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MOLECULAR AND SYNAPTIC MECHANISMS

The roles of TRPV1, TRPA1 and TRPM8 channels in chemical and thermal sensitivity of the mouse oral mucosa

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Abstract

Spices in food and beverages and compounds in tobacco smoke interact with sensory irritant receptors of the transient receptor potential (TRP) cation channel family. TRPV1 (vanilloid type 1), TRPA1 (ankyrin 1) and TRPM8 (melastatin 8) not only elicit action potential signaling through trigeminal nerves, eventually evoking pungent or cooling sensations, but by their calcium conductance they also stimulate the release of calcitonin gene-related peptide (CGRP). This is measured as an index of neuronal activation to elucidate the chemo- and thermosensory transduction in the isolated mouse buccal mucosa of wild types and pertinent knockouts. We found that the lipophilic capsaicin, mustard oil and menthol effectively get access to the nerve endings below the multilayered squamous epithelium, while cigarette smoke and its gaseous phase were weakly effective releasing CGRP. The hydrophilic nicotine was ineffective unless applied unprotonated in alkaline (pH9) solution, activating TRPA1 and TRPV1. Also, mustard oil activated both these irritant receptors in millimolar but only TRPA1 in micromolar concentrations; in combination (1 mM) with heat (45 °C), it showed supraadditive, that is heat sensitizing, effects in TRPV1 and TRPA1 knockouts, suggesting action on an unknown heat-activated channel and mustard oil receptor. Menthol caused little CGRP release by itself, but in subliminal concentration (2 mM), it enabled a robust cold response that was absent in TRPM8$^{-/-}$ but retained in TRPA1$^{-/-}$ and strongly reduced by TRPM8 inhibitors. In conclusion, all three relevant irritant receptors are functionally expressed in the oral mucosa and play their specific roles in inducing neurogenic inflammation and sensitization to heat and cold.

Introduction

Almost everywhere in the world, people like to add spices such as chili peppers, hot mustard or strong mint extracts to their food or drinks, evoking pungent or cooling sensitizations. Also, tobacco smoking or chewing exposes the oral mucosa and its trigeminal sensory nerves to acrid irritants such as nicotine and unsaturated aldehydes (Stanfill *et al.*, 2011). The active chemical ingredients in these natural products act by binding to neuronal *transient receptor potential* (TRP) ion channels commonly named after the chemicals activating them, that is, the capsaicin receptor TRPV1, the mustard oil receptor TRPA1 and the menthol receptor TRPM8 (Caterina *et al.*, 1997; McKemy *et al.*, 2002; Peier *et al.*, 2002; Story *et al.*, 2003). Nicotine primarily binds and activates the nicotinic acetylcholine receptor channel (nAChR) and in high concentrations TRPA1 and TRPV1 (Kichko *et al.*, 2013). Binding of the specific agonist opens the ion channels which allow cations, that is sodium and calcium (TRPs only), to flow into the nerve endings, depolarizing and eventually eliciting action potential discharge activity. The TRP channels are polymodal transducers accepting a multiplicity of exogenous and endogenous chemicals as agonists and, in addition, are activated by temperature changes. TRPV1 belongs to the noxious heat-activated ion channels, TRPA1 is activated by noxious cold and TRPM8 is a low-threshold cooling receptor with a wide dynamic range. Chemical and thermal sensitivities interact with each other, for example, capsaicin increases the TRPV1 responsiveness to heat, hydrogen peroxide sensitizes TRPA1 to cold and menthol sensitizes TRPM8 to mild cooling (Peier *et al.*, 2002; Everaerts *et al.*, 2011; Moparthi *et al.*, 2016). The chemical concentrations required to sensitize are often lower than those needed to overtly activate the TRP channels.

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The sensory nerves expressing the TRP and nAChR channels mostly combine a long-distance signaling function and a more local neurosecretory function which is to release, upon depolarization, vasoactive neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP), inducing plasma extravasation and vasodilatation, cardinal symptoms of neurogenic inflammation. A majority of the fine unmyelinated and thinly myelinated (C and Aδ) sensory nerve fibers of the oral mucosa show immunocytochemical labeling for several types of TRP channels (Abe *et al.*, 2005; Urata *et al.*, 2015) and likely express nAChR. Whether these receptors are fully functional and accessible by chemical and thermal stimuli was to be elucidated.

TRPs essentially control the vesicular exocytosis of CGRP by virtue of their inherent calcium ion conductance (Spitzer *et al.*, 2008); nAChR channels depolarize the nerves and then require support from voltage-gated calcium channels to trigger CGRP release (Shen & Yakel, 2009). Employing isolated organ preparations, such as flaps of buccal mucosa as used in this study, with the nerves in their natural environment, CGRP release can be measured by enzyme immunoassay (EIA) (Averbeck & Reeh, 2001). This measure, thus, provides a highly integrated index of sensory neuron activation such as by established receptor-channel agonists or thermal stimuli. A drawback thereby is that except for capsaicin, none of the chemical or thermal stimuli is selective for any one particular TRP channel. This problem can be tackled using gene-deleted mouse models.

The aim of this study was therefore to gain an insight into the functional irritant receptor channels in the oral mucosa that are targets of the pertinent flavor compounds, of cigarette smoke and hot or cold stimuli.

Materials and method

Animals

The husbandry and usage of the animals were carried out in accordance with the guidelines of the International Association for the Study of Pain (Zimmermann, 1983), approved and registered by the Animal Protection Authority, District Government Mittelfranken, Ansbach, Germany. Institutional supervision was provided by the university's Department for Animal Care (head M. Ziegelmann, Mvet D). According to German law, no formal approval is required for the competent sacrifice of experimental animals.

Adult C57BL6 (n = 105), TRPA1$^{-/-}$ (n = 18), TRPV1$^{-/-}$ (n = 16), TRPM8$^{-/-}$ (n = 6) knockout mice and double-knockouts TRPA1/TRPV1$^{=/=}$ (n = 24) were used. Breeding pairs of heterozygous TRPV1 and TRPA1 knockout mice were obtained from Dr John Davis (Davis *et al.*, 2000) and Dr David Corey (Kwan *et al.*, 2006) and continuously backcrossed to C57BL/6. Double-knockout animals were generated in our animal facility by cross-mating knockouts of both strains. TRPM8$^{-/-}$ mice were from Dr Ardem Patapoutian (The Scripps Research Institute, La Jolla, CA, USA). All mice were housed in group cages in a temperature-controlled environment on a 12 h light/dark cycle and were supplied with water and food *ad libitum*. Mice of either sex (body weight 15–25 g) were killed by exposure to a rising CO_2 concentration. *Ex vivo* several different tissue samples were excised for experiments in other laboratories of the department.

Buccal mucosa preparation immersed

The oral cavity was opened by midline incision and separation of the lower jaws; from both cheeks of the animals, oval-shaped buccal mucosa flaps of uniform area (~25 mm^2) were excised sparing larger salivary glands and the fascia of the masticatory muscles. The buccal mucosa preparation of one side was used as a control and the other side for sensory stimulations (Kichko *et al.*, 2013).

The tissue samples were placed in carbogen-gassed (95% O_2, 5% CO_2, obtaining pH7.4) synthetic interstitial fluid (SIF) inside a thermostatic shaking bath for a washout and resting period of 30 min at 37 °C. SIF contained (in mM) 107.8 NaCl, 3.5 KCl, 1.53 $CaCl_2$, 0.69 $MgSO_4$, 26.2 $NaHCO_3$, 1.67 NaH_2PO_4, 9.64 sodium gluconate. Following the washout period, all preparations were consecutively passed through a set of four (or five) glass tubes containing 125 µL SIF, each incubation step lasting 5 min. The first two incubation steps were to determine basal CGRP release and its variability. The third tube contained the stimuli at different concentrations or temperatures diluted in SIF. The final tube was for washout and to check for reversal.

Buccal mucosa superfusion

After the initial washout, the preparations were placed open air on a silicone rubber-covered triangular aluminum block at 38 °C (mucosal surface up) and continuously superfused for 30 min with SIF (30 µL/min) which was delivered from a calibrated syringe pump (as typically used for microdialysis). Every 5 min, the superfusate was collected from a trough for the CGRP enzyme immunoassay procedure (Kichko *et al.*, 2015). After two 5 min periods to determine baseline CGRP release, the mucosa flaps were exposed to full cigarette smoke or its gaseous phase for 5 min, followed by two washout periods.

Smoking machine

For cigarette smoke (CS) stimulation, a calibrated one-channel smoking machine was used, essentially a piston pump mimicking the average smoking habit (Burghart, Wedel, Germany), and the exhaust fumes were directly blown through an inverted pipette tip (10 or two puffs of 2s at 30-s intervals in 5 min) onto the mucosal epithelium as previously described in detail. To eliminate all aerosol particles (containing the nicotine), a Cambridge glass fiber filter was interposed between cigarette and smoking machine in some experiments (Kichko *et al.*, 2015). Control stimulation was performed by puffing room air onto the mucosal surface which did not evoke any change in CGRP release, like in our previous experiments on the mouse trachea (Kichko *et al.*, 2015).

Immunocytochemical preparations

To visualize the diffusion barrier in our preparations, we sectioned freshly dissected murine buccal mucosa samples and stained with goat polyclonal (Abcam, Cambridge, UK; cat. #36001) primary antibodies (1:200) against CGRP and rabbit polyclonal against substance P (1:2000; Peninsula/BMA, Augst, Switzerland; cat. #T-4107) which were in turn labeled with fluorescent secondary antibodies (1:1000): goat anti-rabbit IgG, Alexa 448-tagged (cat. # A32731), donkey anti-goat IgG, Alexa 555-tagged (cat. # A21432), both from Invitrogen through Life Technologies, Darmstadt, Germany. These immunocytochemical buccal preparations were evaluated using a confocal laser scanning microscope as previously described in detail (Lennerz *et al.*, 2008; Bergua *et al.*, 2013).

CGRP enzyme immunoassay (EIA)

The CGRP content of the incubation fluid or superfusate was measured using commercial enzyme immunoassay (EIA) kits with a

detection threshold of 5 pg/mL (Bertin Pharma, Montigny-le-Bretonneux, France). For this purpose, 100 µl sample fluid was stored on ice and immediately after the buccal mucosa exposure period mixed with 25 µL of fivefold concentrated commercial CGRP-EIA buffer that contained a proprietary cocktail of peptidase inhibitors (Averbeck & Reeh, 2001).

The further CGRP-EIA procedures were run after the end of the experiment; the antibody reactions took place overnight. The EIA solutions in 96-well plates were measured photometrically using a microplate reader (Dynatech, Channel Islands, UK). All results are presented as measured by the EIA in pg CGRP/mL SIF. For reducing the interindividual and day-to-day baseline variability, the data were referred to the second individual baseline value (before stimulation). This value (7–15 pg/mL in all animals) was subtracted from all four (or five) data points of a typical experiment so that only the absolute change in CGRP release (Δ picogram per milliliter) is displayed in the figures.

Chemicals and cigarettes

The following chemicals were purchased from Sigma (Taufkirchen, Germany): capsaicin; allyl isothiocyanate (AITC); L-menthol; the TRPA1 antagonist HC030031 (2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl) acetamide; (+/−) camphor. (−) Nicotine was obtained from Acros (Geel, Belgium). Potassium chloride was obtained from Roth GmbH (Karlsruhe, Germany), and A700485 (TRPM8 blocker) was a kind gift from Abbott (Chicago). The TRPV1 and TRPM8 inhibitor BCTC, N-(4-t-butylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carboxamide, was obtained from Biomol (Cologne, Germany), (Madrid *et al.*, 2006).

Initial stock solutions were made in ultrapure H_2O (Millipore, Darmstadt, Germany) except for camphor, capsaicin, BCTC, menthol and mustard oil (dissolved in 100% ethanol) and were stored at −24 °C. The final solutions ready to use were freshly diluted in SIF (see above) before each experiment. The camphor 2 mM solution contained 0.2% ethanol, and the other final solutions (BCTC, capsaicin, AITC, menthol) contained 0.1% ethanol or less.

The cigarettes used with the smoking machine were commercial Marlboro Red cigarettes purchased in Germany (10 mg tar, 0.8 mg nicotine, 10 mg CO).

Statistical methods

Statistical comparisons were performed using Statistica 7 software (Statsoft, Tulsa, USA). All time series of experimental values were first analyzed for the effect of temperature or chemical stimulation as compared to baseline CGRP secretion using the nonparametric Wilcoxon matched pairs test.

The baseline-corrected (i.e., Δ pg/mL) CGRP values were entered into a one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test, focusing on the peak values of stimulated CGRP release to compare the different chemically stimulated responses in WT, TRPA1$^{-/-}$, TRPV1$^{-/-}$ and TRPM8$^{-/-}$ knockout mice or TRPA1, TRPV1$^{=/=}$ double-knockouts. *P* and *F* values are provided. Time courses of responses are shown as line plots where data points and vertical bars represent means ± SEM of the given number (*n*) of experiments on different animals. In addition, nonparametric box plots show the distribution of response magnitudes with the median and percentile values and occasional outliers.

Results

To illustrate the histological preconditions of chemosensing in the oral cavity, two immunostained sections through the buccal mucosa of the mouse are displayed. Figure 1A shows the thick multilayered squamous epithelium bare of nerve fibers and below it a network of substance P (SP, green), CGRP (red) and co-staining (yellow) fibers. Further below is a large mast cell (orange) surrounded by several SP$^+$ nerves. Figure 1B shows an isolated taste bud embedded in the epithelium and surrounded by a multitude of double-labeled nerve fibers that only in the moat of the bud come close to the surface.

Capsaicin and KCl activate buccal CGRP release concentration-dependently

The TRPV1 agonist capsaicin (0.1 µM; 1 µM) was very effective and potent in a concentration-dependent way to induce CGRP release from the sensory nerves of the immersed buccal mucosa at 37 °C in wild-type mice C57Bl/6 ($F_{1,10}$ = 19.385, P = 0.0013) but capsaicin was completely ineffective in the null mutants TRPV1$^{-/-}$ ($F_{1,6}$ = 27.734, P = 0.002; Fig. 2A). For comparison, unspecific depolarization of the sensory neurons by KCl (60 and 100 mM), inducing calcium influx through voltage-gated calcium channels (Spitzer *et al.*, 2008), was applied and also tendentially resulted in concentration-dependent CGRP release (Fig. 2B).

Buccal mucosa responses to nicotine, menthol and combination

The concentration of 100 µM (−)-nicotine is maximally effective on the isolated mouse trachea to induce CGRP release through

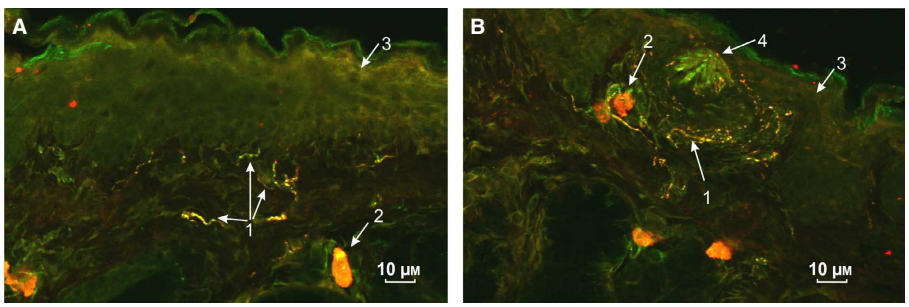

FIG. 1. Sections through mouse buccal mucosa, intraoral surface at the top. CGRP-positive unmyelinated nerve fibers red (Alexa 555), SP-positive ones green (Alexa 448), co-staining yellow. (A) Nerve fibers (1) and mast cells (2) (orange) below the multilayered squamous epithelium (3). (B) Taste bud (4) surrounded by a network of double-labeling neuropeptidergic fibers that approach the surface in the moat of the bud. Mast cells surrounded by SP$^+$ axons. [Colour figure can be viewed at wileyonlinelibrary.com].

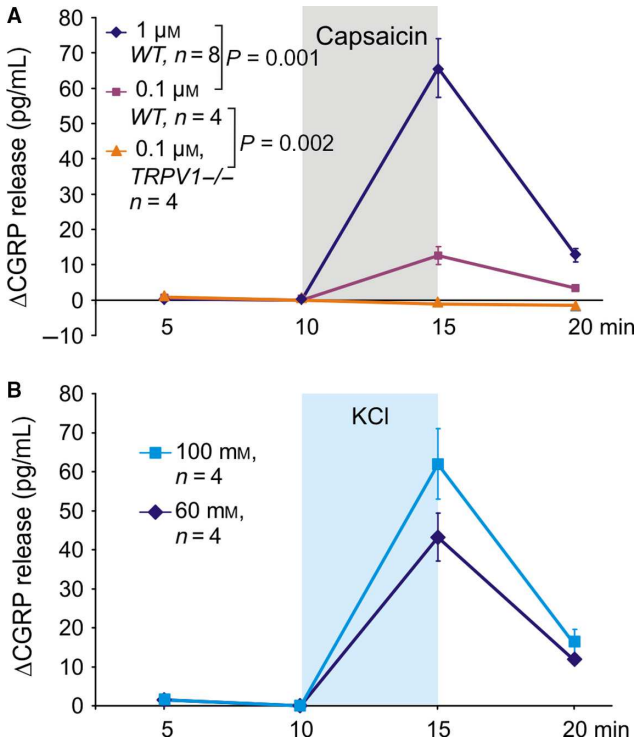

FIG. 2. Stimulated CGRP release from mouse buccal mucosa. (A) Concentration-dependent capsaicin-induced buccal CGRP release in wild type and its absence in TRPV1$^{-/-}$. *P* values refer to peaks of CGRP release (one-way ANOVA, LSD post hoc tests). (B) Concentration-dependent KCl-induced buccal CGRP release. [Colour figure can be viewed at wileyonlinelibrary.com].

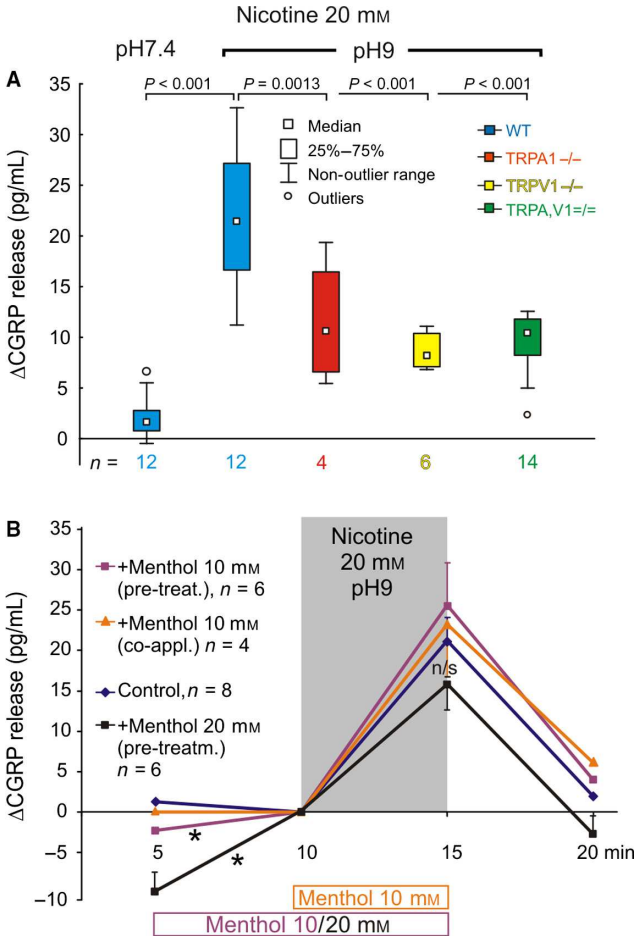

FIG. 3. Effect of supramaximal nicotine 20 mM at pH9 and of menthol (A) The nicotine effect is minimal at neutral pH 7.4 and involves TRPA1 and TRPV1 channels at alkaline pH9 without an additive deficit in double-knockouts. (B) Menthol 10/20 mM pre-treatment or co-application does not alter the nicotine 20 mM (pH9) response, but menthol itself causes little CGRP release during pre-treatment (asterisks refer to $P = 0.03$ Wilcoxon test). [Colour figure can be viewed at wileyonlinelibrary.com].

activation of nicotinic acetylcholine receptor channels (nAChR) (Kichko *et al.*, 2013). This concentration of nicotine 100 μM (as well as 1 mM) was completely ineffective on the mouse buccal mucosa (data not shown). At pH 7.4, the nicotine concentration of 20 mM that occurs in the saliva of consumers of oral tobacco products and nicotine-containing chewing gums (Talavera *et al.*, 2009; Stanfill *et al.*, 2011) evoked a minute buccal CGRP release just exceeding baseline secretion (2 ± 0.5 pg/mL, $P = 0.03$, $n = 12$, Wilcoxon test, Fig. 3A). However, using nicotine 20 mM at pH9, when nicotine is deprotonated, uncharged and lipophilic, we achieved a robust buccal nicotine response that allowed differentiating the causative participation of TRPA1 and TRPV1. Using different knockout mice (TRPA1$^{-/-}$, TRPV1$^{-/-}$ and double-knockouts TRPA1/TRPV1$^{=/=}$), we found that about half of the nicotine response was lost in any of the three mutant strains ($F_{3,34} = 15.218$; $P < 0.001$) whereby the deficits were not additive in the double-knockouts. In analogy to the very similar previous results from the trachea, one may assume that part of the retained nicotine response in the double-knockouts is due to activation of nAChR and another part to unknown mechanism(s) of sensory irritation (Kichko *et al.*, 2013).

Numerous types of oral tobacco products, lozenges and chewing gums contain menthol as a flavor and cooling agent. Micromolar concentrations of L-menthol did not activate any buccal CGRP release (data not shown), but 10 and 20 mM caused minimal increases over baseline (3.4 pg/mL, $P = 0.03$ and 10 pg/mL, $P = 0.03$, Wilcoxon, Fig. 3B) and were significantly different in effect (see Fig. 6B). Menthol 20 mM had previously been reported to reduce lingual heat pain (Albin *et al.*, 2008), but in our hands, the buccal nicotine response was not altered.

Cigarette smoke effects on the superfused buccal mucosa

In view of the very high nicotine concentration and unphysiological pH required to evoke buccal CGRP release, the question arose whether full cigarette smoke would release CGRP, using the superfused oral mucosa preparation and a smoking machine. The amount of full smoke-induced CGRP release was an order of magnitude smaller (Fig. 4A) than in the superfused trachea preparation (Kichko *et al.*, 2015).

As in the previous trachea experiments, two cigarette puffs during the first minute of the 5 min sampling period provided about the same amount of CGRP release as ten puffs regularly distributed over the 5 min; this suggests a marked tachyphylaxis or desensitization from puff to puff (Fig. 4A). The other similarity with the trachea preparation was that there was no significant difference between full smoke and Cambridge-filtered gas phase after ten puffs; just during the first washout period, a difference appeared but was not significant too (Fig. 4B). The cigarette gas phase contains a multitude of volatile TRPA1 agonists but hardly any nicotine (Kichko *et al.*, 2015).

Buccal mucosa responses to mustard oil, heat and combination

To classify the nicotine and cigarette smoke results in the context of other sensory irritant effects, we investigated the classical,

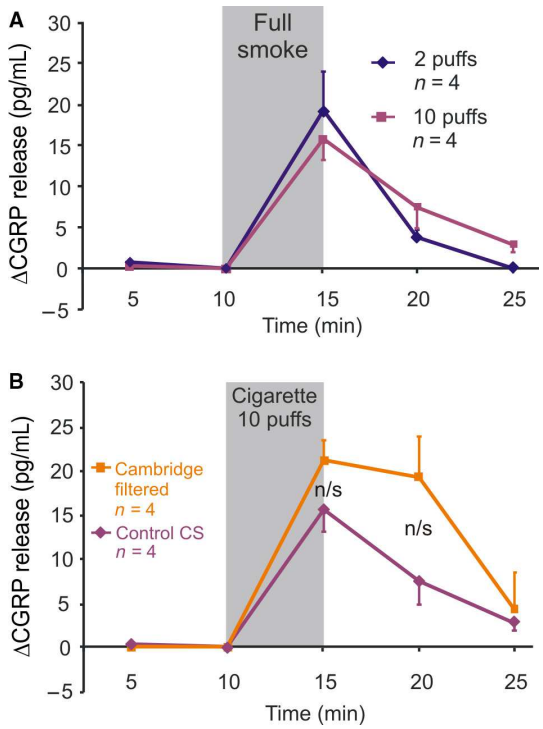

FIG. 4. Effect of cigarette smoke and its gas phase (A) Full cigarette smoke puffed onto the surface of superfused buccal mucosa causes CGRP release; no difference between 10 and 2 puffs in 5 min, suggesting tachyphylaxis. (B) Cambridge-filtered particle (nicotine)-free gas phase of CS essentially as effective as full smoke (control). [Colour figure can be viewed at wiley onlinelibrary.com].

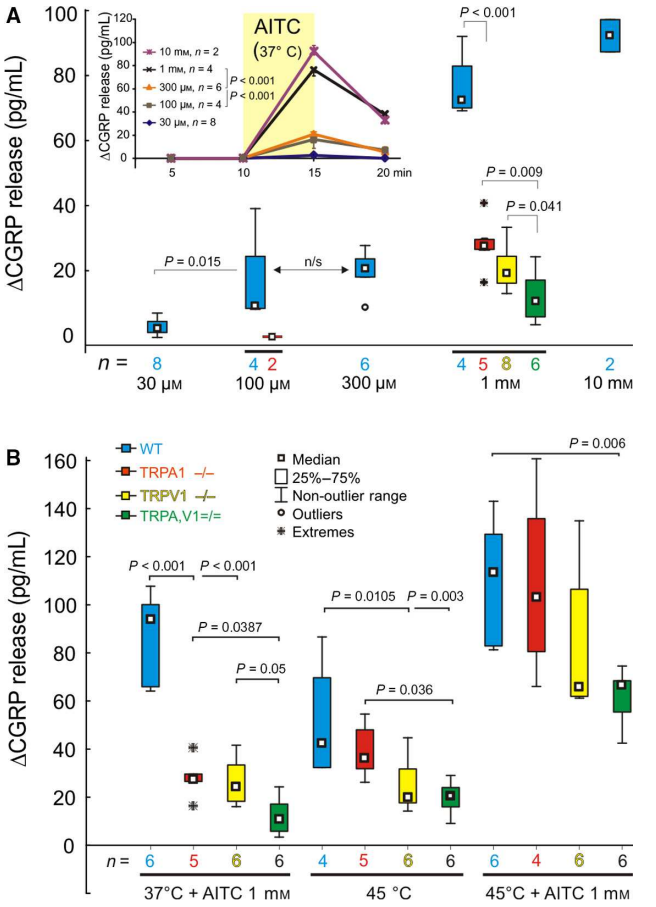

FIG. 5. Effect of AITC and heat stimulation and their combination. (A) AITC 100 and 300 µM significantly stimulate CGRP release in comparison with baseline ($P = 0.01$ and $P = 0.028$; Wilcoxon test) which effect is fully TRPA1 dependent at 100 µM. At 1 mM concentration, the dependence on both TRPA1 and TRPV1 is obvious, and double-knockouts show further significant reductions in the response. The insert shows the time courses of concentration-dependent AITC responses in wild types. (B) AITC 1 mM and heat responses of the different mouse strains; the combination of the stimuli reveals supraadditive effects, that is sensitization to heat, absent in WTs but present in TRPA1 and TRPV1 knockouts, most prominently in the double-knockouts. [Colour figure can be viewed at wileyonlinelibrary.com].

however unselective, TRPA1 agonist allyl isothiocyanate (AITC or mustard oil, MO), the hot principle in mustard and horseradish. It exhibited in wild-type mice a bimodal concentration-response relationship with a threshold between 30 and 100 µM, tendentially increasing response magnitudes at 100 and 300 µM, and a much greater buccal CGRP release at 1 and 10 mM ($F_{4,19} = 84.756$, $P < 0.001$; Fig. 5A). The effects in the lower concentration range appeared dependent on TRPA1, as the null mutants did not respond to 100 µM AITC. At 1 mM AITC, all knockout strains showed great response deficits vs. wild types ($F_{3,19} = 58.09$, $P < 0.001$), and the double-knockouts retained only 15% of the wild-type response, significantly less than TRPA1$^{-/-}$ ($P = 0.009$) and TRPV1$^{-/-}$ ($P = 0.041$). Thus, AITC activated both TRP channels and an additional, unknown, mechanism to release CGRP from the buccal mucosa nerves of mice.

AITC was long known not only to cause cutaneous nociceptor excitation but also a subsequent sensitization to heat (Reeh *et al.*, 1986). To translate these findings from rat to mouse and from action potential discharge to CGRP release, the buccal mucosa preparation was exposed by immersion to moderate noxious heat as a test stimulus (45 °C, 5 min). As expected, the TRPV1 knockouts and the double-knockouts (TRPA1, TRPV1$^{=/=}$) presented with a significant and about equal reduction in the heat response ($P = 0.0105$ and $P = 0.003$, respectively), whereas the reduction of the heat response in TRPA1−/− was not significant (Fig. 5B). To provide actual data for comparison, we repeated the experiments applying 1 mM AITC (at body temperature) once more in the different mouse strains. The graded AITC response deficits in the mutants were fully reconfirmed (ranking of response magnitude: WT>TRPA1$^{-/-}$>TRPV1$^{-/-}$>TRPA1/V1$^{=/=}$).

The co-application of the two effective stimuli AITC 1 mM and 45 °C then revealed in WT a considerably less than additive effect in comparison with the arithmetic sum of heat plus AITC response. This may signify an occlusion by simultaneous activation of one and the same transduction channel (e.g., TRPV1) with AITC and heat. In contrast, in all three mutant strains, the combined stimuli clearly exerted more than additive effects, which may be interpreted as a retained AITC-induced sensitization to heat. In TRPA1−/−, this sensitization fully compensated for the partial loss of AITC responsiveness and can be attributed to an AITC-induced increase in the TRPV1 heat response (Alpizar *et al.*, 2014). In TRPV1$^{-/-}$, the opposite could be postulated: The recently discovered contribution of TRPA1 to heat transduction could be augmented by AITC (Hoffmann *et al.*, 2013; Moparthi *et al.*, 2016). This phenomenon of agonist (AITC)-induced sensitization of TRPA1 has recently been scrutinized but remained mechanistically unclear (Meents *et al.*, 2016). The double-knockouts, having lost most of the AITC and heat responsiveness, still showed a marked supraadditive, thus sensitizing, effect of the AITC and heat combination. This can only be explained by postulating, at least, one other, yet unknown, AITC receptor and heat transducer.

TRPM8 mediates cold-induced CGRP release in the presence of menthol

The first publication on the cold and menthol receptor-channel TRPM8, cloned from sensory neurons, denied its co-expression with CGRP (Peier *et al.*, 2002). However, our pre-treatments (prior to nicotine stimulation) with high menthol concentrations (Fig. 3B) already suggested menthol-induced CGRP release from the buccal mucosa. Such experiments were repeated and confirmed significant and concentration-dependent CGRP release in response to 10 and 20 mM ($F_{1,10} = 7.136$, $P = 0.0234$) but not 2 mM L-menthol (Fig. 6B). The other way to activate TRPM8, that is cooling from body temperature to 14 °C, did not result in any change in CGRP release (Fig. 6A). It has long been known that menthol sensitizes cold-sensing (lingual) nerve fibers, and TRPM8 has been demonstrated to be the integrator of these chemical and thermal stimuli (Schäfer *et al.*, 1986; Voets *et al.*, 2007). Accordingly, the cold stimulus applied in the presence of 2 mM menthol, ineffective by itself, did reproducibly evoke robust CGRP release ($F_{2,19} = 15.991$, $P = 0.00008$). The acute co-application of menthol and cold was more effective than the pre-treatment with menthol, suggesting some exposure time-dependent decline in the sensitizing menthol effect.

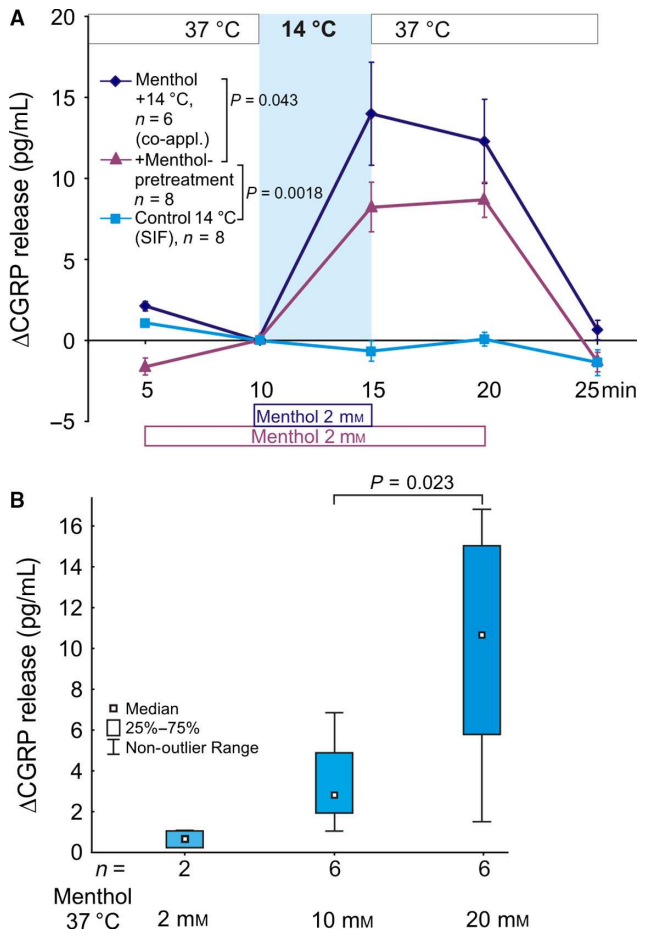

FIG. 6. Menthol-induced buccal CGRP release and sensitization to cold. (A) A step decrease in temperature from 37 °C to 14 °C significantly enhances buccal CGRP release in the presence of subliminal menthol 2 mM concentration, while the cooling step alone is an insufficient stimulus. (B) Concentration-dependent menthol-induced CGRP release from immersed buccal mucosa of wild-type (WT) mice; the *P* value refers to peaks of CGRP release. Menthol 10 and 20 mM raised CGRP release over baseline (both $P = 0.03$, Wilcoxon test). [Colour figure can be viewed at wileyonlinelibrary.com].

Both micromolar menthol and cooling are not selective stimuli for TRPM8 but can also activate TRPA1. However, millimolar menthol concentrations should safely block the murine TRPA1 (but not human TRPA1; Xiao *et al.*, 2008). To ascertain the TRPM8 role in the buccal mucosa, we employed the pertinent null mutant mouse strains. Wild-type C57BL/6, TRPM8$^{+/+}$ littermates and TRPA1$^{-/-}$ knockouts, all showed about the same sensitized cooling responses in the presence of menthol (2 mM), but no increase in CGRP release occurred in the TRPM8 knockouts ($F_{1,8} = 37.903$, $P = 0.0003$; Fig. 7). For a pharmacological cross-check, two established inhibitors of each pertinent TRP channel were employed, using wild-type mice. The TRPA1 antagonists HC030031 and camphor were ineffective, whereas the TRPM8 inhibitors BCTC and A700484 both strongly reduced the cooling response in the presence of menthol ($F_{1,6} = 12.086$, $P = 0.0132$ and $F_{1,6} = 7.669$, $P = 0.0324$, respectively; Fig. 8). Thus, TRPM8 is not only co-immunostaining with CGRP in trigeminal sensory nerves of the oral cavity (Abe *et al.*, 2005; Kim *et al.*, 2014) but actively mediates menthol-sensitized cold-induced CGRP release.

Discussion

Our results delineate the chemosensory responsiveness of the buccal mucosa to popular irritants contained in foodstuff, drinks and cigarette smoke. Exemplary chemicals were employed, such as capsaicin, nicotine, mustard oil and menthol that are known to activate TRPV1, TRPA1 and TRPM8, respectively. Stimulated release of the neuropeptide CGRP was measured as an index of sensory neuron activation. Co-expression of these calcium-conducting TRP channels with CGRP in trigeminal C- and Aδ-fibers of the oral cavity had previously been established (Abe *et al.*, 2005; Wang *et al.*, 2011; Kim *et al.*, 2014; Urata *et al.*, 2015; Yajima *et al.*, 2015).

Capsaicin is by far the most selective and most potent of the here chosen TRP channel agonists, activating exclusively TRPV1 which is almost exclusively expressed in primary afferent neurons (Macpherson *et al.*, 2006; Cavanaugh *et al.*, 2011). The sensation capsaicin evokes in the mouth is well known from chewing hot chili peppers. In all innervated tissues, the TRPV1 channel controls the vesicular exocytosis of CGRP by its superior calcium conductivity (Bernardini *et al.*, 2004). CGRP is the most potent and long-acting vasodilator and in the oral cavity contributes to neurogenic inflammation (Jensen *et al.*, 2016). TRPV1 null mutant mice do not

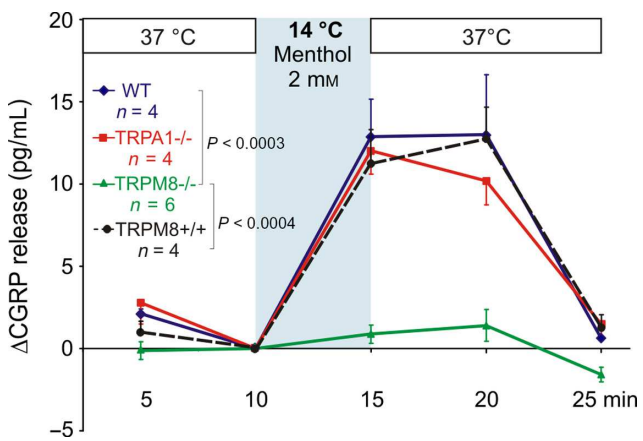

FIG. 7. Cold stimulation in the presence of menthol activates CGRP release through TRPM8 but not TRPA1. TRPM8−/− knockout mice show no response to cold in the presence of menthol 2 mM in contrast to WT, TRPM8+/+ and TRPA1 knockouts. [Colour figure can be viewed at wileyonlinelibrary.com].

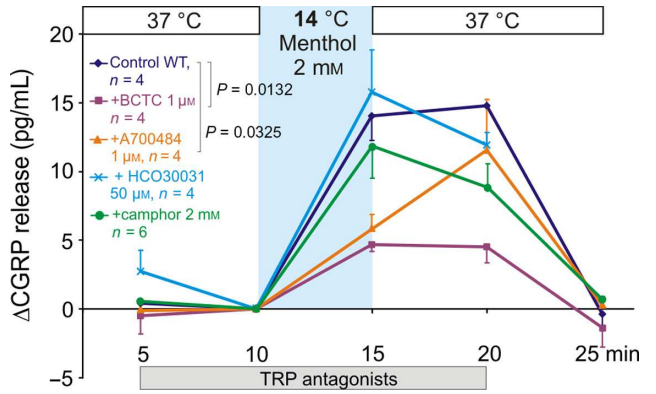

FIG. 8. Pharmacological differentiation of menthol-sensitized cold responses. The *P* values refer to the peaks of CGRP release. The TRPM8 blockers BCTC and A700484 decrease the response by 63% and 55%, respectively. The TRPA1 blockers HC030031 and camphor are ineffective. [Colour figure can be viewed at wileyonlinelibrary.com].

respond to capsaicin in any respect (Caterina *et al.*, 2000), as was the case in our experiments on the buccal mucosa.

Although capsaicin was potent and effective on the thick buccal mucosa preparation, it was moderately less potent and released less CGRP than in the isolated trachea preparation where the peptidergic nerve endings are separated from the surface by only one layer of respiratory epithelium (Kichko & Reeh, 2009). This moderate difference in tissue penetration also applies to the other hydrophobic compounds AITC and menthol that required somewhat higher concentrations in the buccal mucosa than trachea to become effective (T. Kichko and P. Reeh, unpublished data). Nicotine, however, provided a much greater tissue-specific difference, due to its hydrophilicity at neutral pH and to the long diffusion distance through the squamous epithelium of the buccal mucosa, where taste buds (as in Fig. 1B) with peptidergic nerve endings approaching the surface are actually a rare finding. Probably, as a result of the hydrophobic diffusion barrier, the whole bell-shaped concentration-response range (10–500 μM) of nicotine acting through nAChRs in the trachea was missing in the buccal mucosa (Kichko *et al.*, 2013). Even the supramaximal concentration of 20 mM nicotine was ineffective at pH 7.4 when the majority of the nicotine molecules is protonated and less permeable through lipid membranes. At pH 9, the millimolar nicotine concentration, now able to diffuse transcellularly, caused a moderate buccal CGRP release which depended to about equal parts on TRPA1 and TRPV1 activation, like in the trachea. The contributions of these TRP channels were not additive to each other, as the double-knockouts showed no greater deficit in nicotine-evoked CGRP release than the single knockouts (again like in the trachea). This phenomenon is possibly due to the self-limiting influx of desensitizing calcium ions through either TRP channel which determines the mutual cross-desensitization of the largely co-expressed channels (Ruparel *et al.*, 2008).

The cooling compound menthol has recently been shown to reduce the respiratory irritation in mice elicited by the TRPA1 agonists acrolein and cyclohexanone (Ha *et al.*, 2015). With respect to the oral cavity, 20 mM menthol was reported to reduce lingual heat pain in humans, although this is in contrast to another report on enhanced warm sensation by such a high, irritant, menthol concentration (Green, 1985; Albin *et al.*, 2008). Commercial mouthwashes contain 30 mM and higher menthol concentrations (Ali *et al.*, 2015). In our hands, the irritant, CGRP-releasing action of nicotine (20 mM, pH 9), activating TRPA1 and TRPV1, was not influenced by 20 mM menthol.

The effects of cigarette smoke on the superfused buccal mucosa preparation were essentially the same as on the trachea and larynx, just an order of magnitude smaller (Kichko *et al.*, 2015). In the trachea, full smoke largely and the particle-free gas phase entirely acted through TRPA1; only a small contribution of nAChRs could be pharmacologically demonstrated, because the nAChR is increasingly inhibited by nicotine concentrations higher than 100 μM (Kichko *et al.*, 2013). The cigarette gas phase contains a multitude of well diffusible, largely gaseous, small molecules such as H_2O_2, formaldehyde, acrolein, crotonaldehyde, toluene, which are all identified TRPA1 activators (c.f. Kichko *et al.*, 2015). However, when orally inhaled by humans, the Cambridge-filtered cigarette gas is not perceived as irritant even by non-smokers (Lee *et al.*, 2007). This can be explained by the ample deposition of gas phase components in mouth and throat. Thereby, the highly water-soluble constituents of the gas phase can dissolve in the saliva and get diluted to eventually non-irritant concentration (Asgharian *et al.*, 2012). The irritancy of full smoke and gas phase in the mouth is minor, as provided by the present results compared to the trachea.

AITC is the most volatile of the isothiocyanates contained in horseradish, wasabi and mustard (seeds), which property determines the nasal predominance of the burning sensation it evokes, and together with lipophilicity it accounts for the fast and painful penetration of mustard oil into the skin (Reeh *et al.*, 1986; Koltzenburg *et al.*, 1993). AITC activates recombinant and native TRPA1 at μM concentrations and blocks the channel in the mM range (IC_{50} 4.1 mM); in addition, it activates TRPV1 (EC_{50} 3 mM) and even TRPM8 in mM concentrations, as most recently shown (Everaerts *et al.*, 2011; Janssens *et al.*, 2016). Our results are well in accord with this, in general, bimodal concentration-response relationship, showing a TRPA1-dominated μM range and a transition at 1 mM AITC, where TRPA1 still plays a role but TRPV1 prevails and the genetic deletion of both channels further reduces the AITC-evoked CGRP release. The small AITC response retained in the double-knockouts could now be attributed to TRPM8, which would be consistent with the small proportion (9%) of AITC-responsive DRG neurons in these animals (Janssens *et al.*, 2016).

The interpretation of our results from combining AITC (MO) and heat stimulation is less straightforward, because both stimuli alone release CGRP from the buccal mucosa. The combination clearly elicited a greater response than the individual stimuli, consistent with a sensitization to heat, but the magnitude was less than expected from the mere addition of the individual effects. We interpreted this as an occlusion, either by a saturating calcium influx through both TRPA1 and TRPV1 or by AITC and heat both acting through the same, TRPV1, receptor. In favor of this occlusion theory, all three knockout strains, missing one or the other or both calcium entry channels and/or the common AITC and heat receptor, clearly showed supraadditive, truly sensitized, responses to the combined stimuli. In the double-knockouts, the combined effect was almost 90% greater than expected from arithmetic addition. This led us to postulate the existence of yet another AITC and heat transducer. TRPM8 was most recently shown to be also activated by AITC (Janssens *et al.*, 2016), but at first glance, it appears hard to imagine that this established cold transducer could turn into a heat-activated ion channel upon binding AITC. However, Clapham & Miller (2011) derived from elegant thermodynamical considerations that any cold-sensing channel must also be hot-sensing by identical conformational changes in the protein. This theoretical prediction has recently been fulfilled by purified hTRPA1 inserted into artificial lipid bilayers and studied by single-channel recordings (Moparthi *et al.*, 2016). This channel had long been considered to respond only, if at all, to

noxious cold stimulation (Karashima *et al.*, 2009). Now, as predicted by the theory, a U-shaped temperature-response relationship was revealed with a minimum (T_0) of hTRPA1 single-channel activity around 23 °C and an ascending limb of the curve up to body temperature ($Q_{10} \sim 6$). The cold-sensing TRPM8, however, had previously been challenged by temperatures up to 50 °C without responding to heterologously expressing CHO cells (Peier *et al.*, 2002). The above theory also accounts for such an apparently one-sided behavior, conceding that the required temperatures may not be reachable for experimental restrictions. In this case, it may be useful that the theory predicts 'dramatic' changes in temperature-response relationship, if T_0 is shifted by altering the 'molecular interactions within the protein'. Such changes in temperature-sensing have indeed been achieved in hTRPA1 by altering the redox status of the protein and by agonist binding (Moparthi *et al.*, 2016). Whether the postulated heat responsiveness of TRPM8 makes an appearance upon agonist binding has not yet been experimentally tested to our knowledge. A positive result would help understanding the well-known paradoxical cold sensation elicited by sudden exposure to noxious heat. This phenomenon has first been underpinned in the feline oral cavity by lingual nerve recordings from mechanically insensitive, low-threshold cold-sensing fibers (Dodt & Zotterman, 1952). Such unmyelinated nerve fibers are densely innervating the cornea, and it is in these neurons that the essential role of TRPM8 in physiological cold transduction has been demonstrated with single-fiber recordings (Parra *et al.*, 2010).

The initial denial of neuronal TRPM8/CGRP co-expression has long been overcome, most clearly by studies on transgenic mice expressing eGFP (enhanced Green Fluorescent Protein) under the Trpm8 promoter (Kim *et al.*, 2014). This work focused on the trigeminal innervation where the highest Trpm8 mRNA expression among the sensory ganglia had previously been found (Vandewauw *et al.*, 2013). Among all $TRPM8^{GFP}$-positive trigeminal neurons, 26% stained for CGRP and among them 93% also for SP, projecting predominantly through the mandibular nerve to intraoral structures. Given this, at least a small CGRP-releasing effect of the lipophilic TRPM8 agonist menthol was to be expected and occurred concentration-dependently (10–20 mM) at concentrations that would in humans for instance result from using a diluted commercial mouthwash. Compared to capsaicin and AITC, the menthol effects were very small and appeared at concentrations that safely block the murine TRPA1; μM concentrations (max. 70 μM) could activate mTRPA1 but were ineffective on the buccal mucosa (Xiao *et al.*, 2008). Millimolar menthol also activates TRPV3, expressed in keratinocytes but not in sensory ganglia, and it elicits calcium release from intracellular stores, which could also induce CGRP exocytosis (Macpherson *et al.*, 2006; Mahieu *et al.*, 2007). However, menthol has a specific effect at concentrations that do not directly activate TRPM8-expressing neurons or transfected cells, that is to sensitize these cells to cold stimulation (McKemy *et al.*, 2002; Peier *et al.*, 2002). In our experiments, such a concentration was 2 mM menthol which did not evoke buccal CGRP release at body temperature but enabled a robust response upon cooling the preparation to 14 °C, cooling itself being an insufficient stimulus. Pre-treatment with menthol was somewhat less effective than acute co-application with cooling, probably because menthol effects show a tendency to self-desensitize, seen also in psychophysical experiments (Cliff & Green, 1994; Dessirier *et al.*, 2001). The null mutants and our pharmacological tools confirmed that the menthol-sensitized cold-induced buccal CGRP release was due to TRPM8 but not TRPA1. The menthol-induced sensitization to cold has recently been attributed to an agonist-evoked rapid and transient translocation of prefabricated TRPM8 homotetramers into the neuronal plasma membrane, increasing the functional surface expression of the receptor channel (Toro *et al.*, 2015).

In the mouse skin, TRPM8 is functionally expressed in two distinct subclasses of cold-sensing neurons, that is the classical mechanoinsensitive low-threshold cold fibers and mechanosensitive C-fibers that require stronger cooling; the latter not only respond to menthol but often also to other irritants, and some are additionally excited by noxious heat (Milenkovic *et al.*, 2014; Toro *et al.*, 2015). These polymodal nociceptors are most likely the trigeminal neurons that coexpress $TRPM8^{GFP}$ and the vasoactive neuropeptides CGRP and SP. What, however, would a sensitized cold-induced CGRP release mean in physiological respect? Cooling of tissues causes local vasoconstriction, and reheating leads to excess vasodilatation; an experimental model for this reaction is the postocclusive vasodilatation. Lembeck & Donnerer (1981) showed that this phenomenon results from the accumulation of a neuropeptide released from sensory nerve fibers. They assumed it was the long known substance P, simply because the co-expressed and much more potent vasodilator CGRP was only discovered in 1983 (Rosenfeld *et al.*, 1983). Still later, it was established that most of the postocclusive dilatation depends indeed on CGRP but not SP (Fromy *et al.*, 2000). The biological purpose of the homeostatic reaction was obvious – fast recovery from hypoxic stress and nutrient deprivation of the re-perfused tissue. Our result now shows that, in case of cold-induced lack of blood flow, it is not just passive accumulation of CGRP due to insufficient clearance but active neurosecretion evoked by cold activation of TRPM8. The TRPM8-sensitizing role of menthol could – in real life – been taken over by lysophospholipids released from lipid membranes by hypoxic activation of phospholipase A2 (Lambert *et al.*, 2006; Andersson *et al.*, 2007).

Conclusions

Our results from the oral mucosa show that lipophilic irritants such as capsaicin, mustard oil and menthol contained in foodstuff and drinks easily gain access to trigeminal sensory nerves to activate TRPV1, TRPA1 and TRPM8, respectively, which sensitize to heat or cold, respectively, and mediate the release of vasodilatory CGRP (and SP). The hydrophilic nicotine requires a strongly alkaline condition to access and weakly activate TRPA1 and TRPV1, while full cigarette smoke (with nicotine) and its filtered gas phase (without nicotine) exert moderate and about equal effects likely through TRPA1.

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Conflict of interests

No conflict of interests, financial or otherwise, is declared by the author(s).

Author contributions

T.I.K., G.K. and P.W.R. conceived and designed the research. T.I.K., W.N. and P.W.R. performed experiments. T.I.K. and P.W.R. analyzed data. T.I.K., W.N., G.K. and P.W.R. interpreted results of

experiments. T.I.K. prepared figures. T.I.K. and P.W.R. drafted manuscript. T.I.K., G.K. and P.W.R. approved the final version of manuscript.

Data accessibility

Raw data files in form of Excel tables are available upon request to the authors.

Abbreviations

AITC, allyl isothiocyanate (pungent ingredient of mustard oil); BCTC, TRPV1/TRPM8 antagonist; CGRP, calcitonin gene-related peptide; CS, cigarette smoke; eGFP, enhanced Green Fluorescent Protein; MO, mustard oil; SP, substance P; TRPA1, transient receptor potential ankyrin 1; TRPM8, transient receptor potential melastatin; TRPV1, transient receptor potential vanilloid type 1.

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