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The 'headache tree' via umbellulone and TRPA1 activates the trigeminovascular system

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The California bay laurel or *Umbellularia californica* (Hook. & Arn.) Nutt., is known as the 'headache tree' because the inhalation of its vapours can cause severe headache crises. However, the underlying mechanism of the headache precipitating properties of *Umbellularia californica* is unknown. The monoterpene ketone umbellulone, the major volatile constituent of the leaves of *Umbellularia californica*, has irritating properties, and is a reactive molecule that rapidly binds thiols. Thus, we hypothesized that umbellulone stimulates the transient receptor potential ankyrin 1 channel in a subset of peptidergic, nocioceptive neurons, activating the trigeminovascular system via this mechanism. Umbellulone, from μM to sub-mM concentrations, selectively stimulated transient receptor potential ankyrin 1-expressing HEK293 cells and rat trigeminal ganglion neurons, but not untransfected cells or neurons in the presence of the selective transient receptor potential ankyrin 1 antagonist, HC-030031. Umbellulone evoked a calcium-dependent release of calcitonin gene-related peptide from rodent trigeminal nerve terminals in the dura mater. In wild-type mice, umbellulone elicited excitation of trigeminal neurons and released calcitonin gene-related peptide from sensory nerve terminals. These two responses were absent in transient receptor potential ankyrin 1 deficient mice. Umbellulone caused nocioceptive behaviour after stimulation of trigeminal nerve terminals in wild-type, but not transient receptor potential ankyrin 1 deficient mice. Intranasal application or intravenous injection of umbellulone increased rat meningeal blood flow in a dose-dependent manner; a response selectively inhibited by systemic administration of transient receptor potential ankyrin 1 or calcitonin gene-related peptide receptor antagonists. These data indicate that umbellulone activates,

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through a transient receptor potential ankyrin 1-dependent mechanism, the trigeminovascular system, thereby causing nociceptive responses and calcitonin gene-related peptide release. Pharmacokinetics of umbellulone, given by either intravenous or intranasal administration, suggest that transient receptor potential ankyrin 1 stimulation, which eventually results in meningeal vasodilatation, may be produced via two different pathways, depending on the dose. Transient receptor potential ankyrin 1 activation may either be caused directly by umbellulone, which diffuses from the nasal mucosa to perivascular nerve terminals in meningeal vessels, or by stimulation of trigeminal endings within the nasal mucosa and activation of reflex pathways. Transient receptor potential ankyrin 1 activation represents a plausible mechanism for *Umbellularia californica*-induced headache. Present data also strengthen the hypothesis that a series of agents, including chlorine, cigarette smoke, formaldehyde and others that are known to be headache triggers and recently identified as transient receptor potential ankyrin 1 agonists, utilize the activation of this channel on trigeminal nerves to produce head pain.

Keywords: TRPA1; umbellulone; CGRP; migraine; neurogenic vasodilatation

Abbreviations: CGRP = calcitonin gene-related peptide; DMSO = dimethyl sulphoxide; TRPA1 = transient receptor potential ankyrin 1; TRPV1 = transient receptor potential vanilloid 1

Introduction

Umbellularia californica (Hook. & Arn.) Nutt., a tree indigenous to southwestern Oregon and Northern California, is the only species of the genus *Umbellularia* (Peattie, 1953). Inhalation of the scent from the leaves of *U. californica* has been reported to cause sinus irritation, sneezing, headache and even unconsciousness (Drake and Stuhr, 1935; Peattie, 1953). The major volatile constituent of *U. californica* is the irritant monoterpene ketone umbellulone, which can affect respiration, heartbeat and blood circulation in laboratory animals, eventually causing death (Drake and Stuhr, 1935). As known by native Americans and first reported more than 100 years ago (Heamy, 1875), exposure to *U. californica* (commonly known as the 'headache tree') can trigger violent headache crises (Drake and Stuhr, 1935). In this regard, we recently described a case of cluster headache-like attacks triggered by the inhalation of the scent of *U. californica* (Benemei *et al.*, 2009). However, the mechanisms underlying the headache-inducing properties of *U. californica* are unknown.

Migraine and cluster headache have been related to the activation of specific brain areas and the trigeminovascular system, with release of calcitonin gene-related peptide (CGRP) seemingly playing a prominent role. Indeed, CGRP levels are increased in the cranial circulation during migraine and cluster headache attacks (Goadsby *et al.*, 1990; Goadsby and Edvinsson, 1994), and intravenous administration of CGRP triggers migraine attacks in migraineurs (Lassen *et al.*, 2002). More importantly, two chemically unrelated CGRP receptor antagonists have shown clinical efficacy in migraine (Olesen *et al.*, 2004; Ho *et al.*, 2008*b*). The 37 amino acid neuropeptide CGRP is mainly expressed by peripheral neurons, including somatosensory neurons of the dorsal root, vagal and trigeminal ganglia, and exerts remarkable cardiovascular effects and profound arterial vasodilatation (Brain and Grant, 2004). CGRP release from sensory nerve terminals results from the activation of multiple mechanisms, including some members of the transient receptor potential (TRP) family of channels. Peptidergic neurons are characterized by the expression of the capsaicin-sensitive vanilloid 1 (TRPV1) channel (Caterina *et al.*, 1997), and the mustard oil-sensitive ankyrin 1 (TRPA1) channel (Jordt *et al.*, 2004). TRPA1 is also stimulated by cold temperatures (Story *et al.*, 2003; Karashima *et al.*, 2009), by a series of chemically diverse and highly reactive environmental agents and by various electrophilic natural products (Nilius *et al.*, 2007) that covalently modify cysteine residues of the channel (Hinman *et al.*, 2006; Macpherson *et al.*, 2007). This unusual activation is produced also by some endogenous mediators generated at sites of inflammation and tissue injury, including α,β-unsaturated aldehydes, acrolein, 4-hydroxy-2-nonenal and electrophylic prostaglandins (Bautista *et al.*, 2006; Trevisani *et al.*, 2007; Materazzi *et al.*, 2008).

During fractionation of the essential oil from *U. californica*, we observed that inhalation of umbellulone caused a painful cold sensation in the nasal mucosa, somewhat reminiscent of that produced by mustard oil. Despite the β,β-dialkyl substitution, a structural feature known to suppress Michael reactivity (LoPachin *et al.*, 2008), we found that umbellulone reacts quickly with thiols (Avonto *et al.*, 2011). It has been recently reported that topical application of TRPA1 agonists to the nasal mucosa elicits CGRP-dependent and neurogenic meningeal vasodilatation (Kunkler *et al.*, 2011). Altogether, these findings led us to speculate that, umbellulone targets the TRPA1 channel, and through this mechanism, causes nocioceptive responses and CGRP release from trigeminovascular endings. We also hypothesized that activation of TRPA1 in CGRP-containing trigeminal neurons may represent the hitherto unknown, underlying mechanism that explains headache precipitation by a variety of environmental agents.

Materials and methods

Isolation of umbellulone

Dried leaves of *U. californica* (500 g) were steam-distilled using a Clevenger apparatus. An offensive and colourless oil (22 g, 4.4%) was obtained, which was then adsorbed on RP18 silica gel (350 g) and purified by vacuum chromatography on RP18 silica gel (500 g) using water-methanol (1:1) as eluent. The fractions containing umbellulone were combined, saturated with solid NaCl, and extracted with

petroleum ether, obtaining 11.3 g (2.3%, 99% purity by gas chromatography) umbellulone as a colourless oil.

Reaction of umbellulone with cysteamine

Umbellulone (0.1 mmol) was treated with cysteamine (1.2 mmol) in dimethyl sulphoxide (DMSO)-d_6 (0.4 ml), resulting in the instantaneous formation of a Michael adduct, characterized by 2D nuclear magnetic resonance measurements. ^{1}H (500 MHz) and ^{13}C (125 MHz) nuclear magnetic resonance spectra were measured on a Varian INOVA spectrometer. Homonuclear ^{1}H connectivities were determined by the COSY (correlation spectroscopy) experiment. One-bond heteronuclear ^{1}H-^{13}C connectivities were determined with the HSQC (heteronuclear single quantum correlation) experiment. Two- and three-bond ^{1}H-^{13}C connectivities were determined by HMBC (heteronuclear multiple bond correlation) experiments optimized for a $^{2,3}J$ of 7 Hz. Through-space ^{1}H connectivities were evidenced using a ROESY (rotating-frame overhauser effect spectroscopy) experiment with a mixing time of 500 ms. Two and three bond ^{1}H-^{13}C connectivities were determined by gradient 2D HMBC experiments optimized for a $^{2,3}J$ of 9 Hz.

Animals

Sprague-Dawley rats (male, 250 g; Harlan Laboratories) were used. Wild-type (*Trpa1*$^{+/+}$) or TRPA1 deficient mice (*Trpa1*$^{-/-}$), generated by heterozygous mice on a C57BL/6 background were used for *in vivo* (Bautista *et al.*, 2006) or *in vitro* (Kwan *et al.*, 2006) studies. Experiments with *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ littermate mice were blinded to the genotype. Animals were sacrificed with a high dose of intraperitoneal sodium pentobarbital (200 mg/kg). All animal experiments were carried out in accordance with the European Union Community Council guidelines and were approved by the local ethics committees.

Reagents

Umbellulone was dissolved in 100% DMSO at a final concentration of 1 M, and further dilutions in aqueous buffer. The TRPA1 selective antagonist, HC-030031, was synthesized as previously described (Andre *et al.*, 2008). For *in vitro* experiments, HC-030031 was dissolved in 100% DMSO (1 mM stock solution) and then diluted in aqueous buffer. For *in vivo* experiments, HC-030031 was prepared in 0.5% carboxymethylcellulose and administered via intragrastic route. BIBN4096BS (olcegepant), kindly donated by Dr H. Doods (Boehringer-Ingelheim), was dissolved in 0.1 N HCl, diluted in isotonic saline and finally adjusted to pH 6.5–7.0 by 1 N NaOH. If not otherwise indicated, all reagents were from Sigma-Aldrich.

Cell culture

Human embryonic kidney (HEK) cells transfected with the complementary DNA of rat TRPA1, generated and grown as previously reported (Materazzi *et al.*, 2008), were kindly donated by N.W. Bunnett (University of California, San Francisco). HEK293 cells stably transfected with the human TRPA1 complementary DNA and kindly donated by A.H. Morice (University of Hull, United Kingdom), and untransfected HEK293 cells (American Type Culture Collection) were cultured as previously described (Sadofsky *et al.*, 2008). HEK293 cells transfected with the human TRPV1, a kind gift of Martin J. Gunthorpe (GlaxoSmithKline), were grown as previously described (Trevisani *et al.*, 2002). HEK293 cells were transiently transfected with human TRPM8 as previously described (Mahieu *et al.*, 2010). A tetracycline-regulated system for inducible expression of TRPA1 in Chinese hamster ovary cells transfected with the complementary DNA of mouse TRPA1 was used, as described previously (Story *et al.*, 2003). Cells were plated onto coverslips coated with poly-L-lysine (18.3 μM) and used for calcium imaging recordings after 1–2 days.

Isolation of primary sensory neurons

For isolation of rat trigeminal ganglia neurons, ganglia were removed, placed in cold Hank's buffered salt solution, and transferred to Hank's buffered salt solution containing 2 mg/ml collagenase type 1 A, 1 mg/ml trypsin, for 35 min at 37°C for enzymatic digestion. Ganglia were then transferred to warmed Dulbecco's modified Eagle's medium containing 10% foetal bovine serum, 10% horse serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and dissociated in single cells by several passages through a series of syringe needles (23–25 G). Medium and ganglia cells were filtered to remove debris and centrifuged. The pellet was resuspended in Dulbecco's modified Eagle's medium with added 100 ng/ml mouse-NGF and 2.5 μM cytosine-β-D-arabino-furanoside free base. Trigeminal ganglia neurons from *Trpa1*$^{+/+}$ or *Trpa1*$^{-/-}$ adult mice (postnatal weeks 8–12) were cultured as previously described (Kwan *et al.*, 2006; Karashima *et al.*, 2007).

Calcium imaging

Calcium fluorescence was measured in rat trigeminal ganglia neurons or in transfected and untransfected HEK293 cells, as previously reported (Andre *et al.*, 2008; Materazzi *et al.*, 2008). Plated cells were loaded with Fura-2AM-ester (5 μM; Alexis Biochemicals) added to the buffer solution (37°C) containing the following (in mM): 2 $CaCl_2$; 5.4 KCl; 0.4 $MgSO_4$; 135 NaCl; 10 D-glucose; 10 HEPES and 0.1% bovine serum albumin at pH 7.4. After 40 min, cells were washed and transferred to a chamber on the stage of a Nikon Eclipse TE2000U microscope for the recording. Cells were excited at 340 nM and 380 nM to indicate relative intracellular calcium ($[Ca^{2+}]_i$), changes by the $F_{340/380}$ ratio recorded with a dynamic image analysis system (Laboratory Automation 2.0; RCSoftware). Cells or neurons were exposed to umbellulone, mustard oil, capsaicin, menthol and the proteinase activated receptor-2 activating peptide (SLIGKV-NH_2) or their vehicles, which were: 1% DMSO, 1% DMSO, 0.01% ethanol, 0.1% DMSO and distilled water, respectively. The vehicle of HC-030031 and capsazepine were 1% DMSO and 0.1% DMSO, respectively. Trigeminal ganglia neurons were challenged with capsaicin to identify primary sensory neurons. Results are expressed as percentage of the maximum response to ionomycin (5 μM) added at the end of each experiment. $[Ca^{2+}]_i$ mobilization in trigeminal ganglia neurons obtained from *Trpa1*$^{+/+}$ or *Trpa1*$^{-/-}$ mice was measured in Fura-2AM-loaded cells with a monochromator-based imaging system as previously described (Vriens *et al.*, 2005).

Electrophysiology

Ionic currents were recorded in the whole-cell and the cell-attached configurations of the patch-clamp technique. Data were sampled at 5–20 kHz and were filtered off-line at 1–5 kHz. We used 400 ms voltage ramps from a holding of 0 mV from −100 to +100 mV applied every 2 s. The following solutions were used for whole cell measurements in the bath (in mM): 150 NaCl; 6 KCl, 1 $MgCl_2$, 1,5 $CaCl_2$,

10 HEPES; 10 D-glucose, NaOH to pH 7.4. The Ca^{2+} free solution contained (in mM): 150 NaCl; 5 $MgCl_2$; 10 HEPES; 10 glucose; NaOH to pH 7.4. Pipette solution contained (in mM): 100 K-Aspartate; 40 KCl; 2 $MgCl_2$; 4 Na_2-ATP, 5 EGTA and 1.2 $CaCl_2$ for buffering at 50 nM free Ca^{2+}, 10 HEPES and KOH to pH 7.2. In cell-attached single channel recordings, this pipette solution was used as bath solution, and vice versa. Extracellular solution for measuring trigeminal ganglia currents was similar as described in (Vriens *et al.*, 2011). A ramp protocol was applied consisting of a voltage step from the holding potential of 0 mV to +120 mV followed by a 400 ms linear ramp to −120 mV. Electrophysiological data were analysed using WinASCD software (ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/winascd.zip). Dose–response fits were performed using Origin 7.0 (OriginLab Corporation).

Calcitonin gene-related peptide measurement

Slices (0.4 mm) of dorsal spinal cord and trigeminal ganglia obtained from *Trpa1*$^{+/+}$ or *Trpa1*$^{-/-}$ mice and of rat dorsal spinal cord, dura mater and trigeminal ganglia were superfused with umbellulone (30–1000 μM), mustard oil (100 μM) and menthol (300 μM) or their vehicles (0.1 and 0.3% DMSO) dissolved in a modified Krebs solution at 37°C and oxygenated with 95% O_2 and 5% CO_2, containing (in mM): 119 NaCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.5 $MgSO_4$, 2.5 $CaCl_2$, 4.7 KCl, 11 D-glucose; 0.1% bovine serum albumin, phosphoramidon (1 μM) and captopril (1 μM). Some tissues were pre-exposed to capsaicin (10 μM) for 20 min to desensitize TRPV1-expressing sensory nerve terminals. Some experiments were performed in a calcium-free medium, containing EDTA (1 mM). Immunoreactivity for CGRP was assayed in 10 min fractions (two before, one during and one after exposure to the stimulus) according to the methods previously reported (Nicoletti *et al.*, 2008). The detection limit was 5 pg/ml. CGRP immunoreactivity release was calculated by subtracting the mean prestimulus value from those obtained during or after stimulation. Stimuli did not cross react with CGRP antiserum.

Eye wiping assay in mice

Ocular instillation (5 μl) of umbellulone (50–250 nmol), mustard oil (10–20 nmol), capsaicin (1 nmol) and menthol (25 nmol) or their respective vehicles (5, 4, 2 and 5% DMSO, respectively) was used to induce an acute nocioceptive response as previously described (De Petrocellis *et al.*, 2010). Animals were placed individually inside a Plexiglas chamber and were acclimatized for 20 min before stimulus. The number of eye wiping movements following the drug instillation into the eye was recorded for a 2-min time period and was considered as index of pungency.

Dural blood flow

Rats were anaesthetized (sodium pentobarbital, 50 mg/kg, intraperitoneally). Body temperature was kept at 37°C with a heating pad. Diastolic and systolic blood pressure was monitored from the tail using a BP-2000 (Visitech Systems). The animal's head was fixed in a stereotaxic frame and the scalp was incised along the midline and the parietal skin pulled aside. Using a manual drill (Harvard Instruments), a cranial window (4 × 6 mm) was made into the parietal bone to expose the dura mater. The probe (needle type, tip diameter 0.8 mm) of a Laser Doppler Flow Meter (Perimed Instruments) was fixed near a branch of the middle meningeal artery (1 mm from the meningeal layers). The cranial window was filled with a modified synthetic interstitial fluid containing (in mM): 135 NaCl; 5 KCl; 1 $MgCl_2$; 5 $CaCl_2$; 10 D-glucose and 10 HEPES. After 45 min of stabilization, intravenous umbellulone (0.2, 0.5, 1 μmol/kg) or mustard oil (1 μmol/kg) or their vehicles (0.1 and 1% DMSO, respectively; 1 ml/kg) were administered and the flow was monitored for 20 min. Ethanol (140 μl/kg, intravenously) was then administered and finally, sodium nitroprusside (1 mM/100 μl) was topically applied into the cranial window. Meningeal blood flow was monitored before and after intravenous infusion (1 ml/kg) of isotonic saline (0.9% NaCl, as control of the volume of 100% ethanol injection) or topical application to the cranial window of sodium nitroprusside vehicle (synthetic interstitial fluid, 100 μl). In another set of experiments, umbellulone (0.2, 0.5, 1 μmol/kg), capsaicin (0.2 μmol/kg) or their vehicles (0.5% DMSO and 0.01% ethanol), were nasally administered. Tested compounds or vehicles (~50 μl) were applied into the nostril over a 30 s period, and the meningeal blood flow was monitored for 20 min. BIBN4960BS (1 mg/kg, intravenously), capsazepine (4 mg/kg, intraperitoneally, 10 ml/kg) or their vehicle (acidified saline and 5% DMSO, respectively) were given 10 min before stimulus administration. HC-030031 (100 mg/kg, intragastrally) or its vehicle (0.5% CMC) was given 1 h before stimulus administration. Baseline flow was calculated by the mean flow value measured during a 5-min period prior to stimulus. The percentage change in blood flow was calculated as reported previously (Nicoletti *et al.*, 2008).

Umbellulone determination on plasma by high performance liquid chromatography–electrospray tandem mass spectrometry

Rat plasma samples were collected at different time (1, 2 and 15 min) after intravenous or intranasally umbellulone administration (0.2 and 1 μmol/kg) and quantified by high performance liquid chromatography–electrospray tandem mass spectrometry. Stock solution and following dilutions of umbellulone were made in methanol. All chemicals and solvents were of the highest purity available from commercial sources. An ABI-Sciex API 4000 Triple-Quad Mass Spectrometer equipped with the TurboIonSpray source was used. The TurboIonSpray source operated under positive ion mode at a voltage of +5500 V and with a 'turbo' gas flow of 10 l/min of air heated at 350°C. All compound potentials were automatically optimized for umbellulone, by the 'quantitation optimization' option. The resulting declustering potential was +50 V. Optimal collision energy and collision exit potential were found at 15 and 13 V, respectively. Mass spectrometry and tandem mass spectrometry umbellulone spectra were collected in continuous flow mode by connecting the infusion pump directly to the TurboIonSpray source. The quantitation experiments were undertaken using a Series 1100 Agilent Technologies CapPump, coupled to an Agilent Micro ALS autosampler, both being fully controlled from the API 4000 data system. Liquid chromatography was performed using a Poroshell 120 EC-C18 2.7 μm, 3 × 100 mm high performance liquid chromatography column (Agilent Technologies). Column flow was 0.5 ml/min, operated in isocratic mode using an aqueous solution of 74% acetonitrile containing 0.05% formic acid. Data were processed using the Analyst 1.4.1 software. Plasma levels of umbellulone after intravenous or intranasal administration are expressed as area under the curve calculated over 1–15 min.

Statistical analyses

Data are presented as means ± S.E.M. We used one-way ANOVA followed by *post hoc* Bonferroni's test for comparisons of multiple groups and the unpaired two-tailed Student's *t*-test for comparisons between two groups. $P < 0.05$ was considered statistically significant.

Results

Umbellulone reacts with thiol groups

Several, but not all, TRPA1 activators promote channel gating through an unusual mechanism involving covalent modification of the channel (Hinman *et al.*, 2006; Macpherson *et al.*, 2007). The chemical properties of umbellulone were studied in order to determine whether the 'headache tree' extract could react with residues of the TRPA1 channel. When treated with equimolecular amounts of cysteamine in DMSO-d_6, umbellulone gave an instantaneous reaction, resulting in the quantitative formation of a Michael adduct (Fig. 1A), that was characterized in terms of constitution and configuration by 2D nuclear magnetic resonance measurements. Under these conditions, the closely related enones (+)-verbenone and (+)-piperitone (Fig. 1A) were totally non-reactive, even after 48 h at room temperature. The reasons underlying the surprising thiophilicity of umbellulone are undoubtedly related to the presence of the cyclopropane ring adjacent to the enone β-carbon, since the opening of this ring, like in piperitone, or replacement with a four-membered ring as in verbenone, re-established the unreactivity with thiols, typical of β,β-dialkylsubstituted enones (Table 1).

Umbellulone activates the recombinant transient receptor potential ankyrin 1 channel

To verify whether umbellulone acts as an agonist at the TRPA1 channel, we first used cells transfected with rat, human or mouse isoforms of the TRPA1 channel. In both rat TRPA1- and human TRPA1-transfected HEK293, but not in unstransfected HEK293 cells, umbellulone caused a concentration-dependent elevation of $[Ca^{2+}]_i$, with a half maximal effective concentration slightly higher than that of the TRPA1 selective agonist, mustard oil (Fig. 1B–D). Verbenone and piperitone (both 300 μM) did not produce any calcium response in rat TRPA1 transfected HEK293 cells (data not shown). In human TRPV1, and human TRPM8 transfected HEK293 cells, umbellulone (100 μM) was ineffective (Fig. 1E). Umbellulone (1 mM) caused a moderate calcium response ($Ratio_{340/380}$ 0.68 ± 0.06, $n = 52$) in human TRPM8 transfected HEK293 cells. This response, (which was ~50% of the response to 100 μM menthol), was however, significantly higher than the response observed in untransfected cells ($Ratio_{340/380}$ 0.14 ± 0.01, $n = 29$). Using whole-cell patch clamp recordings, umbellulone evoked a large inward membrane current (Fig. 1F and G) in mouse TRPA1 transfected Chinese hamster ovary cells. Currents were activated in both the inward and outward direction similar to mustard oil (Fig. 1H). Half maximal activation of TRPA1 by umbellulone was at ~12 μM. Data were obtained by successive application of umbellulone, which is in contrast to mustard oil activation, acts in a reversible fashion. Umbellulone increased the open probability of TRPA1 channels measured in cell attached patches, without changing single channel conductance (Fig. 1I). No response to umbellulone was seen in Vector transfected Chinese hamster ovary cells (data not shown).

Umbellulone selectively targets the native transient receptor potential ankyrin 1 channel

Next we studied native channels expressed in either rat or mouse somatosensory neurons. Umbellulone elevated $[Ca^{2+}]_i$ in cultured rat trigeminal ganglia sensory neurons (Fig. 2A and B), a response that was inhibited by weakly-selective (ruthenium red) or selective (HC-030031) (McNamara *et al.*, 2007) TRPA1 antagonists, but was unaffected by the TRPV1 antagonist, capsazepine (Fig. 2A and C). Umbellulone (100 μM) produced a robust calcium response in 38% of trigeminal ganglia neurons (53 out of 136) obtained from *Trpa1*$^{+/+}$ mice (Fig. 2D and F). All umbellulone-sensitive neurons were activated by mustard oil, indicating that both compounds activated the same neurons. To verify that the response to umbellulone was mediated by activation of TRPA1 channels, the same protocol was repeated in trigeminal ganglia sensory neurons obtained from *Trpa1*$^{-/-}$ mice (Kwan *et al.*, 2006). As shown in Fig. 2E and F, the responses to umbellulone and mustard oil were abolished in trigeminal ganglia neurons derived from *Trpa1*$^{-/-}$ mice (0 responders out of 95). In contrast, the amplitude of the responses and the percentage of trigeminal ganglia neurons responding to capsaicin were unchanged in both *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice (Fig. 2D–F).

Similar results were obtained with whole-cell patch clamp experiments. Stimulation with umbellulone (100 μM) caused an increase in current density in 36% (8 out of 22) of trigeminal ganglia neurons isolated from *Trpa1*$^{+/+}$ mice (Fig. 3A, E and F). All umbellulone-sensitive neurons also showed significant current increases after application of mustard oil (100 μM; representative example Fig. 3A). In contrast, no current increases were observed in trigeminal ganglia derived from *Trpa1*$^{-/-}$ mice after stimulation with umbellulone (100 μM) and mustard oil (100 μM; 0 out 13; Fig. 3C and E). Nevertheless, capsaicin (1 μM) caused a similar increase in current amplitude and a similar percentage of capsaicin-sensitive neurons from *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice (Fig. 3E and F). Thus, within the tested concentrations, umbellulone-evoked $[Ca^{2+}]_i$ signals and current amplitude increases in trigeminal ganglia neurons are mediated exclusively by activation of native TRPA1 channels.

Umbellulone evokes the release of calcitonin gene-related peptide from trigeminal neurons

TRPA1 is expressed by peptidergic primary sensory neurons (Story *et al.*, 2003; Bhattacharya *et al.*, 2008), where its stimulation

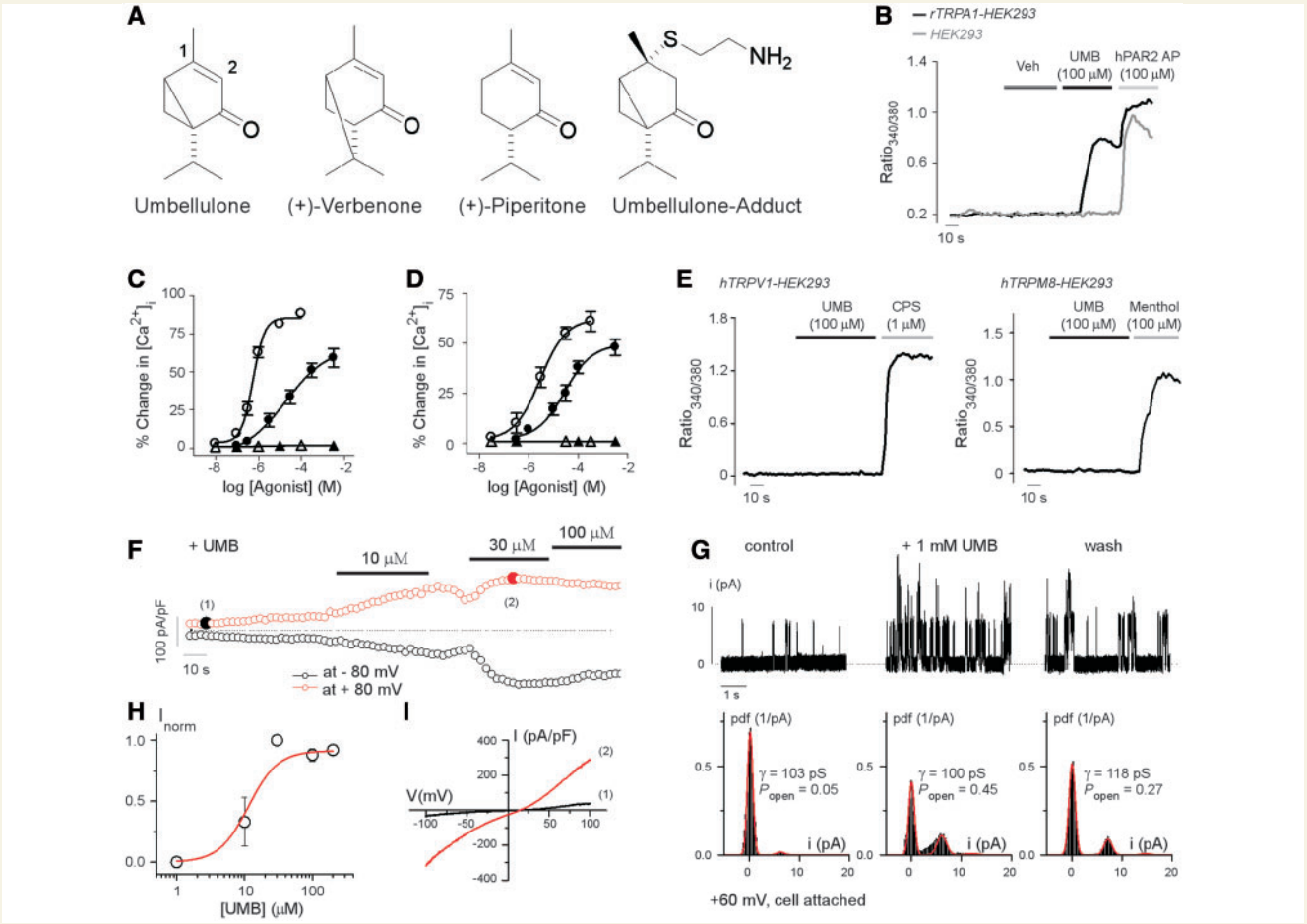

Figure 1 Umbellulone selectively signals to the recombinant TRPA1 channel. (**A**) Chemical structure of umbellulone, (+)-verbenone, (+)-piperitone and umbellulone-adduct with cysteamine. (**B**) Representative traces of intracellular calcium ($[Ca^{2+}]_i$) mobilization evoked by umbellulone and by the activating peptide (SLIGKV-NH_2) of the proteinase activated receptor-2 (hPAR-2 AP) in HEK293 cells transfected with the complementary DNA of rat TRPA1 (rTRPA1-HEK293, black line) or in untransfected-HEK293 cells (HEK293, grey line). (**C**) Concentration–response curves produced by umbellulone (filled circles) and the selective TRPA1 agonist, mustard oil (mustard oil, empty circles) in rTRPA1-HEK293. Half maximal effective concentrations are 18.7 ± 3.5 μM and 0.56 ± 0.06 μM, respectively. No response was seen in HEK293 cells (triangles). Each point represents mean ± S.E.M. of $n > 40$ cells. (**D**) Concentration–response curves produced by umbellulone (filled circles) and mustard oil (empty circles) in HEK293 cells transfected with the complementary DNA of human TRPA1 (hTRPA1-HEK293). Half maximal effective concentrations are 28.5 ± 6.8 μM and 2.9 ± 0.4 μM, respectively. No response was seen in HEK293 cells (triangles). Each point represents mean ± S.E.M. of $n > 30$ cells. (**E**) In HEK293 cells transfected with human TRPV1 complementary DNA (hTRPV1-HEK293) or with human TRPM8 complementary DNA (hTRPM8-HEKT293) umbellulone does not produce any significant response compared to capsaicin (CPS) or menthol, respectively. Typical traces (**F**) and concentration–response curve (**H**) of the activation by umbellulone of mouse TRPA1 expressed in a heterologous system (Chinese hamster ovary cells). Dose–response was calculated from $I_{norm} = I_{norm,max}/(1 + (\text{half maximal effective concentration}/[UMB])^{n}_{H})$, where n = the Hill coefficient. A half maximal effective concentration of 11.6 ± 1.8 μM, a Hill coefficient of 2 and $I_{norm,max}$ of 0.92 was obtained from best fits. Data were normalized on peak amplitude at +80 mV and 30 μM umbellulone. (**I**) Both inward and outward currents are activated (data obtained from the times indicated in panel **F**). (**G**) In cell attached patches, administration of umbellulone from the extracellular side increases the open probability but not the single channel conductance. Holding potential is +60 mV. UMB = umbellulone.

results in a calcium-dependent release of neuropeptides. Exposure to umbellulone caused a concentration-dependent release of CGRP immunoreactivity from the rat dorsal spinal cord (an anatomical area enriched by central terminals of peptidergic afferent neurons) (Fig. 4A). An elevated umbellulone concentration (1 mM), that potentially could activate TRPM8, released CGRP from the dorsal spinal cord of *Trpa1*$^{+/+}$ mice (Fig. 4B). This response was completely absent in preparations obtained from *Trpa1*$^{-/-}$ mice (Fig. 4B). Menthol (300 μM) failed to evoke any significant CGRP immunoreactivity release from rat (Fig. 4A) or mouse dorsal spinal cord (data not shown). Thus, the ability of umbellulone to release CGRP is entirely mediated by TRPA1, and TRPM8 does not contribute to the response. Superfusion with umbellulone or mustard oil (100 μM) increased the outflow of CGRP immunoreactivity from slices of both rat trigeminal ganglia (Fig. 4C and F) and dura mater (Fig. 4D and G), a preparation

Table 1

Pos.	Umbellulone		Umbellulone-Adduct	
	δ_H, mult., *J* in Hz	δ_C, mult[a]	δ_H, mult., *J* in Hz	δ_C, mult.
1		177.8, s		36.5, s
2	5.27, bs	124.1, d	2.43, d, 17.0 1.91, d, 17.0	46.8, t
3		208.1, s		212.0, s
4		40.7, s		49.0, s
5a	1.36, dd, 6.5, 3.2	38.1, t	1.25, dd, 6.5, 6.2	38.3, t
5b	1.12, t, 3.2		1.10, dd, 6.5, 4.5	
6	2.26, dd, 6.5, 3.2	29.0, d	2.04, dd, 6.5, 4.5	20.1, d
7	1.96, hep, 6.9	26.4, d	1.83, hep, 6.9	26.2, d
8	0.96, d, 6.9	20.3, q	0.87, d, 6.9	20.2, q
9	0.92, d, 6.9	19.4, q	0.87, d, 6.9	20.2, q
10	2.07, bs	18.6, q		26.1, q
$-CH_2S$			2.58, t, 7.2	31.9, t
$-CH_2N$			2.68, t, 7.2	41.9, t

^1H (500 MHz) and ^{13}C nuclear magnetic resonance data of umbellulone and its adduct with cysteamine (DMSO-d6).
a Data in $CDCl_3$.
Abbreviations: d = doublet; q = quartet; s = singlet; hep = heptet; dd = doublet of doublets; bs = broad singlet; mult. = multiplet; t = triplet.

enriched with peripheral nerve terminals of trigeminal ganglia neurons. The responses to both umbellulone and mustard oil from both preparations were markedly (> 80%) inhibited by pre-exposure to an elevated capsaicin concentration, which causes selective desensitization of peptidergic sensory neurons (Trevisani *et al.*, 2002), or by superfusion in a calcium-free medium (Fig. 4C, D, F and G). Menthol (300 μM) failed to evoke any significant CGRP immunoreactivity release from slices of rat trigeminal ganglia or dura mater (data not shown). Superfusion with umbellulone (1 mM) or mustard oil (100 μM) increased the outflow of CGRP immunoreactivity from slices of trigeminal ganglia obtained from *Trpa1*$^{+/+}$ mice (Fig. 4E and H). This response was completely absent in preparations obtained from *Trpa1*$^{-/-}$ mice (Fig. 4E and H). Thus, umbellulone evokes a calcium-dependent neurosecretory process from capsaicin-sensitive trigeminal ganglia neurons by a selective TRPA1-dependent mechanism.

Umbellulone evokes a trigeminal acute nocioceptive response

Next, we tested whether umbellulone was able to evoke TRPA1-dependent, acute nocioceptive behaviour in mice. Ocular instillation (5 μl) of the vehicles of umbellulone, mustard oil, menthol or capsaicin caused, during the first 2 min of observation, minimal nocioceptive response (1–2 eye wipings) that was similar in *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice (Fig. 5). Ocular instillation of umbellulone (50–250 nmol) or mustard oil (10–20 nmol) into the mouse eye evoked a concentration-dependent acute nocioceptive behaviour (Fig. 5A and B). Ocular instillation of umbellulone (250 nmol) or mustard oil (20 nmol) caused >15 eye wiping movements in *Trpa1*$^{+/+}$ mice (Fig. 5A and B). In contrast, in *Trpa1*$^{-/-}$ mice the response to umbellulone or mustard oil was practically absent and similar to the response evoked by their respective vehicles (Fig. 5A and B). The robust nocioceptive response produced by capsaicin (1 nmol) was similar in *Trpa1*$^{+/+}$ or *Trpa1*$^{-/-}$ mice (Fig. 5C). Ocular instillation of menthol (25 nmol) evoked eye wiping movements that were similar in *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice (Fig. 5D). These experiments demonstrate that umbellulone elicits an acute nocioceptive response and that, similar to the selective TRPA1 agonist mustard oil, this effect is mostly dependent on TRPA1, most likely expressed in conjunctival trigeminal endings.

Umbellulone increases meningeal blood flow

Activation of peptidergic trigeminal neurons has been associated with vascular responses mainly mediated by CGRP (Brain and Grant, 2004; Sinclair *et al.*, 2010). Baseline meningeal blood flow in rats was neither affected by intravenous infusion (1 ml/kg) of isotonic saline (0.9% NaCl, control of 100% ethanol injection), by vehicles of umbellulone or mustard oil, by intranasal application (50 μl) of vehicles for umbellulone or capsaicin nor by topical application to the cranial window of sodium nitroprusside vehicle (synthetic interstitial fluid, 100 μl) (Fig. 6). Umbellulone (1 μmol/kg, intravenous or intranasal) did not affect systemic blood pressure (data not shown). Umbellulone (0.2, 0.5, 1 μmol/kg, intravenously) increased meningeal blood flow in a dose-dependent manner (Fig. 6B). The response to intravenous umbellulone (1 μmol/kg) was blocked by the CGRP receptor antagonist, BIBN4096BS (1 mg/kg, intravenously) (Doods *et al.*, 2000) and by HC-030031 (100 mg/kg, intragastrally), but was unaffected by capsazepine (4 mg/kg, intraperitoneally) (Fig. 6D). A lower dose of umbellulone (0.5 μmol/kg, intravenously) significantly increased meningeal blood flow, whereas 0.2 μmol/kg of intravenous umbellulone failed to increase meningeal blood flow (Fig. 6B). Mustard oil (1 μmol/kg, intravenously) increased rat meningeal blood flow (Fig. 6E), a response that, similarly to umbellulone, was blocked by BIBN4096BS and HC-030031, but not by capsazepine (Fig. 6E). The increase in meningeal blood flow induced by ethanol (140 μl/kg, intravenously) was inhibited by capsazepine and BIBN4096BS, as previously reported (Nicoletti *et al.*, 2008), but not by HC-030031 (Fig 6F). The increase in blood flow by topical application to the cranial window of the direct vasodilator agent, sodium nitroprusside was unaffected by any antagonist. These data indicate selectivity of the pharmacological interventions (Fig. 6G). Collectively, present results show that umbellulone, via TRPA1 stimulation, produces a CGRP-mediated neurogenic vasodilatation, a response that is considered as a relevant model for migraine (Kurosawa *et al.*, 1995).

Because of its volatile nature, the nasal and airway mucosa is normally exposed to umbellulone, contained in *U. Californica* leaves. To test whether the vascular meningeal response elicited by systemic administration of umbellulone could be recapitulated by nasal delivery of the drug, meningeal blood flow was monitored before and after intranasal administration of umbellulone with doses identical to those used for intravenous administration. Umbellulone by the intranasal route was very effective in increasing meningeal blood flow (Fig. 6A and B). Of note, the intranasal

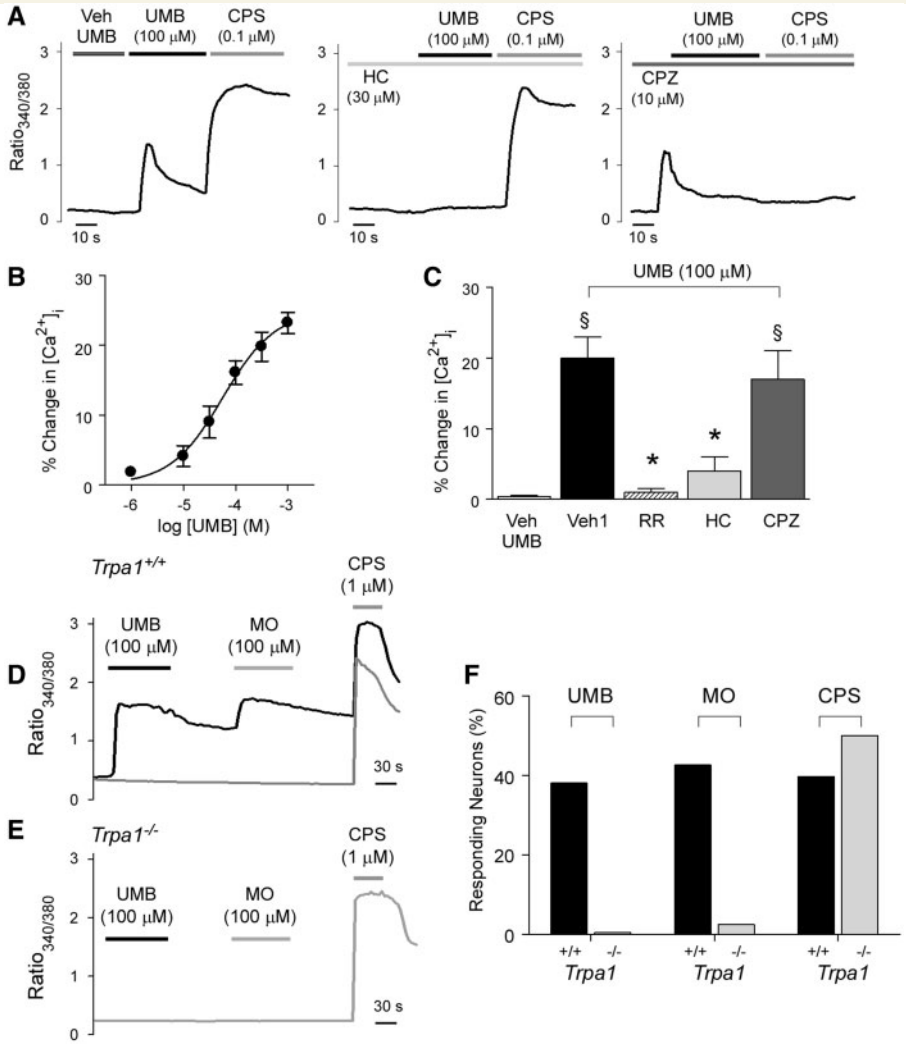

Figure 2 Umbellulone selectively excites the native TRPA1 channel in rodent trigeminal ganglion neurons. (**A**) Representative traces and (**C**) pooled data of $[Ca^{2+}]_i$ mobilization evoked by umbellulone (UMB) and capsaicin (CPS) in rat cultured trigeminal ganglion neurons. $[Ca^{2+}]_i$ mobilization induced by umbellulone in neurons, sensitive to capsaicin is abolished by the unselective, ruthenium red (RR; 3 μM) or selective, HC-030031 (HC; 30 μM), TRPA1 antagonist, and is not affected by the selective TRPV1 antagonist, capsazepine (CPZ; 10 μM). (**B**) Concentration–response curves with a half maximal effective concentration$_s$ of 56.6 ± 8.3 μM of umbellulone in rat trigeminal ganglia neurons. Veh UMB is the vehicle of umbellulone and Veh1 is a combination of vehicles of RR, HC and CPZ. Values are means ± S.E.M. of $n > 24$ neurons. $^{§}P < 0.05$ versus Veh UMB, $^{*}P < 0.05$ versus Veh1. (**D**) Representative traces of the $[Ca^{2+}]_i$ mobilization evoked by umbellulone, mustard oil (MO) and capsaicin in trigeminal neurons isolated from mouse *Trpa1*$^{+/+}$ (black line). Some capsaicin-responding neurons did not respond to the TRPA1 agonist, mustard oil and accordingly to umbellulone (grey line). (**E**) These responses were absent in neurons taken from *Trpa1*$^{-/-}$ mice. (**F**) Percentage of trigeminal neurons responsive to umbellulone, mustard oil and capsaicin in TRPA1 wild-type (*Trpa1*$^{+/+}$; black) or TRPA1-deficient (*Trpa1*$^{-/-}$; grey) mice. Data from >95 neurons.

dose of 0.2 μmol/kg, significantly increased meningeal blood flow (Fig. 6B), whereas when given intravenously, the same dose was ineffective (Fig. 6B). The response evoked by the higher dose of intranasal umbellulone was prevented by systemic administration of BIBN4096BS (1 mg/kg, intravenously) or HC-030031 (100 mg/kg, intragastrally), but remained unchanged after capsazepine (4 mg/kg, intraperitoneally; Fig. 6 A and H). The increase in meningeal blood flow after intranasal application of capsaicin (0.2 μmol/kg) was inhibited by capsazepine and BIBN4096BS, but not by HC-030031, indicating selectivity of the pharmacological interventions (Fig. 6I).

To further investigate the underlying mechanism of the vasodilatatory response evoked by umbellulone we measured the drug plasma levels after both intravenous and intranasally umbellulone administration. The areas under the curve of drug plasma levels resulting from intravenous or intranasal administration of umbellulone (1 μmol/kg) were similar (Fig. 6C). It should be recalled that both intravenous and intranasal umbellulone (1 μmol/kg) produced remarkable and comparable increases in meningeal vasodilatation (Fig. 6B). Administration of a low dose of intravenous umbellulone (0.2 μmol/kg), although yielding detectable drug plasma levels, failed to increase meningeal blood flow (Fig. 6C). In contrast,

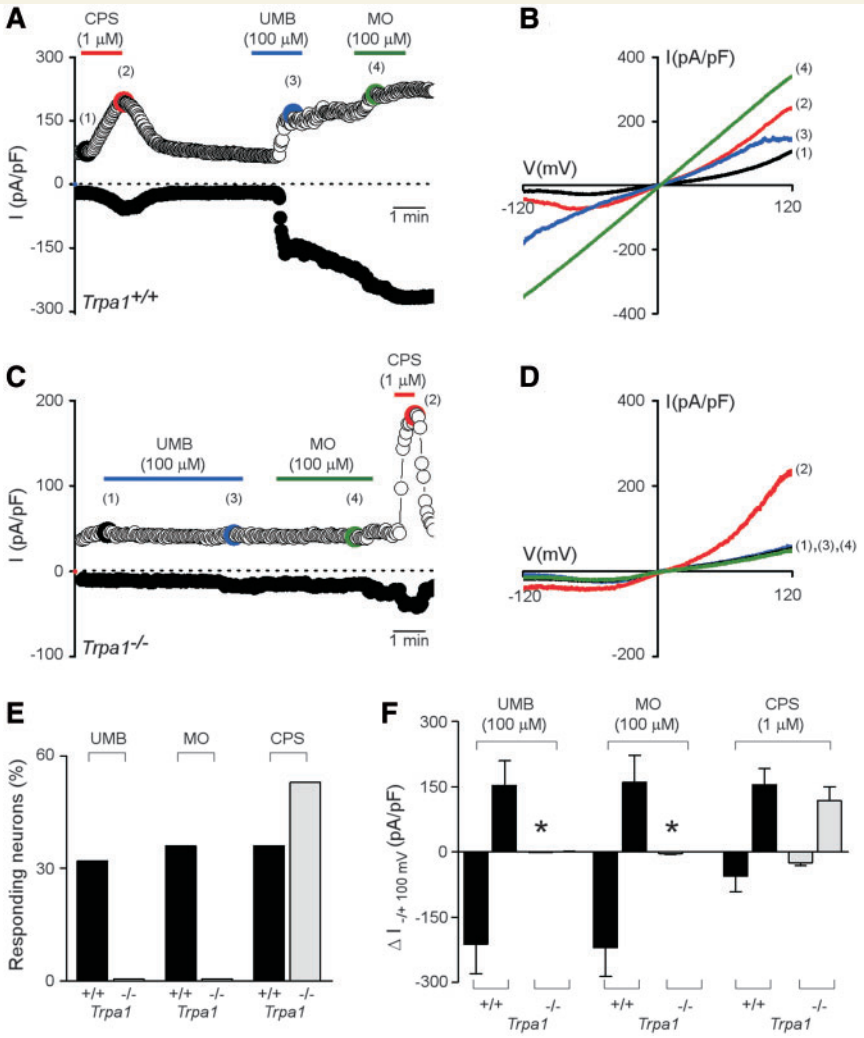

Figure 3 Umbellulone evokes currents consistent with TRPA1 activity in mouse trigeminal ganglion neurons. (**A** and **C**) Time course of trigeminal neuron currents at −/+ 100 mV derived from *Trpa1*$^{+/+}$ (**A**) and *Trpa1*$^{-/-}$ (**C**) mice stimulated with capsaicin (CPS), umbellulone (UMB) and mustard oil (MO). (**B** and **D**) Representative current–voltage relationships obtained from time points indicated by coloured circles (1, basal current; 2, capsaicin; 3, umbellulone; and 4, mustard oil) in trigeminal neuron, illustrating current activation in *Trpa1*$^{+/+}$ trigeminal neuron (**A** and **B**) and no current increase in *Trpa1*$^{-/-}$ trigeminal ganglia neuron (**C** and **D**) after stimulation by umbellulone. (**E**) Comparison of the percentage of cells responding to umbellulone (100 μM), mustard oil (100 μM) and capsaicin (1 μM) from *Trpa1*$^{+/+}$ ($n = 22$) and *Trpa1*$^{-/-}$ mice ($n = 13$). (**F**) Maximal increases in inward-and outward current density at −100 and +100 mV on stimulation with umbellulone, mustard oil and capsaicin in trigeminal neurons taken from *Trpa1*$^{+/+}$ (black) and *Trpa1*$^{-/-}$ (grey) mice. Note that in **F** umbellulone, mustard oil the percentage of responders is zero. *$P < 0.05$ versus umbellulone-*Trpa1*$^{+/+}$ or mustard oil-*Trpa1*$^{+/+}$.

intranasal administration of the low umbellulone dose (0.2 μmol/kg), which yielded undetectable levels in plasma (Fig. 6C) increased blood flow in the meningeal vessels. Thus, it appears that at a low dose, umbellulone is more effective at increasing meningeal blood flow when given by the intranasal than by the intravenous route of administration.

Discussion

The present observation that TRPA1 mediates the trigeminovascular effects of umbellulone is consistent with the recent report (Kunkler *et al.*, 2011) that the intranasal delivery of TRPA1 agonists, mustard oil or acrolein (Bautista *et al.*, 2006), results in meningeal vasodilatation via a TRPA1-dependent mechanism. These findings are also consistent with the epidemiological observation that a series of agents, recently identified as TRPA1 agonists, including formaldehyde, chlorine, ammonium chloride, cigarette smoke and others (McNamara *et al.*, 2007; Andre *et al.*, 2008; Bessac *et al.*, 2008; Fujita *et al.*, 2008), have long been known to be triggers of migraine attacks in susceptible individuals (Courteau *et al.*, 1994; Peatfield, 1995; Wantke *et al.*, 2000; Irlbacher and Meyer, 2002; Kelman, 2007). Chemical inducers of migraine or cluster headache are often compounds with vasodilating properties

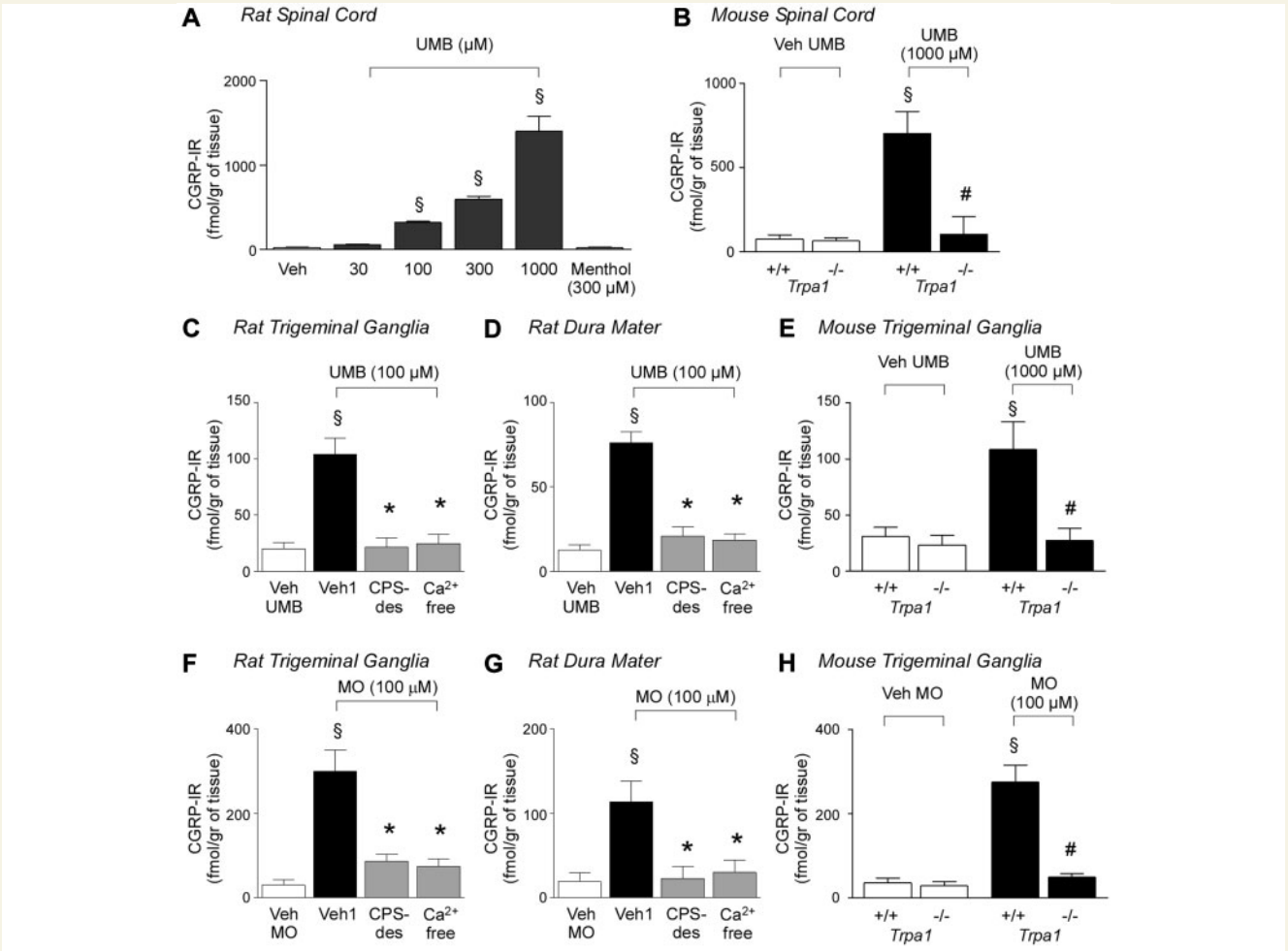

Figure 4 Umbellulone releases CGRP from rodents spinal cord, trigeminal ganglia and meningeal nerve terminals. (**A**) Umbellulone (UMB) increases the outflow of calcitonin gene-related peptide immunoreactivity (CGRP-IR) from slices of rat spinal cord in a concentration-dependent manner. Menthol fails to evoke any significant increase in CGRP immunoreactivity outflow. (**B**) Umbellulone releases CGRP immunoreactivity from dorsal spinal cord slices obtained from *Trpa1*$^{+/+}$ mice, an effect completely absent in preparations taken from *Trpa1*$^{-/-}$ mice. Umbellulone and the selective TRPA1 agonist, mustard oil (MO) elicit a CGRP release from rat trigeminal ganglia (**C** and **F**) or dura mater (**D** and **G**), which contain terminals of trigeminal nerves. CGRP immunoreactivity release evoked by both, umbellulone and mustard oil, is abolished by capsaicin desensitization (CPS-des) or calcium removal (Ca^{2+}-free). Both umbellulone and mustard oil evoke CGRP immunoreactivity release from trigeminal ganglia taken from *Trpa1*$^{+/+}$ mice, an effect completely absent in preparations taken from *Trpa1*$^{-/-}$ mice (**E** and **H**). Veh contains 0.3% DMSO. Veh1 is a combination of vehicles of the various treatments. Values are mean ± SEM of $n = 5$ experiments. $^{§}P < 0.05$ versus respective vehicles, $^{\#}P < 0.05$ versus umbellulone-*Trpa1*$^{+/+}$ or mustard oil-*Trpa1*$^{+/+}$ and $^{*}P < 0.05$ versus Veh1.

(Lance, 1969), such as nitric oxide or ethanol. In particular, these two agents have been identified as capable of releasing CGRP from sensory nerve terminals (Strecker *et al.*, 2002; Nicoletti *et al.*, 2008) and ethanol has been identified as a TRPV1 agonist (Trevisani *et al.*, 2002), which, by this mechanism, releases CGRP and increases meningeal blood flow (Nicoletti *et al.*, 2008). Thus, ethanol and umbellulone, acting on two different molecular targets, TRPV1 and TRPA1, respectively, stimulate trigeminal nociceptors to release CGRP.

Subpopulations of TRPA1-expressing primary sensory neurons, in addition to TRPV1, may also express other TRP channels including TRPV2, TRPV3 and TRPV4 (Caterina *et al.*, 1997, 1999; Xu *et al.*, 2002; Alessandri-Haber *et al.*, 2003). TRPM8 is also expressed in some somatosensory neurons, which however, do not appear to be of peptidergic nature (Bhattacharya *et al.*, 2008). All these channels could, in principle, contribute to neuronal excitation and CGRP release by umbellulone. However, experiments with recombinant TRPV1 excluded the possibility that in the high micromolar range, umbellulone exerts activity on this channel, which is abundantly co-expressed with TRPA1 in peptidergic sensory neurons (Cavanaugh *et al.*, 2011). Similar findings were obtained by pharmacological and genetic approaches both in rat and mouse trigeminal ganglia neurons, where calcium and electrophysiologic responses by umbellulone (up to 100 μM) were completely dependent on the expression of TRPA1. Thus, TRP channels, other than TRPA1, do not contribute substantially

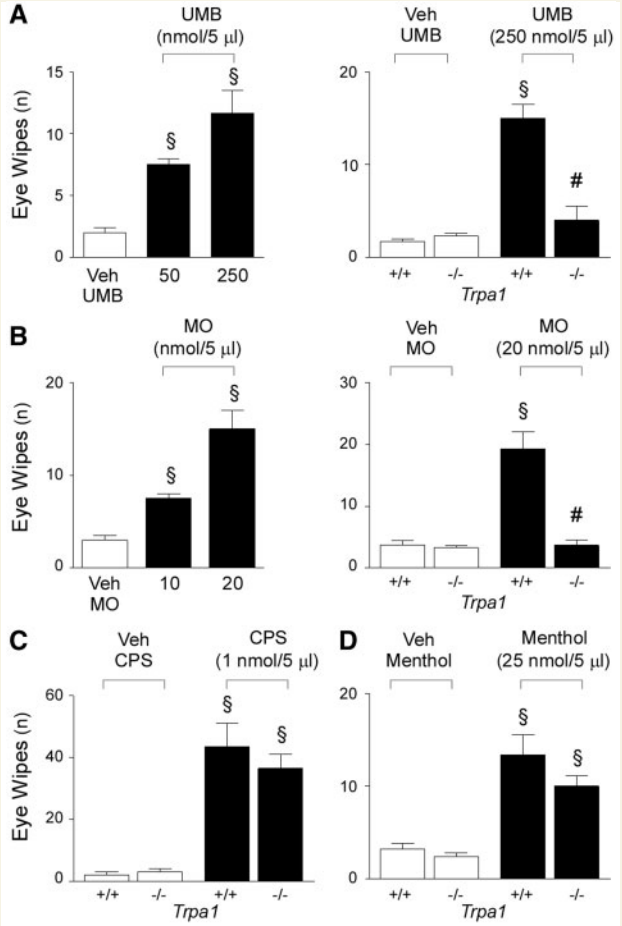

Figure 5 Umbellulone induces an acute nocioceptive behaviour in mice via TRPA1 activation. Right eyes of mice were instilled with umbellulone (UMB) mustard oil (MO), capsaicin (CPS), menthol or respective vehicles and total number of eye wiping movements after the instillation was recorded for a 2 min period. (**A** and **B**) umbellulone and mustard oil cause an acute nocioceptive response in a dose-dependent manner. Responses to umbellulone and mustard oil, observed in *Trpa1*$^{+/+}$ mice, are completely absent in *Trpa1*$^{-/-}$ mice. (**C** and **D**) In contrast, capsaicin and menthol induce in *Trpa1*$^{+/+}$ mice an acute response that is maintained in *Trpa1*$^{-/-}$ mice. Values are mean ± SEM of at least $n \geqslant 6$ *Trpa1*$^{+/+}$ or *Trpa1*$^{-/-}$ mice. $^{\S}P < 0.05$ versus respective vehicles, $^{\#}P < 0.05$ versus umbellulone-*Trpa1*$^{+/+}$ and mustard oil-*Trpa1*$^{+/+}$.

to neuronal excitation by umbellulone. Stimulation of the irritant TRPA1 receptor *in vivo* has been previously reported to produce nocioceptive behaviour in rodents (Trevisani *et al.*, 2002; Bautista *et al.*, 2006). Failure of conjunctival application of umbellulone to evoke wiping behaviour in *Trpa1*$^{-/-}$ mice strengthens the hypothesis that the irritant action of the compound in trigeminal afferents is principally mediated by TRPA1.

Umbellulone is a conjugated enone, but its β,β-dialkyl substitution pattern is expected to inhibit reaction with thiol groups because of both steric (increased hindrance) and electronic (decreased electrophilicity) effects (LoPachin *et al.*, 2008). It was therefore surprising to discover that umbellulone reacted in a 'click-fashion' (quickly and quantitatively) with the biogenic thiol cysteamine, affording a Michael adduct (Fig. 1A). No reaction occurred with the closely related, but not irritant terpenoid enones (+)-verbenone and (+)-piperitone, suggesting that Michael acceptor behaviour could underlie the noxious sensory properties of umbellulone. In accordance with these chemical features, calcium and electrophysiologic experiments indicate that solely the irritant compound, umbellulone, but not the non-irritants, verbenone and piperitone, stimulated the TRPA1 channel in nociceptors.

TRPA1-expressing sensory neurons synthesize (Story *et al.*, 2003; Bhattacharya *et al.*, 2008) and release neuropeptides, including tachykinins and CGRP, from their central and peripheral terminals. In peripheral tissues, including extra- and intracranial vessels, tachykinins and CGRP produce a series of responses, collectively referred to as neurogenic inflammation, which are mainly represented by plasma protein extravasation in post-capillary venules and arterial vasodilatation (Geppetti and Holzer, 1996). The original hypothesis that neurogenic inflammation could be the main contributing factor to migraine mechanism (Moskowitz, 1984) has been strengthened by the paramount observation that two chemically unrelated CGRP receptor antagonists have shown clinical efficacy in migraine (Olesen *et al.*, 2004; Ho *et al.*, 2008*a*). These clinical findings are corroborated by neurochemical evidence that CGRP is released *in vitro* from trigeminal nerve endings of human tissues (Geppetti *et al.*, 1987) and that CGRP is increased in the jugular blood of patients during migraine and cluster headache attacks (Goadsby *et al.*, 1990; Goadsby and Edvinsson, 1994; Lassen *et al.*, 2002; Villalon and Olesen, 2009).

Calcium-dependence and capsaicin-sensitivity of umbellulone-evoked release from both trigeminal ganglia and nerve endings within meningeal structures indicate that umbellulone promotes a secretory process of CGRP from trigeminal nociceptors. Umbellulone failed to release CGRP from the dorsal spinal cord of *Trpa1*$^{-/-}$ mice. Thus, expression of TRPA1 on terminals of nocioceptive neurons is necessary and sufficient to evoke the release of CGRP by umbellulone. In addition, we provide pharmacological and genetic evidence that the *in vitro* CGRP release and the increased blood flow in rat meningeal arterial vessels by umbellulone are both mediated by a common mechanism that results from TRPA1 activation. Activation of perivascular trigeminal nerve endings in meningeal vessels and the resulting CGRP-dependent vasodilatation have been proposed as a predictive model of migraine (Kurosawa *et al.*, 1995). Therefore, our findings suggest that a TRPA1- and CGRP-dependent pathway contributes to the mechanism operated by umbellulone to trigger migraine, and possibly cluster headache.

We recently reported (Benemei *et al.*, 2009) the case of a patient who suffered cluster headaches for 20 years and who, 10 years after the last headache attack, experienced a cluster headache-like attack (accompanied by ipsilateral tearing and nasal obstruction) on the same side as the previous attacks, when he was exposed to a California bay laurel tree. Pain and autonomic symptoms were preceded by a cold sensation in the ipsilateral nostril. Controversial results have been reported regarding the role of TRPA1 as a sensor of cold sensation or cold

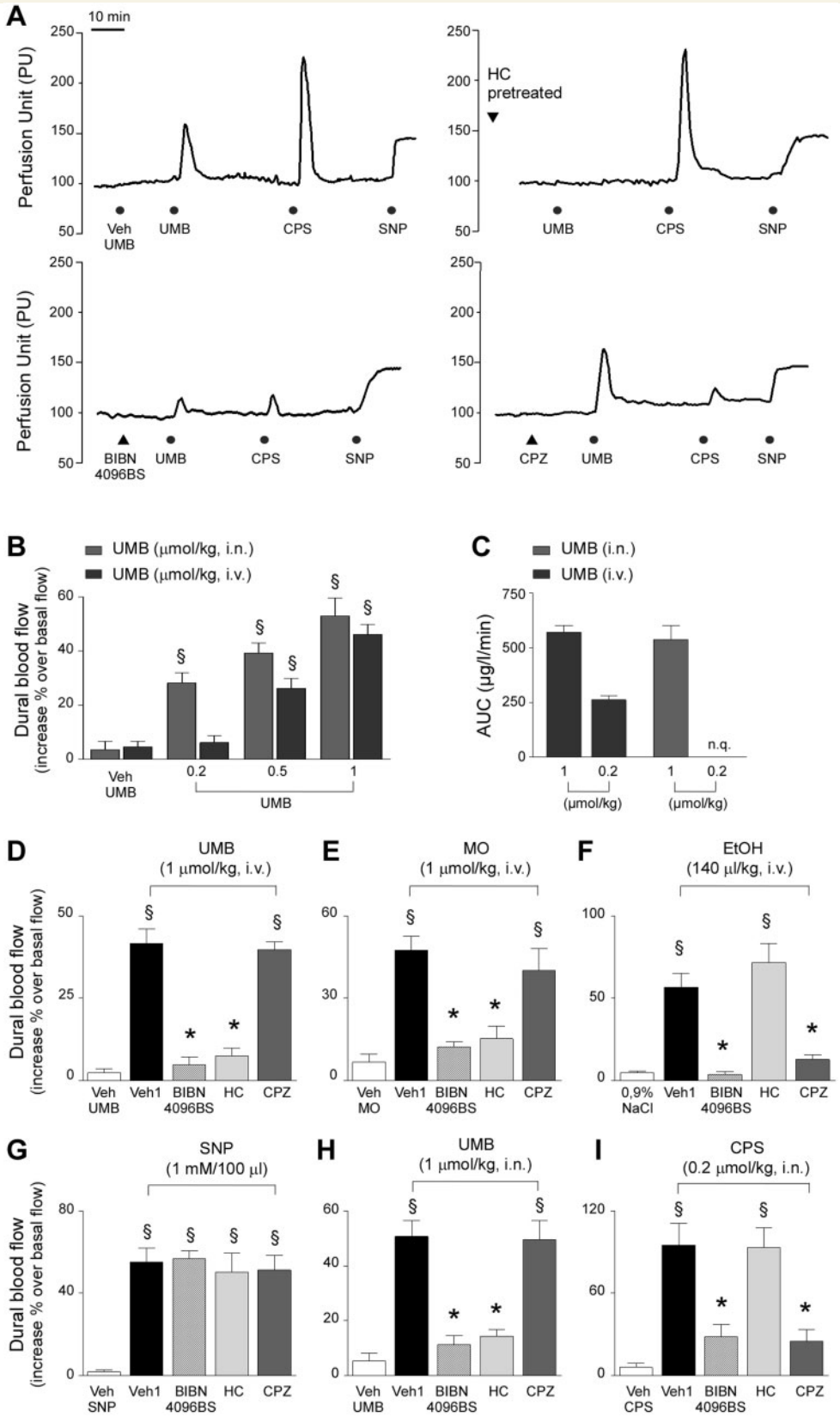

Figure 6 Umbellulone evokes an increase in meningeal blood flow in rats. (**A**) Typical tracings (expressed as perfusion units, PU) of the effect of intranasal (i.n.) administration of umbellulone (UMB). (**B**) intravenous (i.v.) or intranasal (i.n.) administration of umbellulone increases meningeal blood flow in a dose-dependent manner. (**C**) The area under the curve (AUC) (calculated over 1–15 min) of the plasma level of umbellulone after intravenous or intranasal administration. Umbellulone plasma level, after intranasal administration of the low dose of umbellulone, was not quantifiable (n.q.). (**D**) The vasodilatation evoked by the highest dose of umbellulone (intravenously)

(continued)

hypersensitivity (Story *et al.*, 2003; Karashima *et al.*, 2009; Salazar *et al.*, 2011). We showed that at greater than micromolar concentrations of umbellulone may stimulate the cold receptor TRPM8. Thus, we cannot exclude that TRPM8, stimulated by the relatively high concentrations of umbellulone yielded in the nasal mucosa after inhalation of the *U. californica* scent, contributes to the cold sensation described by the patient with cluster headache (Benemei *et al.*, 2009). However, present findings, corroborated by menthol failure to release CGRP, conclusively exclude any contribution of TRPM8, which is not apparently expressed by peptidergic nociceptors (Bhattacharya *et al.*, 2008), to the neurovascular response evoked by umbellulone.

As observed with other TRPA1 or TRPV1 stimulants (Nicoletti *et al.*, 2008; Kunkler *et al.*, 2011) the increase in meningeal blood flow evoked by umbellulone was completely inhibited by CGRP receptor blockade. Because, with the exclusion of the gut, CGRP expression in the PNS is strictly confined to a subset of nociceptive neurons, the contribution to the vascular response evoked by umbellulone by a classical reflex arch arrangement (with an efferent parasympathetic or sympathetic arm, where the released neurochemical substance cannot be CGRP), is unlikely. We found that intravenous or intranasal delivery of a high umbellulone dose resulted in comparable drug plasma levels and produced similar increases in meningeal blood flow. We also found that intravenous administration of a low dose of umbellulone resulted in measurable drug plasma levels, which however, failed to increase meningeal blood flow; whereas the same dose given by the intranasally route increased meningeal blood flow, but did not yield measurable drug levels in plasma. One possible interpretation of these findings is that umbellulone, if the applied dose is sufficiently high, diffuses from nasal mucosa into the systemic circulation to reach the perivascular sensory nerve endings of meningeal vessels, where it activates TRPA1 and releases CGRP. The highly lipophilic nature of umbellulone might explain its quick intranasal absorption, a feature which has been demonstrated also for other essential oil constituents (Guo *et al.*, 2009). Alternatively, as recently proposed for other TRPA1 agonists (Kunkler *et al.*, 2011), umbellulone may stimulate TRPA1 within trigeminal afferents of the nasal mucosa, thus initiating propagated action potentials, which via non-classical reflex pathways (Finger and Bottger, 1993; Meggs, 1995; Ulrich-Lai *et al.*, 2001) eventually result in meningeal vasodilatation. However, this latter hypothesis requires additional, and hitherto lacking, proof. For technical reasons, present meningeal blood flow experiments have been limited to intranasal administration, where absorption occurs only through the nasal mucosa. However, it cannot be overlooked that exposure to *U. californica* scent implies that a substantial proportion of inhaled umbellulone may reach the lower airways and lungs, from which it can diffuse into the blood stream. In this case, the contribution of nasal afferents to the neurovascular meningeal response could be less pronounced.

In conclusion, our observations identify umbellulone, via its selective TRPA1-agonism, as a trigeminovascular stimulator, and provide a possible rationalization for the headache-inducing properties of California bay laurel. Present data also suggest that a similar pathway may represent the underlying mechanism responsible for headache crises triggered in sensitive people by a series of compounds present in environmental pollutants and botanical perfumes/odours (Blau and Solomon, 1985; Kelman, 2007; Friedman and De ver Dye, 2009).

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Figure 6 Continued
is blunted by the selective TRPA1 antagonist, HC-030031 (HC; 100 mg/kg, intragastrally) and by the CGRP receptor antagonist, BIBN4096BS (1 mg/kg, intravenously), but is unaffected by the TRPV1 antagonist, capsazepine (CPZ; 4 mg/kg, intraperitoneally). (**E**) Similarly, mustard oil (MO, intravenously) increases rat meningeal blood flow and the response is blocked by BIBN4096BS and HC-030031, but not by capsazepine. (**F**) The increase in blood flow induced by ethanol (EtOH, intravenously) is inhibited by capsazepine and BIBN4096BS but not by HC-030031. (**G**) HC-030031, capsazepine or BIBN4096BS does not affect the vasodilatation evoked by sodium nitroprusside (SNP, topical administration). (**H**) The vasodilatation evoked by the nasal administration (50 µl, intranasally) of the highest dose of umbellulone is blunted by HC-030031 (100 mg/kg, intragastrally) and by BIBN4096BS (1 mg/kg, intravenously), but is unaffected by capsazepine (4 mg/kg, intraperitoneally). (**I**) The effect of intranasal administration of capsaicin is blocked by capsazepine or BIBN4096BS, but is not affected by HC-030031. Veh1 is a combination of vehicles of the various antagonists. Values are mean ± S.E.M. of at least $n = 8$–10 rats. $^{\S}P < 0.05$ versus respective vehicles and $^{*}P < 0.05$ versus Veh1.

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