

# TRPV1 Receptors and Nasal Trigeminal Chemesthesis

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## Abstract

The trigeminal nerve responds to a wide variety of irritants. Trigeminal nerve fibers express several receptors that respond to chemicals, including TRPV1 (vanilloid) receptors, acid-sensing ion channels, P2X (purinergic) receptors, and nicotinic acetylcholine receptors. In order to assess whether TRPV1 plays a role in responses to a broad array of substances, TRPV1 (along with green fluorescent protein) was expressed in human embryonic kidney cells (HEK) 293t cells which were then stimulated with diverse trigeminal irritants. Calcium imaging was used to measure responses to capsaicin, amyl acetate, cyclohexanone, acetic acid, toluene, benzaldehyde, (–)-nicotine, (R)-(+)-limonene, (R)-(–)-carvone, and (S)-(+)-carvone. Three irritants (acetic acid and the 2 carvones) stimulated nontransfected controls. Two irritants (capsaicin and cyclohexanone) stimulated only transfected cells. The response could be eliminated with capsazepine, a TRPV1 blocker. The 5 remaining irritants were nonstimulatory in both nontransfected and transfected cells. Because all the compounds tested on HEK cells elicited neural responses from the ethmoid branch of the trigeminal nerve in rats, the 5 nonstimulatory compounds must do so by a non-TRPV1 receptor. These results suggest that TRPV1 serves as a receptor for both cyclohexanone and capsaicin in trigeminal nerve endings.

**Key words:** calcium imaging, capsaicin, ethmoid nerve, HEK 293t transfection, irritant

## Introduction

Chemesthetic nerve fibers, originating from the trigeminal and dorsal root ganglia, respond to noxious chemicals, as well as noxious mechanical stimuli and extreme temperatures (Bryant and Silver 2000). Chemesthesis has been most extensively studied in the trigeminal nerve, which innervates the mucosa and skin of the face, nasal cavity, oral cavity, and eyes. In the nasal cavity, trigeminal free nerve endings responsive to noxious stimuli terminate within a few micrometers of the tissue surface (Finger et al. 1990).

Trigeminal nerve fibers in the nasal cavity respond to a wide variety of chemical stimuli (Bryant and Silver 2000), but the receptors and signaling effectors are unclear. Chemesthetic trigeminal nerve fibers contain capsaicin-sensitive, polymodal nociceptors (Silver et al. 1991). Trigeminal chemesthetic nerve endings reportedly contain several different types of ligand-gated receptors, including the vanilloid receptor 1 (TRPV1) (Dinh et al. 2003), the acid-sensing ion channel (ASIC) (Ichikawa and Sugimoto 2002), the purinergic receptor (P2X) (Spehr et al. 2004), and the nicotinic acetylcholine receptor (nAChR) (Alimohammadi and Silver 2000). Trigeminal nerve responses to nicotine, for example, are eliminated in the presence of nAChR blockers (Alimohammadi and Silver 2000).

TRPV1 is a polymodal nociceptor that is sensitive to capsaicin, acids, and heat. It is an ionotropic receptor containing 6 transmembrane domains (Welch et al. 2000) and forms a nonselective cation channel with a preference toward divalent cations, especially calcium (Szallasi and Blumberg 1999).

To determine if TRPV1 may serve as a receptor for other trigeminal irritants, we examined the response of HEK cells transfected with TRPV1 to 10 different irritants that are also shown to activate nasal trigeminal fibers. The effect of the stimuli on TRPV1 transfected HEK cells was monitored using calcium imaging. Our data suggest that TRPV1 serves as a receptor for cyclohexanone in addition to capsaicin.

## Materials and methods

### Trigeminal nerve recording

The procedure for recording whole nerve multiunit responses from the ethmoid branch of the trigeminal nerve was similar to that described previously (Silver et al. 1990). Briefly, adult male Sprague–Dawley rats (250–400 g) (Harlan, Indianapolis, IN) were anesthetized with an intraperitoneal injection

of urethane (ethyl carbamate: 1.0 g/kg). The trachea was exposed and 2 cannulae were inserted, one towards the lungs to allow the rat to breath room air and a second towards the nasopharynx. Rat Ringer's (5.4 mM KCl, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 135 mM NaCl, 1.8 mM CaCl<sub>2</sub>) was pumped through this cannula so that it dripped out of the nose.

After the rat was placed in a head holder, the ethmoid nerve was exposed in the right orbit and placed on 2 platinum-iridium wire hook electrodes. The cavity was then filled with halocarbon oil (Sigma, St Louis, MO) to prevent the nerve from drying out and to provide electrical insulation. Neural activity was amplified (Grass P-511) and monitored using an audio monitor (Grass AM-8). Both the raw neural output and integrated multiunit activity (short time-averaging circuit with a time constant of 0.5 s) were recorded using the AcqKnowledge (v3.7.1) data acquisition program (Biopac Systems Inc., Goleta, CA).

Compounds tested included amyl acetate (AA), cyclohexanone (CY), acetic acid (AC), toluene (TO), benzaldehyde (BE), (–)-nicotine (NI), (*R*)-(+)-limonene (LI), (*R*)-(–)-carvone (RCR), (*S*)-(+)-carvone (SCR), and capsaicin (CA) (all chemicals from Sigma Chemical Co., St Louis, MO). A stock solution for all these compounds was made using rat Ringer's except CA, SCR, RCR, and LI which were made in dimethyl sulfoxide (DMSO). Final dilutions for each compound were made with Ringer's. The final concentration of DMSO was less than 1.0% (v/v). This concentration of DMSO did not elicit responses from the trigeminal nerve (data not shown). The pH of all stimuli tested was approximately 7.0 with the exception of AC which was 4.4. Stimuli were tested at 100 mM except for NI (10 mM) and CA (10 μM).

Rat Ringer's was pumped continuously (Masterflex C/L) through the rat's nasal cavity via the nasopharyngeal tube at a rate of 10 ml/min. The Ringer's solution was allowed to drip freely from the nose. Compounds were delivered in 0.5-cc aliquots using a 3-cc syringe and 23-g needle. The needle was inserted into a rubber septum in the tube close to the nasopharyngeal cannula, and the solutions were smoothly injected into the constant Ringer's flow. All responses were recorded with the AcqKnowledge program.

#### HEK cell preparation and transfection

HEK 293t cells were plated onto lysine-coated culture slides (Becton Dickinson, Portsmouth, NH) and cultured for 24 h. Cells were then washed with sterile phosphate-buffered saline (PBS) and transiently transfected with constructs encoding green fluorescent protein (GFP) (kindly provided by Terrance Egan, St Louis University School of Medicine) and TRPV1 (rodent cDNA kindly provided by David Julius, University of California) using Effectene Transfection Reagent according to the manufacturer's protocol (Qiagen, Valencia, CA). Following transfection, cells were cultured at 37 °C for an additional 24–48 h before they were used for experiments.

#### Calcium imaging

Intracellular Ca<sup>2+</sup> measurements were obtained using ~2 μM fura-2 AM (Molecular Probes, Invitrogen, Carlsbad, CA). Images were acquired with a CCD Sencicam QE camera (Cooke, Inc., Romulus, MI) through a 40× oil immersion objective lens of an inverted Nikon Diaphot TMD microscope. Excitation wavelengths of 350 and 380 nm were used with an emission wavelength ~510 nm. Calcium levels were reported as F350/F380 versus time. Images were captured every 1–5 s using Imaging Workbench 5.2 (Indec Biosystems, Inc., Santa Clara, CA). All solutions were bath applied using a gravity flow perfusion system from Automate Scientific Inc. (San Francisco, CA) and laminar flow perfusion chambers (RC-25F) from Warner Scientific Inc. (Hamden, CT).

Stock solutions for all stimuli were made in Tyrodes (in mM: 140 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 10 HEPES, 10 glucose, and 10 sodium pyruvate, pH 7.4 with NaOH) except CA, SCR, RCR, and LI which were made in DMSO. Final dilutions for each compound were made with Tyrodes. The final concentration of DMSO was less than 1.0% (v/v). This concentration of DMSO did not elicit responses from native or transfected HEK cells (data not shown). The pH of all stimuli tested was approximately 7.0 with the exception of AC which was 4.4. Stimuli were tested at 10 mM, except for NI (1 mM) and CA (0.5 μM). A capsazepine (CZP) stock solution (used to block TRPV1) was also made up in DMSO and diluted to 10 μM with Tyrodes. CZP bathed the cells for 100–200 s. Stimuli were delivered during and after CZP treatment. Only cells that responded to CA or CY were counted in the CZP experiment.

#### Immunocytochemistry

In order to estimate the effectiveness of transfection, we examined transfected cells for the presence of GFP and TRPV1. Following transfection, cells were cultured at 37 °C for an additional 45 h and then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. Cells were washed with PBS and incubated in blocking solution (PBS, Triton X-100, normal goat serum, and bovine serum albumin) for 1–2 h. Next, guinea pig anti-capsaicin receptor antibody (1:1000 in blocking solution; Chemicon, Temecula, CA) was applied to the slides and left in contact with the cells overnight. Following washes with PBS, cells were incubated with rhodamine red-X donkey anti-guinea pig secondary antibody (1:100, Jackson) for 2–3 h. After washing again with PBS, the cells were coverslipped with fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, CA) and stored at 4 °C. To insure specific labeling, 2 controls were done. First, immunocytochemistry was done on nontransfected cells, and there was no specific labeling or GFP fluorescence. Second, during the immunocytochemical processing, some transfected cells were not exposed to primary antibody but were otherwise treated identically to the rest. Again, there was no immunoreactivity, although GFP was expressed in these cells as expected.

## Results

### Trigeminal nerve responses

Neural recordings were obtained from 3 rats. In each of the rats, the ethmoid branch of the trigeminal nerve responded to all 10 of the irritants presented in Ringer's solution. Capsaicin elicited a response at 10  $\mu$ M, NI at 10 mM, and the rest at 100 mM. Responses are shown in Figure 1. With the exception of CA and NI, the mechanism of stimulation for the 8 other compounds (AA, BE, LI, NI, TO, AC, RCR, and SCR) is unknown.

### HEK cell transfection

To specifically test the effects of the above stimuli on the TRPV1 receptor, we transiently transfected HEK 293t cells with TRPV1 constructs. In addition to TRPV1, cells were cotransfected with a construct encoding GFP. The purpose of the GFP construct was to allow easy identification of transfected cells. To insure that GFP was a reliable marker for TRPV1 expression, some cotransfected cells were processed for TRPV1 immunocytochemistry. Approximately 16% of the HEK cells expressed GFP, TRPV1, or both proteins. In general, cells transfected with GFP also expressed the TRPV1 protein as indicated by labeling with the TRPV1 antibody (TRPV1-ir). Twenty four percent of the cells were double labeled with light TRPV1-ir and GFP, and 59% were double labeled with robust TRPV1-ir and GFP. In addition,

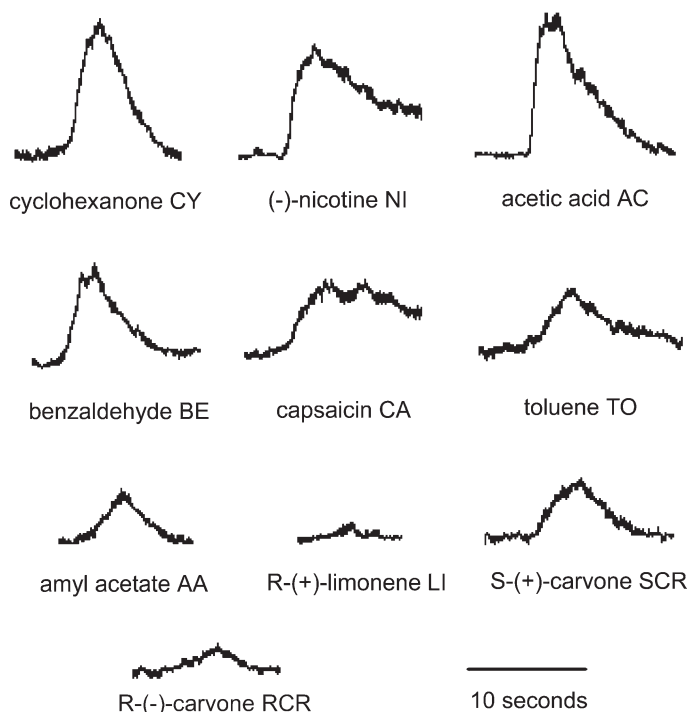
some cells expressed GFP but lacked TRPV1 expression (14%). Three percent of the labeled cells exhibited only TRPV1-ir. A typical group of cells is shown in Figure 2.

### Calcium imaging of HEK 293 cells

Stimulation of TRPV1 by CA increases  $\text{Ca}^{2+}$  influx in heterologous systems (Caterina et al. 1997; Yang et al. 2003). Therefore, we tested the ability of other irritants to elicit a  $\text{Ca}^{2+}$  response in both TRPV1-expressing and nonexpressing HEK 293 cells. Nontransfected cells showed rapid and reversible calcium influx when challenged with AC, SCR, and CRC (Figure 3A). None of the other 7 compounds caused a  $\text{Ca}^{2+}$  response in the nontransfected cells. Application of AA, BE, LI, NI, or TO to TRPV1 transfected cells did not evoke a  $\text{Ca}^{2+}$  response, whereas application of AC, RCR, or SCR resulted in  $\text{Ca}^{2+}$  influx in (Figures 3A and 4A). These results were expected because AC, RCR, and SCR also stimulated  $\text{Ca}^{2+}$  influx in nontransfected cells. Importantly, CA and CY resulted in  $\text{Ca}^{2+}$  influx only in transfected cells, suggesting that these compounds are activating TRPV1 receptors (Figure 3B and C).

We examined responses to CA in 37 GFP-expressing cells and 32 non-GFP-expressing cells. CA elicited calcium increases in 31 GFP-expressing cells (83.8%) but only 6 non-GFP-expressing cells (18.8%). CY produced increases in calcium in 25 of 29 GFP-expressing cells (86.2%) and 6 of 24 non-GFP-expressing cells (25%).

CZP, a TRPV1 blocker, was applied to transfected cells to determine if responses to CA and CY were mediated by TRPV1 (Figure 4). CZP blocked responses to CY in all the 14 transfected cells tested. The response to CA was blocked by CZP in the 15 cells tested. CZP had little, if any, affect on RCR (Figure 4A) SCR, and AC (data not shown), which activates nontransfected cells as well.



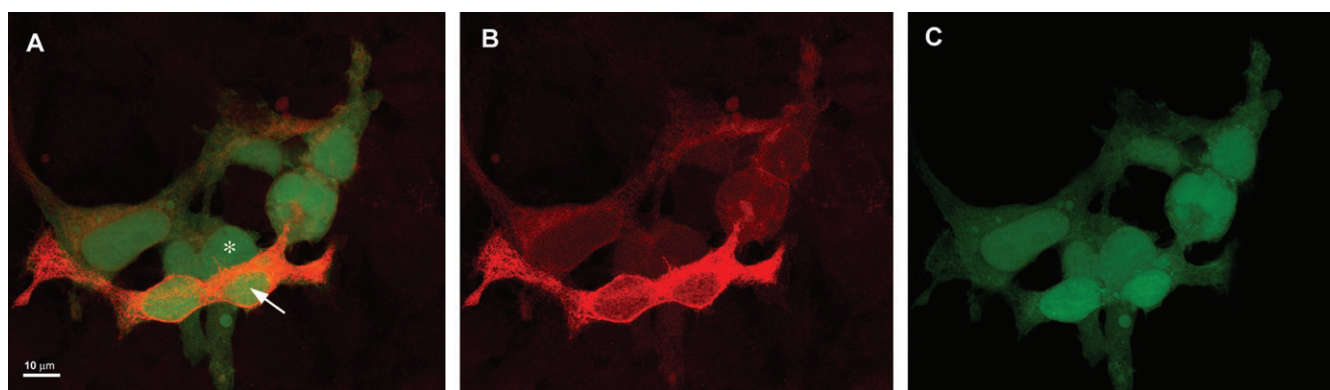
**Figure 1** Multiunit responses from the rat ethmoid nerve. All stimuli, delivered in Ringer's solution to the nasal cavity, were tested at 100 mM except CA (10  $\mu$ M) and NI (10 mM).

## Discussion

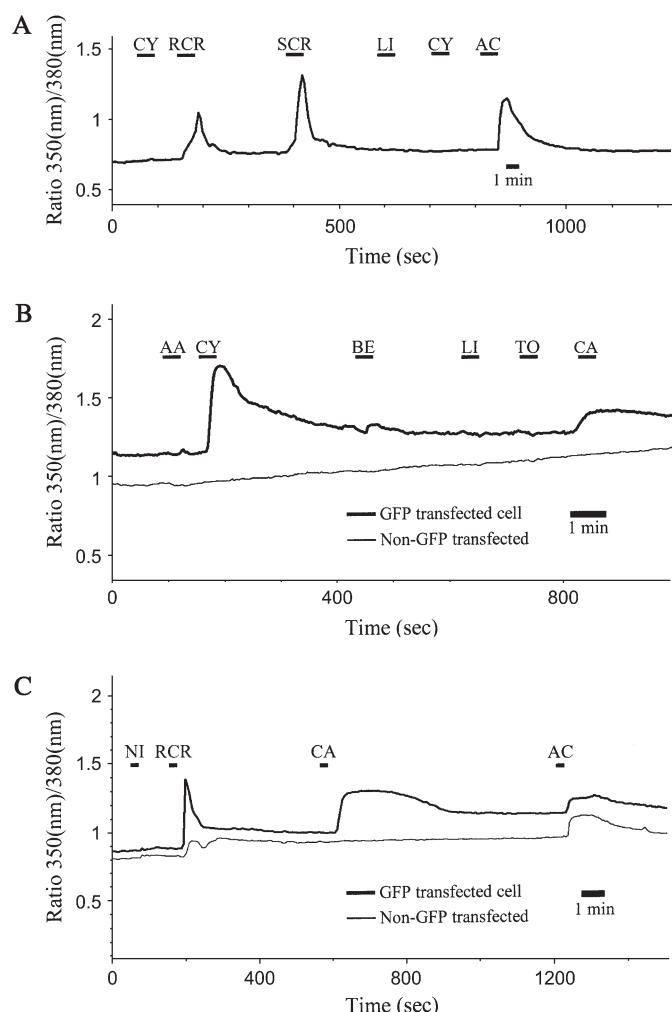
Trigeminal nerve fibers in the nasal cavity respond to a wide variety of chemical stimuli (Bryant and Silver 2000). Most of these compounds also stimulate olfactory receptor neurons (ORNs), although olfactory thresholds are lower than trigeminal thresholds (Tucker 1963; Cometto-Muñiz and Cain 1990). Indeed, with the exception of CA, all the stimuli used in the present study stimulate olfactory receptors. ORNs have numerous receptor proteins which recognize many of the same chemicals that stimulate trigeminal neurons (Buck 2004). These olfactory receptor proteins have not been demonstrated on trigeminal nerves, so the question remains as to the mechanism of action for trigeminal chemostimulation.

Several ligand-gated receptor proteins are located on trigeminal nerve endings. These include the ASIC (Ichikawa and Sugimoto 2002), the purinergic receptor (P2X) (Spehr et al. 2004), the nAChR (Alimohammadi and Silver 2000), and the vanilloid receptor 1 (TRPV1) (Dinh et al. 2003).





**Figure 2** Confocal images of HEK cells transiently transfected with constructs encoding GFP and TRPV1 and then processed for immunoreactivity to TRPV1. **(A)** Combined image showing cells expressing both GFP (green) and TRPV1 (red). White arrow indicates a cell expressing both proteins, and the asterisk indicates a cell expressing GFP only. **(B)** Confocal image showing TRPV1 immunoreactivity. **(C)** Confocal image of GFP fluorescence. Scale bar represents 10  $\mu\text{m}$ .



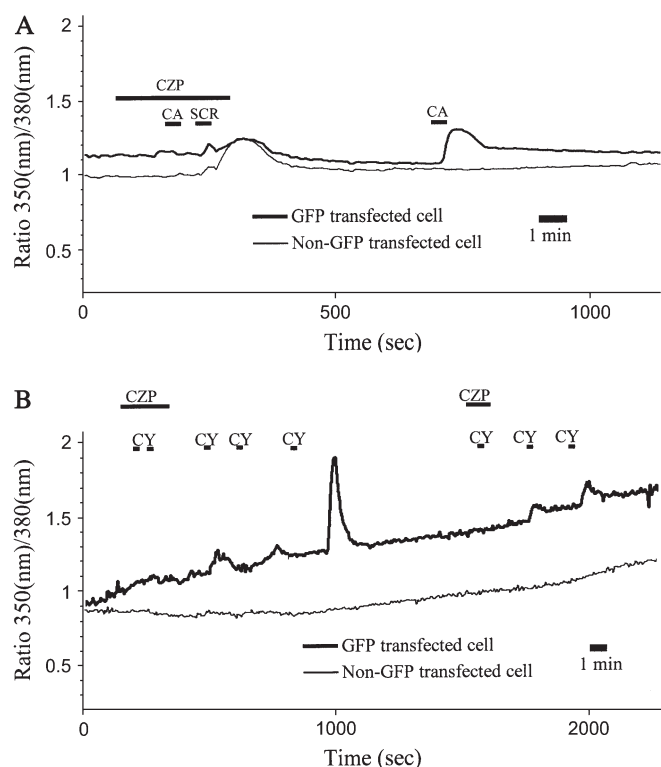
**Figure 3** Calcium imaging of HEK cells. All stimuli were tested at 10 mM except NI (1 mM) and CA (0.5  $\mu\text{M}$ ). **(A)** AC, SCR, and RCR were the only compounds that stimulated control, nontransfected cells. **(B, C)** Only CY and CA stimulated cells expressing GFP and presumably TRPV1. Only RCR and AC were stimulatory in cells expressing and cells not expressing GFP.

ASIC receptors respond to acids and low pH. Purinergic receptors respond to ATP and adenosine, whereas nAChRs are stimulated by NI. TRPV1 responds to a wide variety of seemingly unrelated stimuli (Veronesi and Oortgiesen 2005). These include exogenous chemical irritants like CA, eugenol (Yang et al. 2003), and camphor (Xu et al. 2005), as well as the polyamines, spermine, spermidine, and putrescine (Ahern et al. 2006). In addition, endogenous stimuli, such as anandamide and inflammatory mediators (e.g., lipoxygenase products) can also activate TRPV1s (Holzer 2004).

In the present study, we examined 10 compounds that stimulate the trigeminal nerve in the nasal cavity. Nine of these compounds (excluding CA) stimulated the trigeminal nerve previously when presented in air (Bryant and Silver 2000). In the current study, all these irritants, including CA, stimulated the ethmoid nerve when delivered in Ringer's solution. The concentrations used to stimulate the ethmoid nerve were higher than those used to stimulate HEK cells. Higher concentrations may have been required to elicit responses because in the nerve recording experiments the stimuli were diluted when they were introduced into Ringer's solution flowing into the nasal cavity. In the HEK cell experiments, the stimuli were delivered directly to the dish containing the cells and very little dilution occurred.

Three of the ten compounds (AC, RCR, and SCR) caused an increase in calcium in nontransfected HEK cells. AC presumably activated the ASIC endogenous to the HEK cell (Thomas and Smart 2005). It is not clear how carvone stimulated the native HEK cells. These compounds also increased calcium in HEK cells transfected with TRPV1 but not through TRPV1 because CZP did not affect the response.

Only 2 of the 10 compounds, CA and CY, selectively stimulated HEK cells transfected with TRPV1. Responses to both of these compounds were eliminated in the presence of CZP. CA and CY seem to have little in common. CA, a vanilloid, is the active ingredient in chili pepper and was



**Figure 4** CZP, a TrpV1 blocker, eliminated the response to CA (A) and CY (B) but not SCR (A) in HEK cells transfected with TrpV1.

used to isolate and clone TRPV1 (Caterina et al. 1997; Tominaga et al. 1998). A number of other natural vanilloids including eugenol, gingerol, and piperine also stimulate TRPV1 (Calixto et al. 2005). CY is a 6-membered alicyclic hydrocarbon used primarily as an intermediate in the production of nylon and in aerosol paint concentrates, fungicides, herbicides, pesticides, lubricating oils, and paint thinners (Pazzaglia et al. 2003). It is known as an eye and skin irritant in humans (Chia et al. 1993). It is possible that other irritants structurally unrelated to vanilloids will be found to activate TRPV1.

Five of the stimuli that elicited responses from the trigeminal nerve must have done so through receptors other than TRPV1 because they failed to elicit increased calcium responses from transfected HEK cells. One of those compounds, NI, was reported to sensitize TRPV1 receptors to CA, although it was not stimulatory by itself (Liu et al. 2004). NI has been shown to stimulate trigeminal nerves through nAChRs (Alimohammadi and Silver 1999).

Several other cloned TRP channels respond to various irritant chemicals. Among them are TRPV2 and TRPV3 activated by temperature and 2-aminoethoxydiphenyl borate (Hu et al. 2004). TRPV4 receptors respond to the phorbol ester 4 $\alpha$ -phorbol 12,13-didecanoate which is a semisynthetic product found in some plant seeds (Watanabe et al. 2002). TRPA1 (formerly ANKTM 1) responds to mustard oil (allyl isothiocyanate) and cinnamon oil (cinnamaldehyde)

(Bandell et al. 2004) as well as acrolein (Bautista, et al. 2006). Similarly, TRPM8 responds to menthol (McKemy et al. 2002). Perhaps the 5 compounds that did not activate TRPV1 in the present experiment may stimulate trigeminal nerves through one or more of these receptors or receptors not yet identified.

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