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Eugenol and carvacrol excite first- and second-order trigeminal neurons and enhance their heat-evoked responses

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Abstract

Eugenol and carvacrol from clove and oregano, respectively, are agonists of the warmth-sensitive transient receptor potential channel TRPV3 and the irritant-sensitive TRPA1. Eugenol and carvacrol induce oral irritation that rapidly desensitizes, accompanied by brief enhancement of innocuous warmth and heat pain in humans. We presently investigated if eugenol and carvacrol activate nociceptive primary afferent and higher-order trigeminal neurons and enhance their heat-evoked responses, using calcium imaging of cultured trigeminal ganglion (TG) and dorsal root ganglion (DRG) neurons, and in vivo single-unit recordings in trigeminal subnucleus caudalis (Vc) of rats. Eugenol and carvacrol activated 20-30% of TG and 7-20% of DRG cells, the majority of which additionally responded to menthol, mustard oil and/or capsaicin. TG cell responses to innocuous (39°) and noxious (42°C) heating were enhanced by eugenol and carvacrol. We identified dorsomedial Vc neurons responsive to noxious heating of the tongue in pentobarbital-anesthetized rats. Eugenol and carvacrol dose-dependently elicited desensitizing responses in 55% and 73% of heat-sensitive units, respectively. Responses to noxious heat were briefly enhanced by eugenol and carvacrol. Many eugenol- and carvacrol-responsive units also responded to menthol, cinnamaldehyde and capsaicin. These data support a peripheral site for eugenol and carvacrol to enhance warmth- and noxious heat-evoked responses of trigeminal neurons, and are consistent with the observation that these agonists briefly enhance warmth and heat pain on the human tongue.

Introduction

Eugenol and carvacrol are organic chemicals found in clove and oregano, respectively. These compounds have antiseptic and flavor-additive properties, and are used in a variety of commercial applications. Eugenol has been used in dentistry as a local anesthetic

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(Markowitz et al., 1992) owing to its inhibitory effect on voltage-gated sodium and calcium channels in trigeminal nociceptors (Lee et al., 2005; Park et al., 2006; Chung et al., 2008; Park et al., 2009). Carvacrol has also been reported to have antinociceptive effects (Cavalcante Melo et al., 2012). Additionally, eugenol and carvacrol elicit oral pungency (Cliff Heymann, 1992; Klein et al., 2013) and eugenol activates TRPA1 and TRPV1 (Bandell et al., 2004) that are expressed in nociceptive nerve endings. Eugenol enhanced presynaptic glutamate release in the rat superficial spinal cord dorsal horn via an action at TRPA1 (Inoue et al., 2012). Carvacrol activates human and mouse TRPA1 (Bandell et al., 2004; Xu et al., 2006; Lee et al., 2008; de la Roche et al., 2013). A common feature both of compounds is that they activate TRPV3 (Xu et al., 2006; Vogt-Eisele et al., 2007; Sherkheli et al., 2009), which is expressed in sensory neurons and keratinocytes and is activated by innocuous warming (Xu et al., 2002; Smith et al., 2002; Peier et al., 2002; Chung et al., 2004). Previous reports suggested that TRPV3 also contributes to heat pain in mice (Moqrich et al., 2005; Huang et al., 2008), although this has been disputed since knockout mice lacking TRPV3 exhibited little or no change in thermal preference behavior or acute heat nociception (Huang et al., 2011).

In humans, eugenol and carvacrol elicited oral and nasal irritation consisting of warming, cooling, burning, stinging, pricking, tingling and numbing subqualities (Cliff & Heymann, 1992; Green 2002; Wise et al., 2012; Klein et al., 2013) similar to those elicited by other TRP channel agonists (Dessirier et al., 2001; Albin et al., 2008; Simons et al., 2003; Bennett & Hayes, 2012). Moreover, both eugenol and carvacrol enhanced the perceived intensity of innocuous warmth as well as heat pain on the tongue (Klein et al., 2013). Collectively, these studies suggest that eugenol and carvacrol have both pro- and anti-nociceptive effects via their actions at TRPV3, TRPA1 and TRPV1 expressed in peripheral and central primary afferent terminals.

There are few previous studies of the ability of eugenol and carvacrol to directly excite primary sensory or higher-order trigeminal neurons (Ohkubo & Kitamura, 1997). We presently investigated if these chemicals excite trigeminal ganglion (TG) and dorsal root ganglion (DRG) neurons, including those responsive to thermal stimuli, using the method of flourometric calcium imaging. Since many irritants activate neurons in trigeminal subnucleus caudalis (Vc; Carstens et al., 1998; Zanotto et al., 2007), we also used *in vivo* electrophysiological methods to investigate if eugenol and carvacrol activate Vc neurons and enhance their responses to warmth and/or noxious heat. An abstract of a portion of this work has appeared previously (Klein et al., 2012b).

Materials and Methods

All experiments were conducted under protocols approved by the UC Davis Institutional Animal Care and Use Committee.

Calcium imaging

Trigeminal ganglia (TG) and lumbrosacral dorsal root ganglia (DRG) were extracted from juvenile (2-3 wk) male Sprague-Dawley rats (n= 20). The ganglia were triturated and TG and DRG cells were processed as previously described (Klein et al., 2011a, Klein et al.,

2011b) and plated onto glass coverslips pre-treated with poly-D-lysine. Cells were given fresh media after one hour and imaged within 48 hours.

After loading with 10μM Fura-2AM (F1221, Invitrogen, Grand Island NY) in Ringer's solutions (140 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, 4.54 mL NaOH, and pH adjusted to 7.4), TG and DRG cells were placed in a perfusion chamber (CSC-25, Bioscience Tools, San Diego, CA) on a thermal stage (BTC-S and BTC-100, Bioscience Tools) set at 34°C. Images were taken every 3 sec with NIS Elements software (Nikon Instruments Inc., Melville, NY) at 340/380 nm wavelengths. Solutions were administered by a gravity-fed solenoid operated perfusion system (ValveLink 8.2, AutoMate Scientific; Berkeley, CA) and removed via vacuum line at the other end. Chemicals used included 250 μM menthol (Givaudan Flavors Corporation, Cincinnati OH), 100 μM AITC (allyl isothiocyanate; mustard oil; Sigma-Aldrich Chemical Co., St. Louis MO), 200 μM eugenol (Sigma), 100 μM carvacrol (Sigma), and 1 μM capsaicin (Sigma). All chemicals were dissolved in 0.015% ethanol Ringer's solution. In separate experiments, TG and DRG cells were tested for sensitivity to 1 μM farnesyl phosphopyruvate (FPP, Enzo Life Sciences, Farmingdale, NY) in 100 μM NH_4HCO_3 and 0.0028% ethanol. Chemicals were delivered for 30 sec, with the exception of capsaicin which was delivered for 10 sec. Ringer's solution containing a high K+ concentration (144 mM) was given at the end of the experiment to verify neuronal recordings. Vehicle (0.015% ethanol in Ringer's) did not have any effect (data not shown).

In a separate group of experiments we investigated thermal responses. TG and DRG cells were perfused with Ringer's solution pre-heated to either 39°C or 42°C for thirty seconds using a miniature heater (TC-RD, Bioscience Tools) connected to a separate temperature controller (BTC-1-100, Bioscience Tools). The bath temperature was monitored by a thermocouple within the feedback-controlled thermal stage and also by a separate thermocouple (IT-18, Physitemp Instruments, Inc., Clifton, NJ) placed in the bath just outside the microscopic field of view. The thermocouple was connected to a microprobe thermometer (BAT-12, Physitemp) which was fed via a Powerlab interface (AD Instruments, Colorado Springs CO) to a digital computer and viewed using Chart 5 software. Following delivery of the first heat stimulus (either 39 or 42°C), a second equivalent heat stimulus was delivered 10 min later. The second heat stimulus was preceded 30 sec earlier by bath delivery of either eugenol, carvacrol or no stimulus.

Single Unit Recording

Methods were essentially the same as described previously (Klein et al., 2011c; Zanotto et al., 2007; Zanotto et al., 2008). Eighty-three adult male Sprague-Dawley rats (480 ± 8.3g) were anesthetized with sodium pentobarbital (induction: 65 mg/kg i.p., maintenance: 10 mg/kg i.v.). The caudal medulla was exposed surgically while body temperature was maintained by a heating pad and oxygen delivered via tracheal cannula. The ECG was recorded and displayed continuously using a Powerlab interface (AD Instruments). A tungsten microelectrode (FHC, Bowdoin, ME; 10 MΩ) was positioned using a hydraulic microdrive (David Kopf Instruments, Tujunga CA) to record single Vc units having heat-sensitive lingual receptive fields. We used a noxious heat stimulus (see below) to isolate Vc

units and did not attempt to identify units responsive to innocuous warming that are quite rare (Dostrovsky & Hellon, 1978; Andrew & Craig, 2001). Unit activity was amplified and displayed using Powerlab and Spike 2 (Cambridge Electronic Design, Cambridge, UK) interfaces. In some experiments more than one heat sensitive unit was recorded and discriminated by waveform post-hoc using the Spike 2 software. Only units that responded to noxious heat were selected for further analysis.

Thermal stimuli were delivered using a feedback-controlled Peltier thermode (NTE-2A, Physitemp, Clifton, NJ; 13 mm diameter) attached to a micromanipulator to contact the dorsal anterior surface of the tongue. The Peltier thermode was programmed to deliver noxious heat (up to 53°C) followed 2 min later by cooling (down to 7°C) from an adapting temperature of 35°C (Klein et al., 2012b). The lingual-thermode interface temperature was measured using a fast thermocouple (IT-18, Physitemp) connected to a microprobe thermometer (BAT-12, Physitemp) and was displayed continuously using a Powerlab interface and Chart software (AD Instruments). The heat-cold sequence was delivered twice and unit responses were averaged to provide the baseline thermal response level before chemical application. The Peltier thermode was removed 3 min after the last cold stimulus, followed by chemical stimulation. Chemicals were 0.1-10% eugenol or carvacrol, 1% l-menthol, 10% cinnamaldehyde (CA, Sigma), 0.01% capsaicin (Cap) or vehicle (10% ethanol and 1% Tween-80, Sigma). Chemicals were applied to the anterior dorsal surface of the tongue with a perfusion pump that delivered the chemical at a constant rate (0.5 ml/min). The Peltier thermode was replaced 1 min later and the heat/cold thermal sequence was repeated 2 min later. Three sequences of heating and cooling were delivered 3, 8 and 13 min after chemical application. Mechanical stimulation was performed using von Frey filaments (0.68-288.4 mN) followed by touching and pinching with a blunt forceps before and after vehicle application and after eugenol/carvacrol application.

An electrolytic lesion was made at the conclusion of each experiment. The brainstem was post-fixed in 10% buffered formalin and 50 μm sections cut on a microtome. Lesions were identified microscopically (Klein et al., 2011c).

Data Analysis

For calcium flourometry, each cell's maximum response (i.e., increase in 340/380 nm ratio) during the 60 sec period post chemical application was divided by the maximum ratio during the minute before chemical application (baseline). An increase in fluorescence ratio >20% was considered a positive response. A pivot table was generated to tabulate incidences of cellular responses to chemical application. TG and DRG cell responses to successive heat stimuli were analyzed via paired t-test.

In vivo electrophysiological single unit data were analyzed by summing the number of action potentials during the 30 sec period beginning with noxious heat stimuli, or during the 60 sec period beginning with cold or chemical stimuli. Data were baseline-corrected by subtracting the sum of spontaneously occurring action potentials recorded during a comparable (30- or 60-sec) period prior to the stimulus. An increase in baseline-corrected firing of >30% was considered to be a positive response. Statistical analyses were conducted

using Graph Pad Prism 5 (Graph Pad Software Inc., La Jolla CA) and SPSS 9.0. Error reported is the standard error of the mean (SEM).

Results

Fluorometric calcium imaging

Incidence of sensitivity to eugenol and carvacrol—A total of 240 TG cells and 276 DRG cells were tested for sensitivity to eugenol, carvacrol, menthol, AITC and capsaicin. Eugenol activated 23% of TG cells and 7.6 percent of DRG cells, whereas carvacrol activated 30.4% of TG and 21.3% of DRG cells. The incidences of responsiveness to menthol (28.8% and 25.3% for TG and DRG cells, respectively), AITC (38.3% and 33.4%) and capsaicin (62.9% and 56.5%) were similar to previously published data (Klein et al., 2011a-c). Fig. 1A, B shows an example of a carvacrol-responsive TG cell that also responded to AITC, eugenol and capsaicin but not menthol. Tables 1 and 2 show the incidence of responses of eugenol- and carvacrol-sensitive TG and DRG cells, respectively, to the other chemicals tested. Over 70% of eugenol- and carvacrol-sensitive cells responded to capsaicin. Forty to 61% of eugenol- and carvacrol-sensitive TG and DRG cells also responded to AITC.

In separate experiments, 240 TG and 168 DRG cells were tested for sensitivity to the TRPV3 specific agonist FPP (Bang et al., 2010). Only 5.4% (13/240) of TG and 1.7% (3/168) of DRG cells, respectively, responded to 1 μM FPP. Of all FPP-responsive cells, 75% and 55.6% also responded to eugenol and carvacrol, respectively. Eighty-five percent of TG and 100% of DRG cells that responded to FPP also responded to capsaicin.

Eugenol and carvacrol enhancement of warmth- and noxious heat-evoked responses—Eugenol and carvacrol enhanced innocuous warmth and heat pain sensation on the tongue in humans (Klein et al., 2013). We tested if the responses of TG and DRG cells to innocuous warmth (39°C) and/or noxious heat (42°C) stimuli were enhanced in the presence of eugenol and/or carvacrol. Fig. 2 shows examples of 3 TG cells' responses to an initial application of a 39°C stimulus. Ten min later, eugenol (200 μM) was applied for 30 sec followed by a second 39°C heat stimulus, which elicited larger responses in cells 2 and 3. Since the incidences of responsiveness to chemical (Liu et al., 1996; Bautista et al., 2006; Bautista et al., 2007) and innocuous or noxious thermal stimuli were similar for TG and DRG cells (present data), the data were combined. Overall, 104 of 462 (22.5%) TG and DRG neurons tested with a 39°C heat stimulus and 172 of 354 (48.6%) tested with a 42°C heat stimulus responded. Both eugenol and carvacrol significantly enhanced the responses of TG and DRG cells to the 39°C (Fig. 2C) as well as the 42°C stimuli (Fig. 2D). Reapplication of the 39°C or 42°C heat stimulus in the absence of eugenol or carvacrol elicited responses that did not differ significantly from the initial response (Fig. 2C, D, left-hand pair of bars, respectively). The incidence of heat responsiveness did not change after reapplication of the 39°C or the 42°C stimuli. However, both eugenol and carvacrol increased the incidence of thermal responsiveness. For the 39°C stimulus, the number of thermosensitive cells increased following eugenol (from 32 to 39 of 88 cells tested) and carvacrol (from 23 to 28 of 80 cells tested). With the 42°C stimulus, the number of heat-sensitive cells increased

following eugenol (from 64 to 91 out of 150 cells tested) and carvacrol (from 26 to 30 out of 58 cells tested).

Vc Neurons

Unit Classification—We recorded from a total of 102 Vc units with heat-sensitive lingual receptive fields. Recording sites were located at an average depth of 383 ± 34 μm below the medullary surface, and are shown collectively (Figs. 5C, 6B). Of these, 77.5% also responded to pinch by blunt forceps. Of the pinch-sensitive units, 51% responded at lower frequency to graded innocuous mechanical von Frey filament stimuli (36.3-288.4 mN) and were classified as wide dynamic range (WDR), while the other 26.5% did not and were classified as nociceptive-specific (NS). Of the 22.5% pinch-insensitive units, 14.7% were mechanically insensitive and 7.8% responded to innocuous touch in a non-incrementing manner.

Fig. 3A shows a typical example of a Vc unit that responded to noxious heat, cooling, and eugenol. Following eugenol, heat-evoked responses were enhanced compared to the response prior to eugenol (left-most portion of PSTH in Fig. 3A). Application of vehicle did not affect the unit's firing rate, nor did it affect responses to noxious heat (data not shown). This unit additionally responded to subsequent application of menthol, CA and capsaicin (right-hand portion of PSTH starting at min 92).

Concentration-dependent responses to eugenol and carvacrol—Eugenol and carvacrol elicited concentration-related increases in Vc unit firing rates. Fig. 3B shows mean responses of Vc units to eugenol (•) which elicited a significant increase in firing at 1% compared to vehicle (▲) that did not increase further at 10%. Fig. 3B also shows mean responses to carvacrol (□) that increased in a concentration-related manner. Application of vehicle did not significantly affect firing rate compared to pre-stimulus baseline. Overall, of the noxious heat-sensitive Vc units tested, 55% (29/53) and 73% (38/52) responded to lingual application of eugenol and carvacrol, respectively, in the 1-10% concentration range.

We also investigated the temporal pattern of Vc unit firing elicited by constant-flow application of eugenol and carvacrol over a 10-min period. Fig. 4 shows averaged responses of Vc units to 10% eugenol (Fig. 4A) or 10% carvacrol (Fig. 4B), both of which elicited an initial significant increase in firing that eventually adapted to the pre-stimulus baseline level (one-way ANOVA, $p<0.05$). This desensitizing firing pattern is similar to that observed for eugenol- and carvacrol-evoked oral irritant sensations in humans (Klein et al., 2013).

Eugenol and carvacrol enhancement of noxious heat-evoked responses—Eugenol at a concentration of 0.1% increased firing (by >30%) in 27% (3/11) of Vc neurons. This concentration of eugenol had no significant effect on noxious heat-evoked responses (Fig. 5A). Eugenol at 1% excited 50% (10/20) of Vc units and significantly enhanced the mean heat-evoked response (Fig. 5B, D). The enhancement was only observed for the first heat-evoked response post-eugenol. Eugenol 10% activated 72.7% (8/11) of Vc neurons recorded but did not enhance responses to noxious heat stimulation (Fig. 5C, n = 11). Subsequent heat-evoked responses were not significantly different from the pre-eugenol response (Fig. 5D), indicating that the enhancing effect of eugonol was short-lived.

Similarly, carvacrol (0.1%) excited 50% (3/6) of Vc neurons and had no effect on heat-evoked responses (Fig. 6A). Carvacrol at 1% excited 86.7% (13/15) of heat-sensitive Vc units, but did not enhance unit responses to noxious heat (Fig. 6B). Ten percent carvacrol activated all but one Vc unit tested (92%, 11/12) and also enhanced the mean response to noxious heat (Fig. 6B, * $p<0.05$, one-way repeated measures ANOVA, Tukey post-hoc).

The vehicle did not directly excite Vc neurons, and had no effect on responses to noxious heat (Figs. 6D, 8D, open bars). The average threshold for heat activation before chemical application was 45.2 ± 0.3°C and was not influenced by application of vehicle, eugenol or carvacrol (Table 3).

Cold-evoked responses—Forty-three percent (45/105) of heat-sensitive Vc units also responded to noxious cooling. Eugenol activated 56.5% (13/23) and carvacrol activated 77.3% (17/22) of the cold-sensitive neurons. Application of neither vehicle, eugenol nor carvacrol affected the cold-evoked neuronal responses (data not shown).

Responses to additional stimuli—Of those units responsive to eugenol (Table 4) or carvacrol (Table 5), over 90% responded to menthol, AITC and /or capsaicin with 38% and 32% of eugenol- and carvacrol-sensitive units responding to all 4 of the chemical irritants tested (Tables 4, 5). Of all heat-responsive Vc units tested, 44.7% (47/105) responded to 1% menthol, 52.3% (55/105) to 10% CA and 80% (84/105) to 0.01% capsaicin.

Application of vehicle, eugenol or carvacrol did not significantly change unit responsiveness to mechanical stimulation (data not shown).

Discussion

The present results show that eugenol and carvacrol activated substantial percentages of TG and Vc neurons, including those that responded to innocuous warmth and/or noxious heat and irritant chemical stimuli. In addition, both eugenol and carvacrol briefly enhanced responses of TG and DRG neurons to innocuous warming as well as noxious heat, and Vc neuronal response to noxious heat, consistent with our human psychophysical study showing that these agonists briefly enhanced the magnitude of perceived warmth and heat pain (Klein et al., 2013). These results indicate that the chemical enhancement of warmth and heat pain perception, and heat-evoked responses of Vc neurons, is due to a peripheral enhancement of thermosensitivity of primary sensory neurons.

Eugenol and carvacrol have been previously reported to directly activate TRPV3 expressed in heterologous cell lines (Xu et al., 2002; Xu et al., 2006). Carvacrol also activated cells expressing mouse TRPA1 and human TRPA1 (de la Roche et al., 2013). There are few previous studies of the effects of eugenol and carvacrol on primary sensory neurons (Ohkubo and Kitamura, 1997). We presently observed that eugenol excited 23% and 8% of TG and DRG cells, respectively, and carvacrol excited 30% and 21%. Nearly all (>96%) eugenol- and carvacrol-sensitive TG and DRG cells responded to one or more additional TRP agonists, and over 70% responded to capsaicin, implying that eugenol and carvacrol excite nociceptive sensory neurons.

The selective TRPV3 agonist, FPP (Bang et al., 2010), activated much smaller percentages of TG and DRG cells. Given a role for TRPV3 in innocuous warmth transduction, we speculated that FPP-sensitive TG and DRG cells may represent TRPV3-expressing warm receptors. FPP was reported to excite keratinocytes prior to DRG cells in co-culture (Bang et al., 2010), suggesting that sensory effects of TRPV3 activation may be mediated largely via keratinocyte-to-TG/DRG communication, in addition to direct activation of a small subset of FPP-sensitive TG and DRG cells. FPP also reduced thermal paw withdrawal thresholds and elicited nocifensive behavioral responses when injected in the inflamed (but not control) hindpaw of mice (Bang et al., 2010), consistent with a role for TRPV3 in inflammatory pain.

That eugenol and carvacrol ostensibly excite thermal nociceptors and warm receptors is consistent with our report that these chemicals elicit oral irritation, characterized by subqualities of numbing, warming, tingle, burning, stinging and cooling (Klein et al., 2013). These subqualities are similar to those elicited by other chemesthetic TRP channel agonists (Cliff and Heymann, 1992; Green, 2002; Bennett and Hayes, 2012). This suggests that chemesthetic sensations, formerly referred to as the “common chemical sense”, are generated mainly by chemical excitation of polymodal nociceptors. Subtle differences in the sensory subqualities elicited by different chemesthetic agents may be attributed to the simultaneous excitation of other chemosensitive primary afferent fibers including warm and/or cold receptors.

A large proportion of TG and DRG cells (Table 1 and 2) and a high percentage of noxious heat-sensitive Vc neurons was excited by eugenol, carvacrol, and other TRP agonists including AITC, capsaicin and menthol (Tables 4 and 5), consistent with previous studies (Carstens et al., 1998; Zanotto et al., 2007; Klein et al., 2011c, d). The broadly-tuned chemesthetic sensitivity of Vc neurons presumably reflects input from primary afferent trigeminal nociceptors that exhibit similar broad tuning. Vc unit responses to TRPA1 and TRPM8 agonists exhibited desensitization over time (Zanotto et al., 2007; Simons et al., 2004) similar to human psychophysical reports of a temporally desensitizing pattern of oral irritation elicited by repeated lingual application of menthol, AITC, cinnamaldehyde, eugenol and carvacrol (Dessier et al., 2001; Simons et al., 2003; Klein et al., 2013). Capsaicin and AITC also enhanced heat pain, and menthol enhanced cold pain, even after chemical desensitization had been established (Merrill et al., 2007; Zanotto et al., 2007; Albin et al., 2008; Klein et al., 2013). That the same TRP agonist sensitized thermal gating while desensitizing chemical gating of the channel implies separate molecular mechanisms for these two contrasting effects (Carstens & Mitsuyo, 2005).

TRP channels can be activated by many stimuli, including chemicals, voltage, temperature and in some cases mechanical force. Mutagenesis experiments conclude that the mechanisms of temperature and chemical activation of TRPV3 (Grandl et al., 2008) and TRPV1 (Yang et al., 2010; Grandl et al., 2010) are different. Like many TRP channels, TRPV1 rapidly desensitizes upon repeated chemical application (Bhave et al., 2002). However, TRPV3 sensitizes to repeated chemical stimulation (Xu et al., 2002) in a calcium calmodulin- and ATP-dependent manner (Xiao et al., 2008; Phelps et al., 2010). Repetitive mechanical stimulation of heat-and-mechanosensitive polymodal nociceptors results in rapid desensitization, even though responses of the some of these same C-fibers to noxious heat

are subsequently enhanced (Peng et al. 2003). Repeated chemical application could lead to desensitization of TRP channels in a calcium-dependent manner, while calcium activation of protein kinases (e.g. PKA) or other cellular mediators could simultaneously sensitize a different part of the channel involved in thermal transduction. Our data showing desensitizing responses to chemical application and sensitized thermal responses are consistent with this possibility.

We propose that the effects of eugenol and carvacrol to enhance perceived warmth and heat pain (Klein et al., 2013), as well as heat-evoked responses of Vc neurons (Figs. 5, 6), are due to their peripheral enhancement of thermally-evoked responses of TG and DRG cells (Fig. 2). Presumably, the ability of eugenol and carvacrol to enhance thermally-evoked responses is via their action at TRPV3, TRPV1 and/or TRPA1 expressed in lingual nociceptors and warm receptors. Eugenol, carvacrol and thymol enhanced the responses of TRPV3-expressing epithelial cells to warming (Xu et al., 2006). Eugenol was also reported to activate TRPV1 (Bandell et al., 2004) although at much higher concentrations compared to capsaicin (Yang et al., 2003). Both eugenol and carvacrol also activate TRPA1 (Xu et al., 2006; Bandell et al., 2004; de la Roche et al., 2013); however, the concentration required for carvacrol was much higher compared to AITC (Bessac et al., 2008) and the TRPA1 antagonist HC-030031 does not antagonize carvacrol-evoked responses of human endothelial cells (Earley et al., 2010).

Eugenol and carvacrol sensitized both primary afferent (TG, DRG cells) and central (Vc) neuronal responses to heat. Previous studies have also suggested that TRPV3 indirectly mediates the sensation of heat (Moqrich et al., 2005) via activation of TRPV3-expressing keratinocytes (Chung et al., 2004) that release second messengers such as ATP or prostaglandin E2 to excite sensory nerve fibers (Tominaga et al., 2001; Mandadi et al., 2009; Huang et al., 2011). TRPV3 is highly expressed in the lingual epithelium (Xu et al., 2002; Xu et al, 2006). However, we observed that eugenol and carvacrol enhanced responses of TG and DRG cells to both innocuous warmth (39°C) and noxious heat (42°C), implying sensitization of thermal responses in the absence of mediators from keratinocytes or other epithelial cells. As previously noted, eugenol and carvacrol could enhance thermally-evoked responses via their action at TRPA1 or TRPV1. Potentially, TRPV1/TRPV3 heteromers expressed in sensory nerve endings could also explain the heat enhancement observed presently (Smith et al., 2002; Cheng et al., 2011). It could be speculated that the effect of eugenol and carvacrol to enhance heat-evoked responses of sensory neurons is due to an allosteric influence on specific amino acid residues localized to the extracellular surface of TRPV3 and TRPV1 that are essential for thermotransduction (Kim et al., 2013). Finally, it is possible that eugenol and carvacrol enhance thermal gating of other heat-sensitive channels such as TRPV1 (Caterina et al., 1997), TRPM3 (Vriens et al., 2011), Anoctamin 1 (Anoc1; Tian et al., 2012; Cho et al., 2012), TREK-1 (KNCK2)(Maingret et al. 2000) or TRPV4 (Guler et al., 2002).

TRPA1 agonists sensitized spinal cord neuronal responses to noxious heat (Simons et al., 2004; Sawyer et al., 2009), and briefly enhanced human perception of lingual heat and cold pain (Albin et al., 2008). Eugenol and carvacrol have both been reported to activate and desensitize TRPA1 (see Introduction). TRPA1 has also been implicated in cold transduction

(Bandell et al., 2004; Karashima et al., 2009), although this has been disputed (for review, see Caspani & Heppenstall, 2009). Human subjects receiving cutaneous application of TRPA1 agonists reported a burning quality but no cold sensation (Namer et al., 2005). The lack of effect of eugenol and carvacrol on cold perception and cold-evoked responses of Vc neurons suggests that these chemicals do not affect lingual cold transduction, regardless of whether TRPA1 is involved or not.

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Highlights

- Eugenol and carvacrol activated 7-30% of trigeminal and dorsal root ganglion cells
- Eugenol and carvacrol enhanced sensory neuronal responses to warmth and noxious heat
- Eugenol and carvacrol excited heat-sensitive trigeminal caudalis (Vc) neurons
- Eugenol and carvacrol enhanced Vc neuronal responses to noxious heat
- Eugenol, carvacrol act peripherally to enhance lingual warmth and heat sensitivity

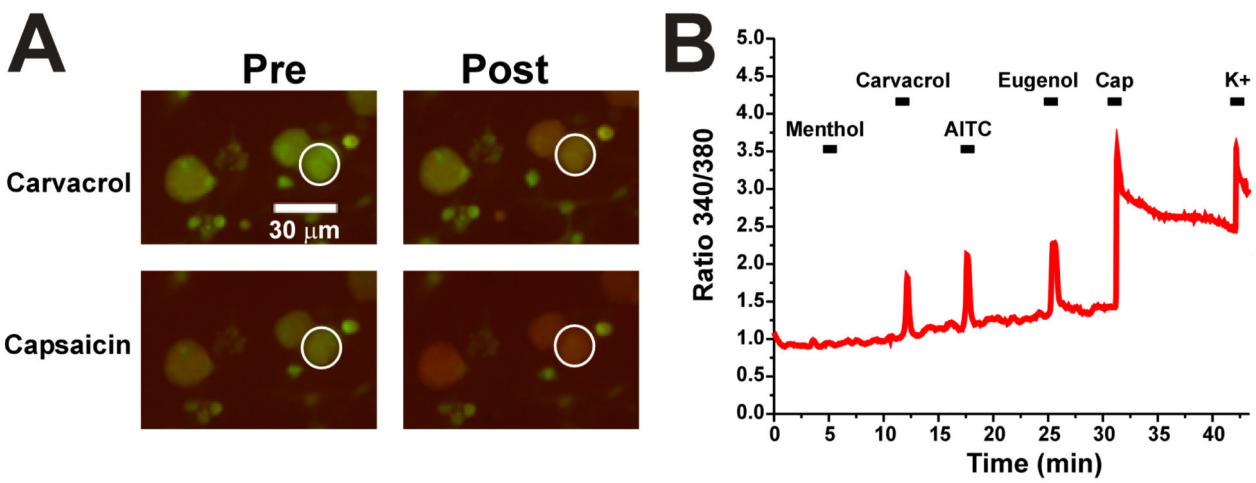

Fig. 1.

TG and DRG cell responses to eugenol, carvacrol and other agonists. A. Example of TG cell responses to carvacrol and capsaicin. B. Graph plots the ratio of 340/380 nm vs time for the encircled TG cell in A. TG cell responded to carvacrol (100 μM), AITC (100 μM), eugenol (200 μM), capsaicin (Cap; 1 μM), and a high potassium solution (K+) but not menthol (250 μM). Chemical applications indicated by black bars above the trace.

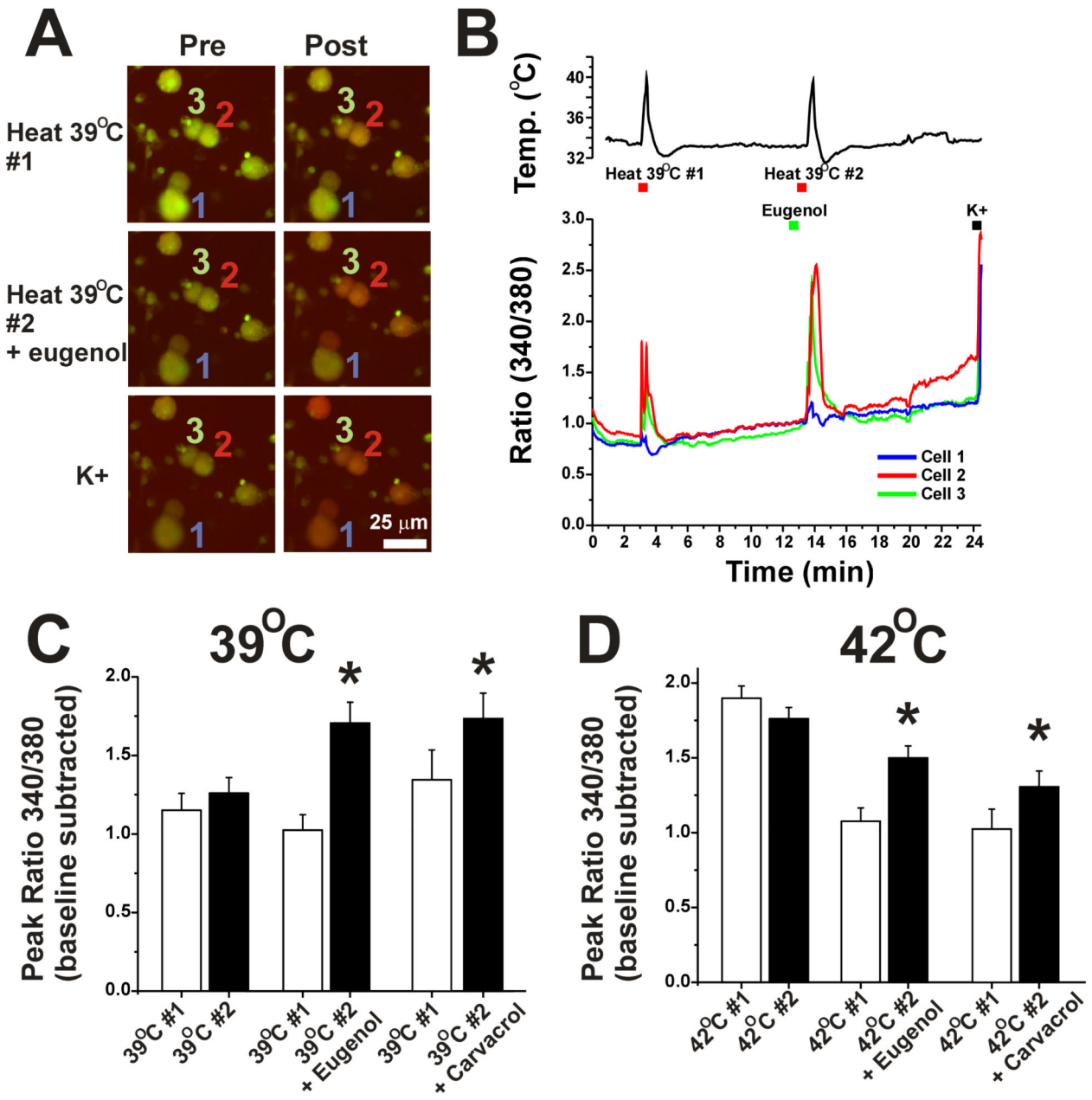

Fig. 2.

Eugenol enhances TG cell responses to noxious heat stimulation. A. Photomicrographs of TG cells before (Pre) and after (Post) 39°C heat alone (upper row) or 39°C immediately preceded by bath application of 200 μM eugenol (middle row). Bottom row shows same TG cells before and after application of high K+ solution. B. Upper trace: bath temperature recorded near the TG cells. Lower trace: Responses of cells 2 and 3 to successive heat stimuli and K+; cell 1 responded to K+ but not heat. Note heightened response to the second heat stimulus preceded by eugenol. C. Enhancement of innocuous warmth (39°C) – evoked responses. Graph plots mean baseline-corrected responses of TG and DRG cells to two successive 39°C warm stimuli delivered at a 10 min interstimulus interval. Open bars: first

response. Filled bars: second response, without (left; n=49 [39 DRG, 10 TG cells]) or when preceded 30 sec earlier by 200 μM eugenol (middle; n=39 [8 DRG, 31 TG cells] or 100 μM carvacrol (n = 28 [12 DRG, 16 TG cells]). *: significantly different compared to preceding warm stimulus ($p < 0.05$, paired t-test). D. 42°C heat stimulus. Format as in C for successive 42°C heat stimuli without (n=82 [62 DRG, 20 TG cells]) or when preceded 30 sec earlier by 200 μM eugenol (n = 91 [53 DRG, 38 TG cells]) or 100 μM carvacrol (n = 30 TG cells). *: significantly different compared to preceding heat stimulus ($p < 0.05$, paired t-test).

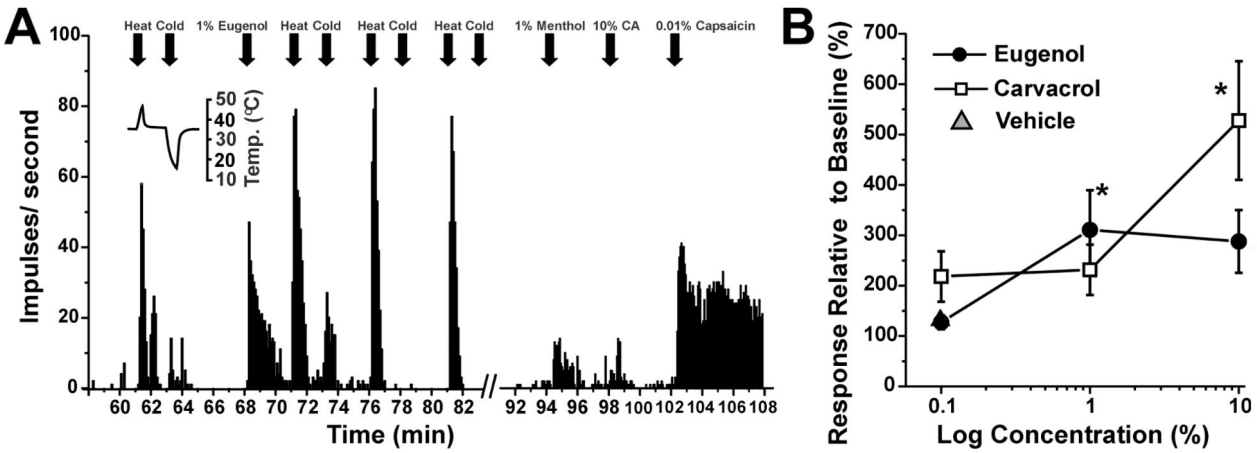

Fig. 3.

Dose-dependent activation of Vc neurons by eugenol and carvacrol. A: Individual example. Shown are peristimulus time histograms (PSTH, bins: 1 sec) of a Vc unit's responses to noxious heat and cold stimuli, 1% eugenol application (100 μl), followed by noxious heat and cold stimulation three times post-eugenol application. This unit also responded to additional chemical applications (100 μl) of 1% menthol, 10% CA and 0.01% capsaicin. Upper left-hand inset shows temperature trace recorded at the thermode-tongue interface. B. Dose-dependent activation of Vc neurons by eugenol and carvacrol. Graph plots mean responses of heat-sensitive Vc neurons to 0.1, 1 and 10% eugenol and carvacrol applied lingually (100 μl). Responses normalized to average baseline firing rate (100%) immediately preceding chemical application. One percent eugenol and 10% carvacrol were significantly greater than vehicle treatment. (*; $p<0.05$, one-way ANOVA, Tukey post-hoc test). Vehicle, n = 74. Eugenol: 0.1%, n = 11; 1%, n = 20; 10%, n = 11. Carvacrol: 0.1%, n = 6; 1%, n = 15; 10%, n = 12.

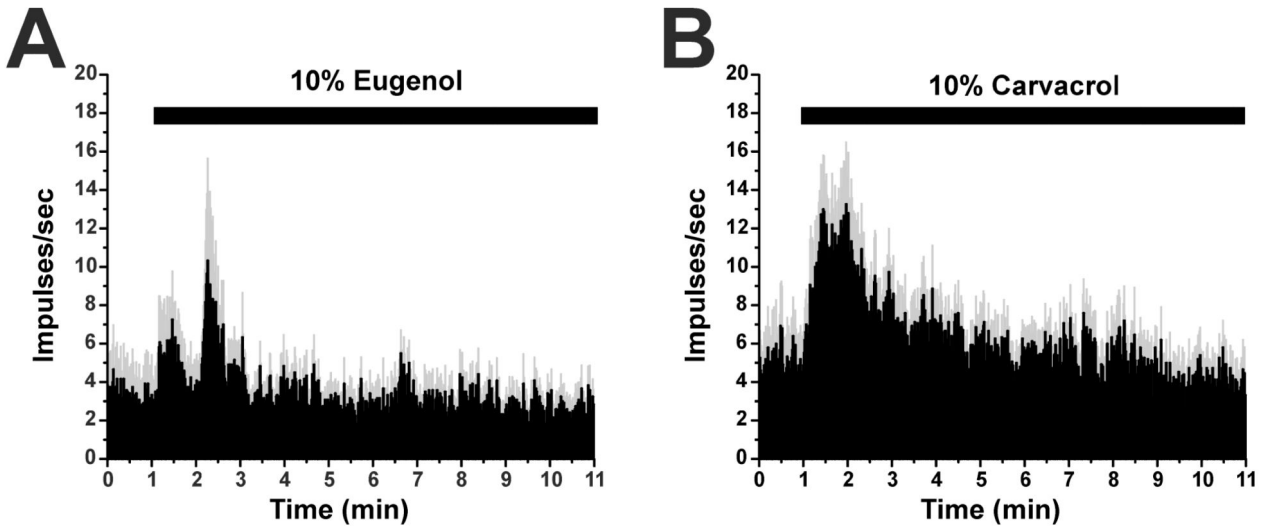

Fig. 4.

Temporally desensitizing responses of Vc neurons to eugenol and carvacrol. A: Eugenol. Averaged PSTH of response to 10% eugenol superfused over the anterior dorsal surface of the tongue at a constant rate (0.5 ml/min) for 10 min. There was a significant change in firing rate over time, characterized by an initial increase that adapted to the pre-stimulus baseline level ($p<0.05$, one-way ANOVA, n=11). Gray error bars: SEM. B. Carvacrol (format as in A). Carvacrol (10%) similarly elicited a significant initial increase in firing that adapted to the pre-stimulus baseline level during the 10 min application period ($p<0.05$, one-way ANOVA, n=15).

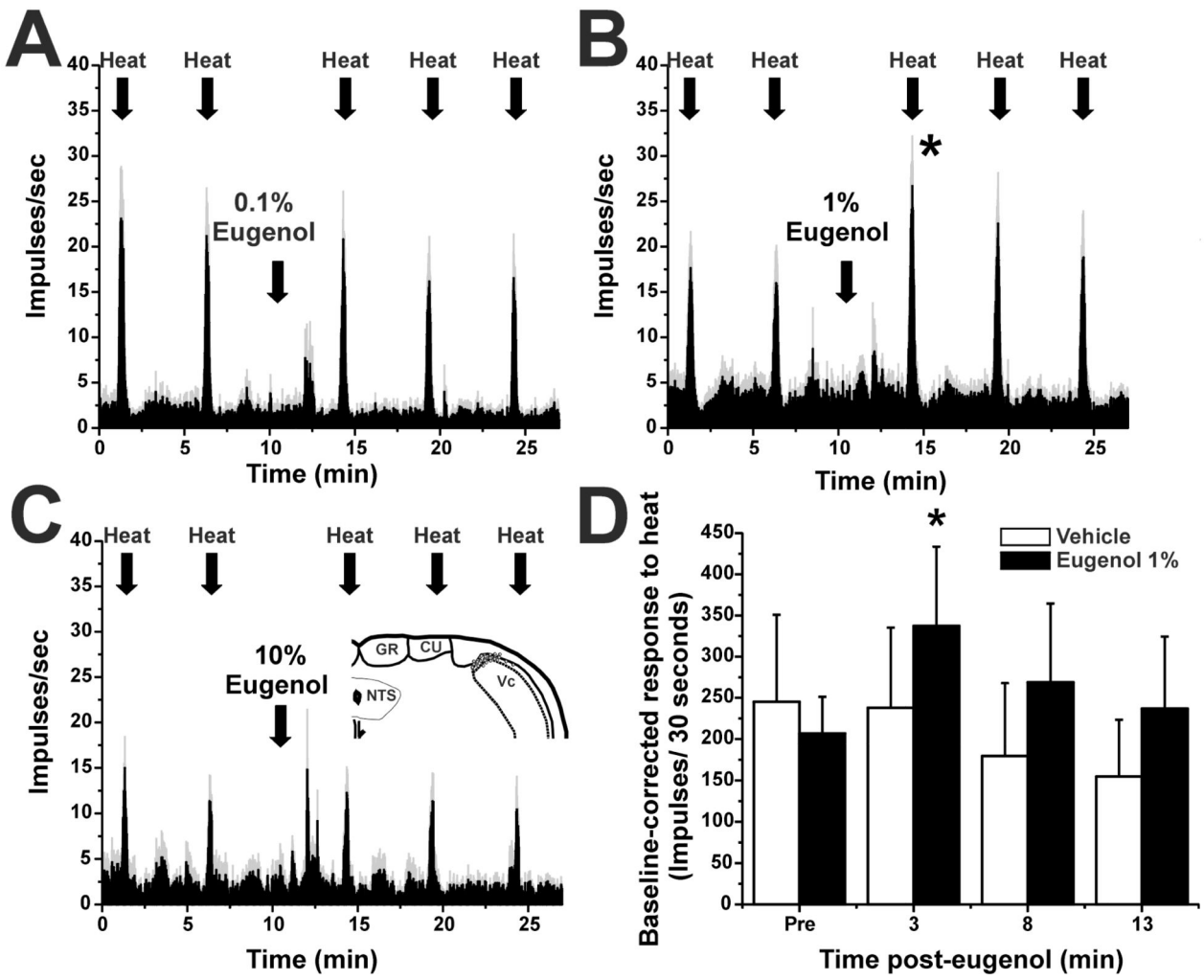

Fig. 5.

Eugenol enhancement of Vc neuronal responses to noxious heat. A: Eugenol 0.1% had no effect on heat-evoked responses. Shown is the averaged PSTH of 11 Vc units to heat stimuli (arrows), before and following topical application of 0.1% eugenol to the tongue. Gray error bars: SEM. B. 1% eugenol significantly enhanced heat-evoked responses 3 min post-application compared to averaged values pre-application (*, $p<0.05$, repeated-measures ANOVA, Tukey post hoc, n=20). See panel D. C. 10% eugenol had no effect on heat-evoked responses (n=11). Inset shows histologically recovered Vc units. Abbreviations: CU, cuneate n., GR, n. gracilis; NTS, n. of solitary tract; Vc, trigeminal subnucleus caudalis. D. Summary of enhancement of heat-evoked responses following 1% eugenol. Bar graph plots mean noxious heat-evoked responses of Vc units. Open bars: response before (pre) and following application of vehicle. Filled bars: response before (pre) and following application of 1% eugenol. * $p<0.05$, ANOVA; see panel B).

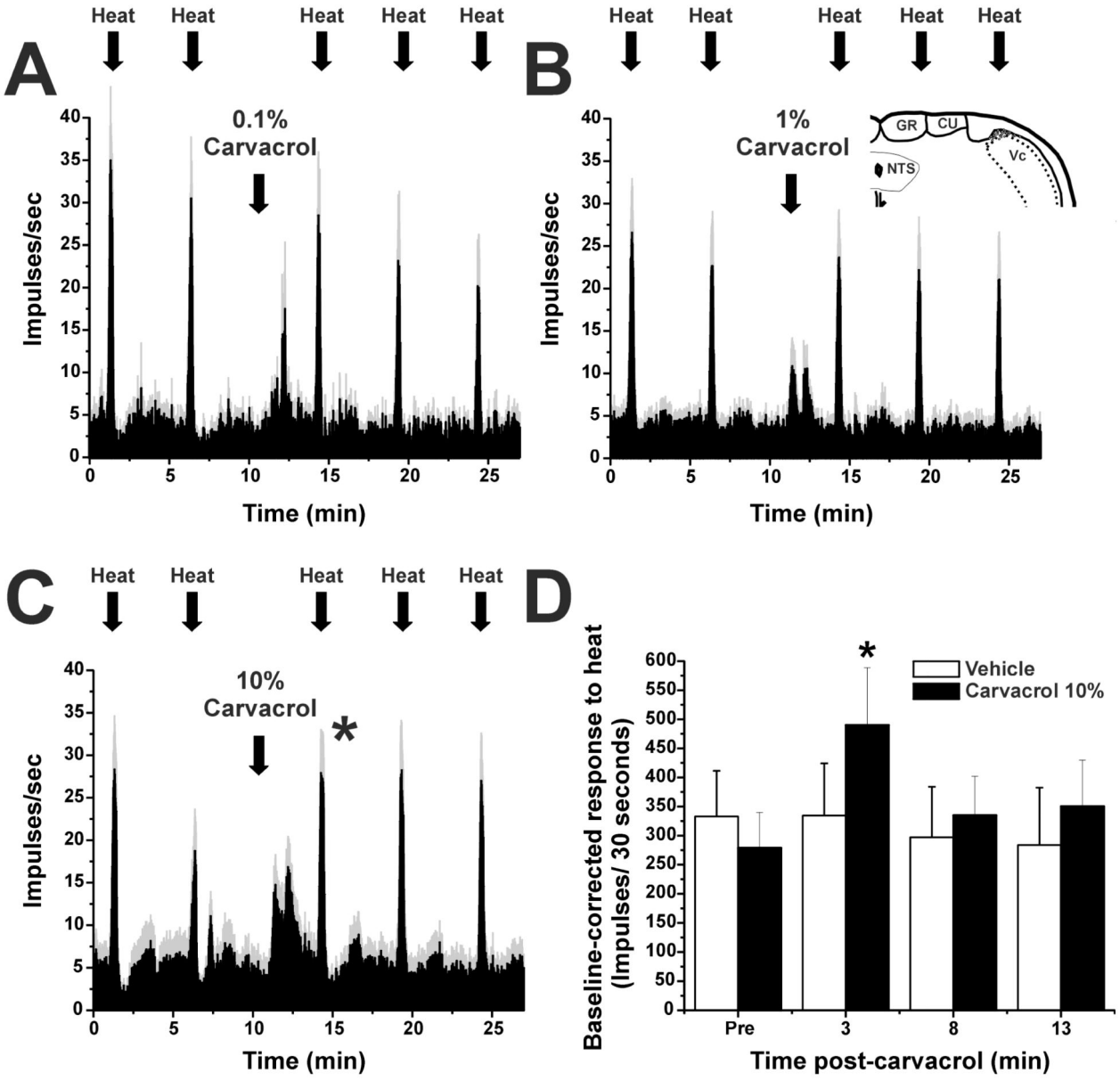

Fig. 6.

Carvacrol enhancement of Vc neurons to noxious heat (format as in Fig. 5). A. carvacrol 0.1% did not affect heat-evoked responses (n=15). B: Carvacrol 1% did not affect heat-evoked responses (n=15). C: Carvacrol 10% significantly enhanced heat-evoked responses at 3 min post-application compared to averaged values pre-application (*, $p<0.05$, repeated-measures ANOVA, Tukey post hoc, n=12). D: Summary of data with 10% carvacrol (format as in Fig. 5D).

Table 1

Incidence of responses of eugenol-sensitive TG and DRG cells to other irritants. +, responded; 0, did not respond.

	Menthol	AITC	Capsaicin	n	Percent (%)
TG cells					
	0	0	0	1	2.3
	+	0	0	0	0
	+	+	0	3	7
	0	+	+	15	34.9
	+	0	+	4	9.3
	+	+	+	9	20.9
	0	+	0	6	13.6
	0	0	+	5	11.6
DRG cells					
	0	0	0	2	11.1
	+	0	0	0	0
	+	+	0	1	5.6
	0	+	+	8	44.4
	+	0	+	3	16. 7
	+	+	+	2	11.1
	0	+	0	0	0
	0	0	+	2	11.1

Table 2

Incidence of responses of carvacrol-sensitive TG and DRG cells to other irritants. +, responded; 0, did not respond.

	Menthol	AITC	Capsaicin	n	Percent (%)
TG cells					
	0	0	0	2	3.8
	+	0	0	4	7.5
	+	+	0	6	11.3
	0	+	+	14	26.4
	+	0	+	4	7.5
	+	+	+	11	20. 8
	0	+	0	7	13.2
	0	0	+	5	9.4
DRG cells					
	0	0	0	1	1.8
	+	0	0	0	0
	+	+	0	2	3.6
	0	+	+	8	14.5
	+	0	+	28	50.9
	+	+	+	12	21.8
	0	+	0	0	0
	0	0	+	4	7.3
	0	0	0	1	1.8

Table 3

Vc unit heat thresholds. Thresholds are in °C ± SEM. Vehicle: n = 75. 0.1% eugenol: n = 11. 1% eugenol: n = 20. 10% eugenol: n = 11. 0.1% carvacrol: n = 6. 1% carvacrol: n = 15. 10% carvacrol: n = 12.

Chemical	Averaged Pre-Chemical Threshold (°C)	Post-Chemical Threshold #1	Post-Chemical Threshold #2	Post-Chemical Threshold #3
Vehicle	44.3 ±0.4	44.0 ± 0.4	45.2 0.4	45.5 ± 0.4
0.1% Eugenol	43.5 ± 0.9	42.8 ± 1.1	44.4 ± 1.2	45.4 ± 1.1
1% Eugenol	44.9 ± 1.1	45.0 ± 1.0	45.9 ± 1.0	48.2 ± 1.2
10% Eugenol	46.8 ± 1.0	46.6 ± 1.2	44.9 ± 1.3	44.8 ± 1.1
0.1% Carvacrol	43.7 ± 1.0	44.7 ± 1.5	45.7 ± 1.8	45.0 ± 2.7
1% Carvacrol	45.9 ± 1.2	45.1 ± 1.0	45.3 ± 1.1	47.3 ± 1.3
10% Carvacrol	47.1 ± 1.0	46.7 ± 1.5	47.6 ± 1.1	47.9 ± 1.0

Table 4

Responses of eugenol-responsive Vc units to other chemical irritants. +, responded; 0, did not respond.

Menthol	Cinnamaldehyde	Capsaicin	n	Percent (%)
0	0	0	3	10.3
+	0	0	1	3.4
+	+	0	1	3.4
+	+	+	11	37.9
0	+	+	6	20.7
0	0	+	1	3.4
0	+	0	0	0
+	0	+	6	20.7

Table 5

Responses of carvacrol-responsive Vc units to other chemical irritants. +, responded; 0, did not respond.

Menthol	Cinnamaldehyde	Capsaicin	n	Percent (%)
0	0	0	3	7.9
+	0	0	0	0
+	+	0	1	2.6
+	+	+	12	31.6
0	+	+	7	18.4
0	0	+	11	28.9
0	+	0	2	5.3
+	0	+	2	5.3