003; Bautista et al. 2005; Karashima et al. 2007).

GIV1- and GIV2-sensitive TG and DRG cells were separated into 4 groups: those additionally activated only by menthol (Figure 4A–D, green areas), those activated by menthol as well as CA and/or capsaicin (Figure 4A–D, blue, teal, and magenta), those not activated by menthol or CA or capsaicin (Figure 4A–D, black area), and those not activated by menthol but activated by CA and/or capsaicin (Figure 4A–D, red, yellow, and dark yellow). We compared the magnitude of mean responses of these 2 groups, pooling TG and DRG cells because they exhibited similar peak amplitudes and time courses of response to the GIV compounds. Mean responses are shown for menthol-sensitive cells in Figure 5A and for menthol-insensitive cells in Figure 5B. Cells responsive to menthol but not other TRP channel agonists exhibited a significantly larger mean peak response to GIV1 compared with menthol-sensitive cells that responded to multiple TRP channel agonists (Figure 5A). Menthol-insensitive cells did not exhibit any significant differences in magnitude of mean response to either GIV compound, regardless of their sensitivity to other TRP channel agonists (Figure 5B).

#### Vc unit recordings

A total of 33 cold-sensitive Vc units was recorded. These cells were located superficially in the dorsomedial aspect of Vc at a mean depth of 133.3  $\mu$ m  $\pm$  21.2 (standard error of the mean) (Figure 6B inset). Twenty out of 33 units tested also responded to noxious heating (54 °C). Overall, 44% of the units (14/32) responded to menthol and 47% (15/32) responded to CA, and 78% of menthol-sensitive cells also responded to CA. Thus, many Vc units presumably received input from primary afferent fibers that express TRPM8 and/or TRPA1. Application of

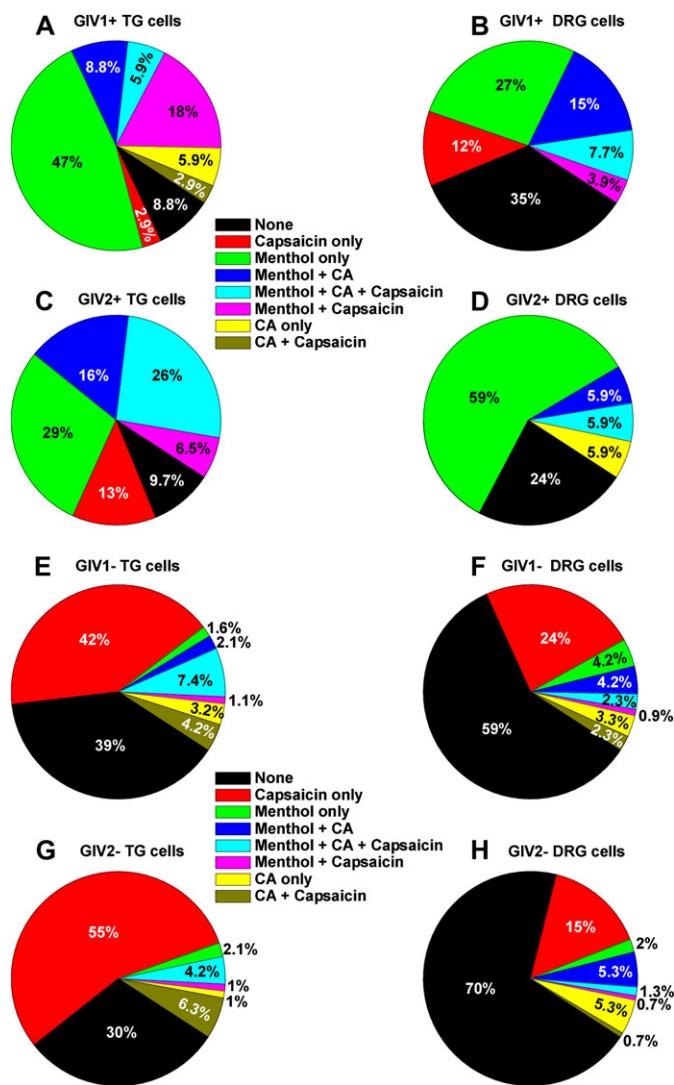
vehicles PG ( $n = 25$  units) or DMSO ( $n = 24$  units) did not result in a significant change in mean firing rate and did not significantly affect thermal responses (data not shown).

In the first set of experiments, GIV1 did not significantly affect overall firing in any of the 12 units tested but significantly enhanced responses to the cold-water stimulus applied 2 min post-GIV1 (Figure 6A). GIV1 also significantly attenuated the mean response to the noxious hot-water stimulus (Figure 6A). GIV2 excited 8/12 (66.6%) Vc units to result in a significant overall increase in firing but did not significantly affect cold- or hot water-evoked responses (Figure 6B).

In the second set of experiments, cold and heat stimuli were applied by Peltier thermode and responses following application of GIV1 and GIV2 were recorded over an 18-min period before thermal stimuli were reapplied. Eleven out of 17 and 11/14 Vc units responded to cooling at thresholds below 15 °C prior to application of GIV1 (Figure 7A) and GIV2 (Figure 7B), respectively. GIV1 significantly increased firing rate in 7/17 units at some point during the 20-min period following its application but did not significantly affect overall firing rate. Twenty minute post-GIV1, the mean cold-evoked response was again significantly enhanced (Figure 6C), accompanied by a significant decrease in cold threshold (toward warmer temperatures) (Figure 7A). Fourteen of the 17 units exhibited a decrease in threshold post-GIV1, including 9 of the 11 low-threshold units (Figure 7A). The mean heat-evoked response was not significantly affected following GIV1.

GIV2 excited 8/14 Vc units, although the overall increase in mean firing rate failed to reach statistical significance ( $P = 0.067$ ). After application of GIV2, cold-evoked responses (Figure 6D) and cold thresholds (Figure 7B) were not significantly affected. Heat-evoked responses were significantly attenuated (Figure 6D) with no significant change in heat threshold.

Table 1 provides a comparison of the incidence of responsiveness of GIV1- and GIV2-sensitive TG, DRG, and Vc neurons to menthol, CA, and capsaicin. In general, the



**Figure 4** Responses of TG and DRG cells to GIV1, GIV2, menthol, CA, and capsaicin. **A:** Pie chart shows incidence of responsiveness of GIV1-sensitive TG cells ( $n = 34$ ) to each combination of chemicals tested (capsaicin, menthol and CA). **B:** as in A for 26 GIV1-sensitive DRG cells. **C:** as in A for 31 GIV2-sensitive TG cells. **D:** as in A for 17 GIV2-sensitive DRG cells. **E:** as in A for 190 GIV1-insensitive TG cells. **F:** as in A for 215 GIV1-insensitive DRG cells. **G:** as in A for 96 GIV2-insensitive TG cells. **H:** as in A for 152 GIV2-insensitive DRG cells.

primary sensory (TG and DRG) neurons that responded to the GIV cooling compounds exhibited a higher incidence of responsiveness to menthol compared with second-order (Vc) neurons, whereas the latter exhibited a higher incidence of responsiveness to capsaicin indicative of convergence of thermal and nociceptive afferents at the level of Vc.

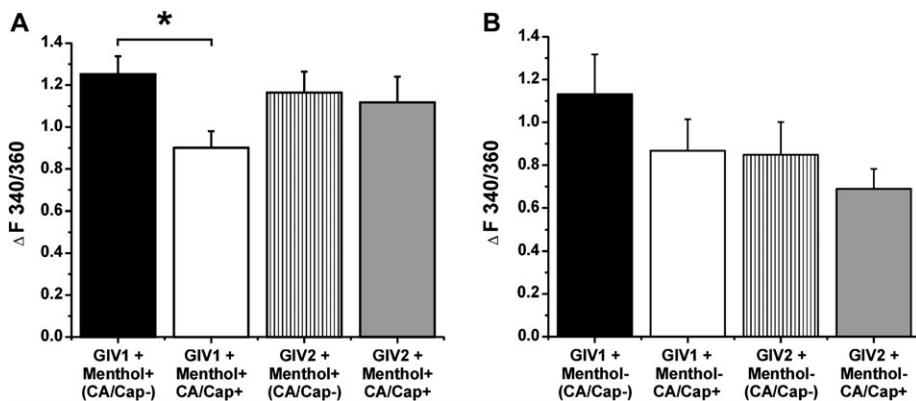
## Discussion

The present results show that novel cooling compounds, GIV1 and GIV2, act primarily at TRPM8 and excite many menthol-sensitive primary sensory TG and DRG neurons. Both GIV1 and GIV2 directly excited some second-order tri-

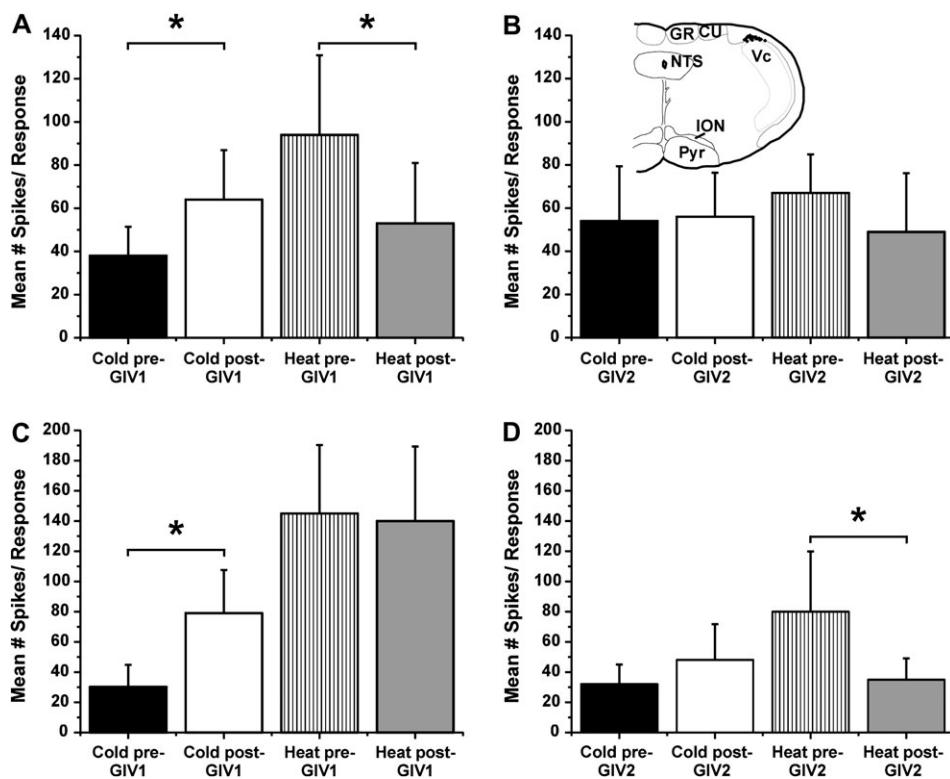
geminal Vc neurons, and GIV1 significantly enhanced neuronal responses to subsequent cooling. This is consistent with previous human psychophysical data (Furrer et al. 2008) showing that GIV1 induced cooling sensations that far outlasted those elicited by GIV2 or WS-3, a commonly used cooling agent also derived from menthol.

Based on the calcium flux data with transfected HEK cells, GIV1 and GIV2 are more potent than menthol or WS-3 in activating hTRPM8. GIV1 and GIV2 also activated hTRPA1, but only at much higher concentrations than those needed to activate hTRPM8, indicating that both compounds exhibit greater selectivity for hTRPM8. The present studies using calcium imaging also revealed a significant co-activation of menthol-sensitive rat TG and DRG cells by GIV1 and GIV2, although the concordance was not 100%. The overall percentages of menthol-sensitive TG (23%) and DRG cells (16%) are comparable to those observed in our recent studies using the same methods (TG cells: 17%, DRG cells: 14.4%; Klein, Carstens, et al. 2011; Klein, Sawyer, et al. 2011). This suggests that prior application of the GIV compounds did not markedly reduce the incidence of menthol sensitivity of TG and DRG cells. That the GIV compounds were able to activate a subset of menthol-insensitive TG and DRG cells suggests that they acted by some currently unknown, TRPM8-independent, mechanism. There was also a significant coincidence of activation of rat TG cells by CA and GIV2. Based on the calcium flux data, we assume that GIV1 and GIV2 act primarily at TRPM8 to directly excite sensory TG and DRG neurons. However, in the calcium imaging studies of rat TG and DRG cells, it is possible that the GIV compounds may have also acted at TRPA1 consistent with prior reports of “promiscuous” activation of TRPA1 by TRPM8 agonists (Macpherson et al. 2006; Karashima et al. 2007). The irritation/burning sensory qualities elicited by these compounds as reported in human sensory trials (Furrer et al. 2008) might be attributable to activity at TRPA1.

In the present study, GIV1 activated a significant proportion of menthol-sensitive TG and DRG cells, directly excited some Vc neurons, and significantly enhanced Vc neuronal responses to cooling. By comparison, GIV2 significantly elevated neuronal activity but did not significantly enhance responses to cooling. This difference may explain the significantly more prolonged subjective oral cooling sensation elicited by GIV1 compared with GIV2 (Furrer et al. 2008). The enhancement of cold-evoked responses of Vc neurons by GIV1 is consistent with our previous study showing that menthol also significantly enhanced Vc neuronal responses to lingual cooling (Zanotto et al. 2007, 2008). Because cold- and menthol-sensitive Vc neurons also respond to noxious heat, capsaicin, and other irritants, they presumably signal pain. A perceptual correlate of enhanced firing of such neurons may be the ability of menthol to increase cold pain on the human tongue (Albin et al. 2008) and to induce cold allodynia on forearm skin (Wasner et al. 2004; Namer et al. 2005; Hatem



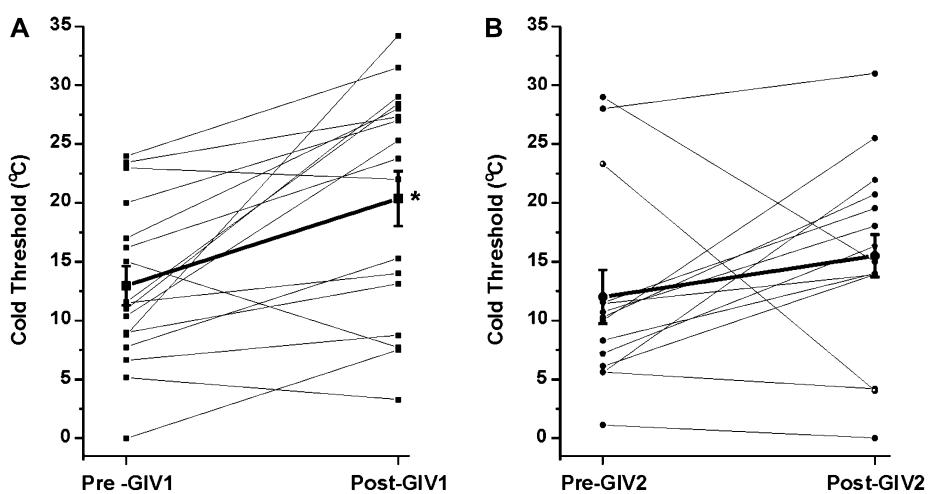
**Figure 5** GIV1 and GIV2 activation of TG and DRG cells, sorted according to responsiveness to menthol and capsaicin. **A:** menthol-sensitive cells. Bar graph plots mean maximal calcium responses (baseline-corrected) to GIV1 and GIV2 for each group. +: responded; -: did not respond. Error bars: standard error of the mean. \*: significant difference ( $P < 0.05$ , unpaired  $t$ -test). **B:** as in A for GIV-sensitive but menthol-insensitive TG and DRG cells.



**Figure 6** GIV1 and GIV2 effects on thermal responses of Vc neurons. **A:** bar graph plots mean responses of Vc neurons to cold and noxious heat stimuli, before and after application of GIV1. Responses to the cold stimulus (ice water) and noxious heat stimulus (53 °C water) were quantified as the sum of action potentials/60 s following application of the thermal stimulus and were baseline corrected by subtracting the number of action potentials occurring during the 60 s prior to application of the thermal stimulus. \*: significant difference between pre- and post-GIV1 ( $P < 0.05$ , paired  $t$ -test). Error bars: standard error of the mean ( $n = 12$ /group). **B:** graph as in A for GIV2. Inset: histologically verified recording sites compiled on representative section through Vc. Abbreviations: Cu, cuneate n., Gr, gracile n.; ION, inferior olivary n.; NTS, n. tractus solitarius; Pyr, medullary pyramid; Vc, trigeminal subnucleus caudalis. (Adapted from atlas of Paxinos and Watson 1998.) **C:** graph as in A for GIV1, except that cold and noxious heat stimuli were delivered via Peltier thermode. Responses to cold were summed over 75-s period of cooling and were baseline corrected by subtracting action potentials that occurred during the 75 s prior to onset of cooling. Responses to heat were summed over 60-s period of heating and baseline corrected by subtracting action potentials that occurred during 60 s prior to onset of heat. \*: significant difference between pre- and post-GIV1 ( $P < 0.05$ , paired  $t$ -test). Error bars: standard error of the mean ( $n = 18$ ). **D:** graph as in C for GIV2. \*: significant difference between pre- and post-GIV1 ( $P < 0.05$ , paired  $t$ -test). Error bars: standard error of the mean ( $n = 14$ ).

et al. 2006). It is noteworthy that GIV1 significantly reduced the cold threshold (i.e., shifted to higher temperature) of Vc neurons such that they could be activated by innocuous cool-

ing. Such neural activity may be relevant to the recently discovered phenomenon of innocuous thermal nociception, whereby an innocuous cool stimulus delivered to normal skin



**Figure 7** GIV1 reduces threshold to cooling of Vc neurons. **A:** graph plots individual (thin lines) and mean (thick line with error bars = standard error of the mean) unit cold thresholds preapplication and postapplication of GIV1. \*: significant difference,  $P < 0.05$ , paired *t*-test. **B:** as in A for cold thresholds preapplication and postapplication of GIV2, which has no significant effect.

**Table 1** Comparison of incidence of responsiveness of GIV1- and GIV2-sensitive TG, DRG, and Vc neurons to menthol, CA, and capsaicin

	GIV1+			GIV2+		
	Menthol	CA	Capsaicin	Menthol	CA	Capsaicin
TG cells	27/34 (79.4%)	8/34 (23.5%)	10/31 (29.4%)	24/31 (77.4%)	13/31 (41.9%)	14/31 (45.2%)
DRG cells	14/26 (41.2%)	6/26 (17.6%)	6/26 (17.6%)	12/17 (70.5%)	3/17 (17.6%)	1/17 (5.9%)
Vc cells	3/6 (50%)	4/29 (13.8%)	1/1 (100%)	7/27 (25.6%)	10/27 (37.0%)	4/6 (66.6%)

Numerator corresponds to number of positive cell responses and the denominator to the total number of cells tested.

can elicit nociceptive (stinging, burning) sensations (Green and Pope 2003; Green et al. 2008).

The enhancement of cold-evoked responses of Vc neurons by menthol and GIV1 may be explained by a peripheral action at epithelial nerve endings expressing TRPM8. Menthol was previously reported to directly activate some cold-sensitive primary afferent fibers and to enhance their response to cooling (Hensel and Zotterman 1951; Dodt et al. 1953; Wang et al. 1993; Lundy and Contreras 1995). Presumably, cold-sensitive afferent fibers express TRPM8, which is gated by decreasing temperature in the innocuous range in a manner that is enhanced by menthol (McKemy et al. 2002; Peier et al. 2002). TRPM8 was reported to be activated by temperatures as high as  $\sim 24$  °C (McKemy et al. 2002; Peier et al. 2002), whereas TRPA1 was originally reported to respond at temperatures below  $\sim 17$  °C (Story et al. 2003). It is noteworthy that many of the presently recorded Vc neurons had cold thresholds  $< 15$  °C, suggesting that they receive input from afferents expressing TRPA1. If so, this implies that GIV1 sensitized TRPA1 as an additional mechanism potentially explaining the ability of GIV1 to enhance cold-evoked responses of Vc neurons.

GIV1 significantly inhibited responses elicited by hot water but not Peltier heating (Figure 6A,C), whereas GIV2 significantly inhibited responses elicited by Peltier heating but not

hot water (Figure 6B,D). Hot-water stimuli were tested 2 min, whereas Peltier heat stimuli were tested 20 min, after application of GIV1 or GIV2. Thus, the asymmetric effects of these compounds on Vc neuronal responses to heat may be explained by a rapid and short-lasting inhibitory effect of GIV1 as compared with a more slowly developing inhibitory effect of GIV2. The inhibitory effects of GIV1 and GIV2 on heat-evoked responses are consistent with previous animal studies showing antinociceptive (Klein et al. 2010) and anti-hyperalgesic (Proudfoot et al. 2006) effects of menthol, as well as human psychophysical studies showing menthol suppression of oral heat sensation (Green 1986, 2005; Albin et al. 2008). The antinociceptive effect of menthol in rats might be explained by a peripheral effect in which menthol inhibits TRPA1 expressed in nociceptive nerve endings (Macpherson et al. 2006; Karashima et al. 2007). However, this cannot explain menthol's effect in humans because human TRPA1 is not inhibited by menthol (Xiao et al. 2008). Menthol also excites peripheral cold receptors that may centrally inhibit spinal nociceptive neurons (Jinks and Carstens 1998). An additional possibility is that menthol activates supraspinal circuits that give rise to descending inhibition of spinal nociceptive neurons.

We presently observed that substantial proportions of TG (13–42%) and DRG cells (10–23%) were activated by both

menthol and CA. Our results are consistent with a previous report that 38% of mouse TG cells responsive to mustard oil (another TRPA1 agonist) also responded to menthol (Karashima et al. 2007). A recent study from our laboratory reported that ~10% of menthol-sensitive rat TG cells also responded to CA (Klein et al. 2010). These findings stand in contrast to other prior studies reporting that virtually no mouse DRG cells responded to both menthol and CA (Hjerling-Leffler et al. 2007) as well as in situ hybridization studies reporting little or no coexpression of TRPM8 and TRPA1 in rat (Kobayashi et al. 2005) or mouse (Story et al. 2003) TG and/or DRG cells. We currently are unable to offer a resolution to these apparent discrepancies. It is also noteworthy that substantial fractions of GIV1- and GIV2-sensitive DRG and TG cells also responded to capsaicin (Figure 4). If the capsaicin- and/or CA-sensitive cells represent nociceptors, then the ability of the GIV compounds to activate these cells may explain the reported pungency of these cooling agents (Furrer et al. 2008).

TRPM8-expressing primary afferents project to superficial laminae of the spinal cord dorsal horn (Dhaka et al. 2008) where partly overlapping populations of neurons are activated by menthol or the TRPA1 agonist allyl isothiocyanate (Wrigley et al. 2009). This is similar to neurons located in superficial laminae of the dosomedial aspect of Vc, many of which respond to menthol as well as other TRP channel agonists (Carstens and Mitsuyo 2005; Zanotto et al. 2007, 2008). That such Vc neurons generally respond to a wider array of stimuli than primary sensory neurons (Table 1) suggests convergence of primary afferent fibers onto Vc neurons.

Given that GIV1, GIV2, and menthol elicit sensations of innocuous cooling (Furrer et al. 2008), it is likely that these agents activate innocuous cold receptors. This is further supported by our observation that the magnitude of calcium response elicited by GIV1 was significantly larger in TG and DRG cells that exclusively responded to menthol but not other TRP channel agonists, compared with cells that were activated by multiple TRP channel agonists (Figure 5A). We speculate that cells only responsive to menthol (and GIV1 or GIV2) represent cold receptors, whereas those sensitive to menthol as well as CA or capsaicin represent nociceptors. Afferent fibers of at least some cold receptors may be synaptically connected to cool-specific Vc neurons that respond to innocuous cooling but not to heat or other noxious stimuli (Dostrovsky and Craig 1996). Cooling-specific Vc neurons were not encountered in the present study and may be less numerous compared with the cold-sensitive nociceptive Vc units recorded from presently that responded to menthol, CA and capsaicin, and thus presumably signal pain sensation.

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