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ACTIVATION OF TRPA1 ON DURAL AFFERENTS: A POTENTIAL MECHANISM OF HEADACHE PAIN

Rebecca M. Edelmayer^a, Larry N. Le^a, Jin Yan^a, Xiaomei Wei^a, Romina Nassini^b, Serena Materazzi^b, Delia Preti^c, Giovanni Appendino^d, Pierangelo Geppetti^b, David W. Dodick^e, Todd W. Vanderah^a, Frank Porreca^a, and Gregory Dussor^a

^aDepartment of Pharmacology, College of Medicine, University of Arizona, Tucson, AZ, USA

^bDepartment of Preclinical & Clinical Pharmacology & Headache Center, University of Florence, Florence, Italy

^cDepartment of Pharmaceutical Chemistry, University of Ferrara, Ferrara, Italy

^dDepartment of Chemical, Alimentary, Pharmaceutical and Pharmacological Sciences, University of Eastern Piedmont, Novara, Italy

^eDepartment of Neurology, Mayo Clinic Arizona, Scottsdale, AZ, USA

Abstract

Activation of transient receptor potential ankyrin-1 (TRPA1) on meningeal nerve endings has been suggested to contribute to environmental irritant-induced headache but this channel may also contribute to other forms of headache such as migraine. The preclinical studies described here examined functional expression of TRPA1 on dural afferents and investigated whether activation of TRPA1 contributes to headache-like behaviors. Whole-cell patch-clamp recordings were performed *in vitro* using two TRPA1 agonists, mustard oil (MO) and the environmental irritant umbellulone (UMB), on dural-projecting trigeminal ganglion neurons. Application of MO and UMB to dural afferents produced TRPA1-like currents in approximately 42% and 38% of cells, respectively. Using an established *in vivo* behavioral model of migraine-related allodynia, dural application of MO and UMB produced robust time-related tactile facial and hindpaw allodynia that was attenuated by pretreatment with the TRPA1 antagonist HC-030031. Additionally, MO or UMB were applied to the dura and exploratory activity was monitored for 30 minutes using an automated open-field activity chamber. Dural MO and UMB decreased the number of vertical rearing episodes and the time spent rearing in comparison to vehicle treated animals. This change in activity was prevented in rats pretreated with HC-030031 as well as sumatriptan, a clinically effective anti-migraine agent. These data indicate that TRPA1 is expressed on a substantial fraction of dural afferents and activation of meningeal TRPA1 produces behaviors consistent with those seen in patients during migraine attacks. Further, they suggest that activation of meningeal TRPA1 via endogenous or exogenous mechanisms can lead to afferent signaling and headache.

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ADDRESS CORRESPONDENCE TO: Gregory Dussor, PhD, Department of Pharmacology, University of Arizona HSC, Tucson, AZ 85724, 520-626-6726 (voice), 520-626-2204 (fax), dussorg@email.arizona.edu.

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Keywords

migraine; TRPA1; mustard oil; umbellulone; allodynia; headache; dura

1. Introduction

The prevalence of migraine and other primary headache disorders in the general population is quite common, yet the mechanisms that initiate the headache pain associated with the attack are poorly understood. Prior work using preclinical animal models indicates that trigeminal nociceptors innervating the cranial dura are sensitive to chemical and mechanical stimulation suggesting that activation of these fibers may trigger the headache. However, the cellular mechanisms by which extracellular stimuli activate dural nociceptors are largely unknown.

TRPA1 is a non-selective cation channel expressed in sensory neurons, including those of the trigeminal branch [27] as well as neurons in the airways [23,39,41,44]. TRPA1 is thought to mediate neuronal responses to a series of byproducts of oxidative and nitrative stress, such as nitrooleic acid [51], 4-hydroxynonenal [53], and reactive prostaglandins [35] as well as many exogenous ligands including pungent plant derivatives like isothiocyanates [27], cinnamaldehyde [2], and allicin [7]. Additionally, environmental irritants such as formaldehyde [37], acrolein [6], chlorine [9] and cigarette smoke extract [1] have been shown to activate the channel. TRPA1 has also been linked to mechanotransduction in sensory afferents [14,29,34] and has been proposed as a noxious cold sensor, although this remains controversial [20,31]. A substantial amount of preclinical work has since established a primary role for TRPA1 in pain transduction [4]. New research has demonstrated that a gain-of-function mutation in TRPA1 results in a heritable episodic pain syndrome [32] clinically validating this channel in human pain conditions. Thus, TRPA1 has become a prime target for pain therapeutics [4].

Recent evidence proposes that TRPA1 signaling may contribute to the headache associated with a variety of primary headache disorders. Clinical reports have indicated that inhalation of TRPA1 irritants can trigger migraine headache with a high frequency in susceptible individuals [15,19,28,45,60]. Exposure to the scent of *Umbellularia californica* (aka “the headache tree”) can produce cluster-like headache attacks in humans [8] and the principal volatile component of the plant, umbellulone, was recently identified as a TRPA1 agonist [42,59]. Notably, activation of trigeminal TRPA1 via exposure to environmental irritants and headache provoking agents contributes to meningeal vasodilation and calcitonin gene-related peptide (CGRP) release in rodents [42], two phenomena implicated as components of migraine pathophysiology.

Although evidence suggests that TRPA1 may contribute to activation of headache-related signaling pathways, pain-like behavioral responses following TRPA1-dependent afferent signaling from the meninges have not been demonstrated. The present studies employed a known TRPA1 agonist, allyl isothiocyanate, and a recently discovered TRPA1 environmental irritant, umbellulone, to investigate the contribution of TRPA1 to nociceptive signaling from the meninges in order to determine whether this channel could play a role in headache pathophysiology. By using a combination of *in vitro* electrophysiology, a validated model of headache-related allodynia, and a novel behavioral activity model this work provides evidence that identified dural afferents express TRPA1 and activation of this channel within the meninges produces pain-like behavioral responses consistent with headache. Thus, these preclinical findings support a role for TRPA1-dependent activity in migraine or other types of environmental irritant-induced headache pain.

2. Methods

2.1. Animals

Adult, male Sprague Dawley rats (200–300g, Harlan) were maintained in a climate-controlled room on a 12 hr light/dark cycle (light on 7am, light off 7pm) with food and water *ad libitum*. All procedures were performed in accordance with the policies and recommendations of the IASP, the NIH guidelines for the handling and use of laboratory animals, and were approved by the IACUC of the University of Arizona.

2.2. Chemicals

Fluorogold (Fluorochrome, LLC) was dissolved in synthetic-interstitial fluid (pH 7.4, 310 Osm) to 4%. Umbellulone oil (UMB) was isolated at the Department of Chemical, Alimentary, Pharmaceutical and Pharmacological Sciences of the University of Eastern Piedmont as previously described [42]. Allyl isothiocyanate (AITC; Mustard oil or MO; Sigma) and UMB were dissolved in DMSO to 10 mM as stock solutions and diluted to their final 100 μ M concentrations with external bath solution for all patch-clamp experiments. The concentration of MO employed is consistent with previously published literature for selective activation of the TRPA1 receptor on rat sensory neurons [18,33]. The final DMSO concentration never exceeded 0.1% for patch-clamp experiments. For the behavioral experiments, 10, 30, and 100% UMB and 10% MO were prepared in mineral oil. MO was prepared at a concentration known to induce dural nociceptor activation in anesthetized animals [5]. The vehicle injection was 100% mineral oil (Min oil). The TRPA1 antagonist HC-030031 was synthesized at the Department of Pharmaceutical Chemistry of the University of Ferrara as previously described [40]. HC-030031 was dissolved in a 0.5% methyl cellulose solution to the final dose of 100 mg/kg for p.o. administration as formerly reported [22]. Sumatriptan succinate (a gift from Dr. John Andrews, Neuraxon) was prepared in saline to the final dose of 0.6 mg/kg for s.c. administration as previously described [21]. The dose of HC-030031 used here is consistent with that used previously to block TRPA1-mediated nociceptive responses [17,20,22,38,47] and the dose of sumatriptan is consistent with the doses known to elicit antinociception in rodents [11,21,54].

2.3. Surgical preparation

2.3.1. Tracer injection—Dural afferents were identified as previously described [56,58]. Briefly, seven days prior to sacrifice animals were anesthetized with a combination of ketamine and xylazine (80 mg/kg and 12 mg/kg, Sigma). Under a dissecting microscope, two holes (3 mm diameter) were made in the skull leaving a thin layer of bone at the bottom of the hole. Fine forceps were used to carefully remove the remaining bone to expose but not damage the dura. Fluorogold (FG, 5 μ l/hole; 4% in synthetic-interstitial fluid) was then applied onto the dura. Gelfoam was placed in each hole to increase dye absorption and prevent spread of the tracer. Holes were covered with bone wax and the incision was closed with silk sutures. Postoperatively, animals received gentamicin (8 mg/kg). The dura was evaluated at the time of sacrifice and only animals with intact undamaged dura were used for further experiments.

2.3.2. Dura cannulation—Dura cannulas were implanted as previously described [21,56,58]. Briefly, animals were anesthetized as described above and an incision was made to expose the skull. A 1 mm hole was made in the skull (2 mm left of the sagittal suture and 2 mm anterior to the lambdoid suture) with a hand-drill (Plastics One) to carefully expose the dura. A custom guide cannula (Plastics One) was inserted into the hole and sealed into place with glue. Two additional 1 mm holes were made in the parietal bones to receive stainless-steel screws (Small Parts), and dental acrylic was used to fix the cannula to the screws. A dummy cannula (Plastics One) was inserted to ensure patency of the guide

cannula. Postoperatively, animals received gentamicin (8 mg/kg). Rats were housed separately and allowed 6–8 days of recovery before behavioral testing.

2.4. Cell culture

Seven days following FG application, trigeminal ganglia (TG) were removed, enzymatically treated, and mechanically dissociated as previously described [56,58]. Rats were anesthetized with isoflurane (Phoenix Pharmaceuticals) and sacrificed by decapitation. Care was taken during all culture procedures to minimize light exposure to the cells. TG were dissected into ice-cold Hanks balanced-salt solution (divalent free). Ganglia were incubated in 20 U/ml Papain (Worthington) followed by 3 mg/ml Collagenase Type II (Worthington), triturated, and plated on poly-d-lysine (Becton Dickinson) and laminin (Sigma) coated plates. Cells were cultured in a room-temperature, humidified chamber in Liebovitz L-15 medium supplemented with 10% FBS, 10 mM glucose, 10 mM HEPES, 50 U/ml penicillin/streptomycin, and 50 ng/ml NGF. Cells were used within 24 hrs post plating.

2.5. Electrophysiology

Whole cell patch-clamp experiments were performed at room temperature (~26 °C) on isolated rat TG using a MultiClamp 700B patch-clamp amplifier and pClamp 10 acquisition software (Axon Instruments). A Nikon TE2000-S Microscope equipped with a mercury arc lamp (X-Cite® 120) was used to identify FG-labeled dural afferents. Voltage-clamp experiments were performed at a holding potential of –70 mV, and recordings were sampled at 5 kHz and filtered at 1 kHz (Digidata 1322A, Axon Instruments). Data were analyzed using Clampfit 10 (Molecular Devices) and Origin 8 (OriginLab). Current density (pA/pF) was analyzed, and the current magnitude was quantified using peak current amplitude from the first application of MO or UMB in all experiments divided by the cell capacitance measured by the amplifier circuitry; data are presented as Mean ± SEM. Pipettes (OD: 1.5 mm, ID: 0.86 mm, Sutter Instrument) were pulled using a P-97 puller (Sutter Instrument) and heat polished to 2.5–4 MΩ resistance using a microforge (MF-83, Narishige). Series resistance was typically <7 MΩ and was compensated 60%. Pipette solution contained (in mM) 140 KCl, 11 EGTA, 2 MgCl₂, 10 NaCl, 10 HEPES, 2 MgATP, 0.3 Na₂GTP, 1 CaCl₂, pH 7.3 (adjusted with *N*-methyl glucamine), and was ~320 mosM. External solution contained (in mM) 135 NaCl, 2 CaCl₂, 1 MgCl₂, 5 KCl, 10 Glucose, 10 HEPES, pH 7.4 (adjusted with *N*-methyl glucamine), and was ~320 mosM. Solutions were rapidly changed during recordings using gravity-fed flow pipes positioned near the cell and controlled by computer driven solenoid valves, the solution exchange time was ~20 ms. Currents were only observed when solutions were switched from external bath to MO- or UMB-containing solution using our drug application system. A minimum of a 4 min washout period was used for recovery between applications of TRPA1 agonist. The order of agonist application was varied during sampling (i.e. MO preceding UMB or vice versa). A cutoff of 20 pA was selected as a minimum amplitude for response for all the experiments based on preliminary studies. Dural afferents were sampled from a total of 22 animals labeled with fluorogold tracer.

2.6. Behavioral testing

2.6.1. Tactile testing—Allodynia was evaluated using behavioral techniques previously validated and described [21,56,58]. Briefly, rats were acclimated to suspended Plexiglas chambers with a wire mesh bottom for 1 hr before treatments and testing. Ten µl of vehicle or testing solution was injected through an injection cannula (Plastics One) cut to fit the dura guide cannula. Some animals were pre-treated with systemic test solutions 1 hr before dura cannula injection. Withdrawal thresholds for the face and hind-paws were determined at 1 hr intervals for 5 hours following dural administration. Pilot experiments determined that all

animals returned to baseline thresholds by 6 hours post injection despite some animals still showing slight allodynia at the 5 hour time point. A behavioral response to von Frey filaments applied to the periorbital region, was indicated by sharp withdrawal of the head. Paw withdrawal thresholds were determined by applying von Frey filaments to the plantar aspect of the hind-paws, and a response was indicated by withdrawal of the paw. The withdrawal thresholds were calculated by the Dixon up-down method. Maximum filament strengths were 8 and 15 g for the face and hind-paws, respectively.

2.6.2. Activity testing—Activity testing was conducted using behavioral techniques similar to those previously described [36,49,55] with the following adaptations. Rats were acclimated to the activity testing room for 1 hr before treatments and testing. Animals were not habituated to the activity chambers before the testing day to simulate a novel environment. Ten μ l of vehicle or testing solution was injected as described above. Rats were immediately placed into individual activity chambers (41 cm long $\times$ 41 cm wide $\times$ 39 cm high, Tru Scan Photobeam System; Coulbourn Instruments) following the dura injection. Each chamber contained two photobeam sensor rings that measured movement in three dimensions. One ring, located near the floor of the chamber, sensed horizontal movement of the animal. For the purposes of these studies, the horizontal beam sensor calculated the total movement time, the total movement distance, the velocity, and total rest time of the animal in the horizontal plane. The second ring was elevated to a consistent height above the chamber floor in order to record vertical movement of the animal, i.e. rearing behavior. The vertical beam sensor calculated the total number of times any part of the animal enters the vertical plane (# of vertical entries) and the total time spent in the vertical plane (Time; sec). Locomotor/exploratory activity was recorded and analyzed using Tru Scan software v.2.01 (Coulbourn Instruments) for a total time of 30 min. Some animals were pretreated systemically (s.c.) with sumatriptan succinate 1 hr before dura cannula injection and activity testing. Other groups were pretreated systemically (p.o.) with HC-030031 2 hrs before dura injection and activity testing. Pilot studies indicated that the large volume necessary for oral administration (approximately 3 ml) had an effect on the rearing behavior that was unrelated to the test compound, as decreased exploratory rearing behaviors were observed in animals receiving p.o. vehicle pretreatment 1 hr prior to testing in comparison to animals treated 2 hrs before dura injection (data not shown). Because this volume effect was less robust with longer time between pretreatment and testing, 2 hrs was used as a pretreatment time point for HC-030031 activity testing.

2.6.3. Locomotor coordination testing—The effects of dural application of TRPA1 agonists and vehicle on locomotor coordination were assessed using a rotarod (Rotamex 4/8; Columbus Instruments). Animals with dural cannulas were trained to walk on the rotating rod and were required to stay on the rod for 180 seconds at a constant speed of 10 rpm to pass a baseline measurement test used to preclude preexisting locomotor deficits. Following baseline testing, rats received 10 μ l of vehicle or testing solution as described above. Fifteen minutes post dural injection rats were tested for coordination deficits. The rats were challenged to again walk for 180 seconds at 10 rpm. Any decrease in the time spent walking on the rod from baseline would be indicative of a decrease in locomotor coordination.

2.7. Data analysis

All electrophysiological data are presented as cell counts or as a percentage of TRPA1-expressing neurons. All behavioral data are presented as means $\pm$ SEM. Allodynia studies were analyzed among groups and across time using two-factor ANOVA. Some allodynia data were converted to area over the time-effect curve to allow for analysis of multiple treatment groups. Activity data were evaluated using either an unpaired t-test for continuous data (time analysis of vertical rearing behavior) or the Mann-Whitney test for non-

parametric ordinal data (counts of vertical entries). Significance was set at $p < 0.05$ for all data analysis.

3. Results

3.1. Cultured dural afferents generate TRPA1-like currents

Patch clamp electrophysiology was performed on cultured TG neurons taken from rats in which fluorogold was previously applied to the dura. Only retrogradely-labeled cells (i.e. fluorogold-positive) were selected for recording. Figure 1A illustrates an example of current evoked from a representative dural afferent following a 2 min step from extracellular bath to 100 μM MO. Additionally, in MO positive dural afferents, activation of TRPA1 receptors produced action potential firing in 4 out of 9 cells (Fig. 1B) indicating that MO is capable of producing firing in dural afferents. Figure 1C illustrates an example of MO and UMB currents evoked on the same representative dural afferent using a 2 min step from extracellular bath to the indicated TRPA1 agonist. Current amplitudes ranged from 20 pA to 2400 pA and 25 pA to 340 pA following MO and UMB application, respectively. The calculated dural afferent mean current density in response to MO was 14.45 ± 1.91 pA/pF ($N = 50$), which is consistent with previous studies using 100 μM MO to stimulate sensory neurons [18]. The calculated dural afferent mean current density for UMB was 1.71 ± 0.28 pA/pF ($N = 20$). The current amplitudes and current densities reported here are from the first application of MO or UMB to the cell. It was observed that subsequent applications produced currents of variable amplitudes, i.e. sometimes smaller or larger than the first application, which may be due to stimulus-induced trafficking of the TRPA1 channel to the plasma membrane and increased functionality of the receptor [46] or desensitization on subsequent applications. Among 119 dural afferents recorded, 42% exhibited MO-evoked currents (Table 1). Among 52 dural afferents recorded, 38% generated currents in response to UMB (Table 1). Nearly all of the dural afferents that exhibited MO-evoked currents also exhibited UMB-evoked currents (Table 1) indicating that these TRPA1 agonists activate currents on the same population of neurons. The percentage of TRPA1 expressing dural afferents reported here are comparable to values that have been described in the literature using immunohistochemical analysis that approximately 32% of all trigeminal neurons express the TRPA1 receptor [30]. These data suggest that a substantial fraction of dural afferents express TRPA1, and that activation of the receptor can contribute to nociceptive signaling from the dura to central neurons following stimulation.

3.2. Mustard oil and umbellulone oil applied to the dura provoke headache-related cutaneous allodynia

Application of 10% MO to the cranial meninges produced delayed facial (Fig. 2A) and hindpaw (Fig. 2B) cutaneous allodynia ($p < 0.001$) in rats that was evident over a 5 hr time course compared to the mineral oil vehicle application. In addition, application of 10% UMB, but not mineral oil vehicle, to the cranial meninges produced significant facial (Fig. 2C) and hindpaw (Fig. 2D) cutaneous allodynia ($p < 0.001$) that was similar in time course to MO treated animals. The maximal reductions in withdrawal thresholds, in response to tactile stimuli, occurred between 2–3 hrs following dural application with response thresholds significantly different from controls and with thresholds returning towards baseline at 5 hrs (Fig. 2).

MO- and UMB-induced cutaneous allodynia was evaluated for 5 hrs following treatment with the TRPA1 antagonist HC-030031 or vehicle. Systemic 1 hr pretreatment (oral) with HC-030031 (100 mg/kg) significantly prevented the development of generalized allodynia in animals that received 10% MO on the dura in comparison to animals that were pretreated with vehicle before 10% MO application (* $p < 0.001$) (Fig. 3A and B). Additionally,

systemic 1 hr pretreatment (oral) with HC-030031 (100 mg/kg) significantly prevented the development of facial (* $p = 0.002$) and hindpaw (* $p < 0.001$) allodynia in animals that received 10% UMB on the dura in comparison to animals that were pretreated with vehicle before 10% UMB application (Fig. 3A and B). The antagonist and its oral vehicle control injection did not induce allodynia in mineral oil treated animals (Fig. 3). These data demonstrate that both MO- and UMB-induced cutaneous allodynia are likely to result from activation of TRPA1 within the meninges.

3.3. Mustard oil and umbellulone oil applied to the dura decrease exploratory rearing activity in a novel environment

A computer operated photobeam tracking system was used to monitor exploratory behaviors for 30 min in rats immediately following dural application of MO or UMB. Following dural MO or UMB, rats did not significantly or consistently alter the amount of time spent or distance traveled while exploring the bottom/horizontal surface of the novel activity chamber although changes in these parameters were sometimes noted (data not shown). However, the amount of time spent and number of times the animals reared (i.e. stood on their hind legs in a vertical position) changed significantly following dural stimulation with TRPA1 agonist. Dural injection (mineral oil) did not significantly affect rearing behavior when compared to naïve or surgically cannulated animals (i.e. without any dural injection) (Fig 4A and B). Application of 10% MO to the dura significantly decreased the number of vertical entries (* $p = 0.002$) and the time spent rearing (* $p < 0.001$) in comparison to mineral oil treated animals (Fig. 4A and B). The most significant behavioral change between groups that received MO and those that received mineral oil occurred during the first 15 minutes following dural injection (Fig. 4C and D), an earlier time course than that observed with the development of allodynia. The method for activity testing used here likely captures the immediate behavioral manifestation of primary nociceptor activation within the dura, whereas the tactile allodynia testing reveals the development of central sensitization over time following dural afferent activation. Although 10% UMB caused cutaneous allodynia in rats over a 5 hr time course, this concentration of UMB did not cause a significant change in exploratory rearing behavior within 30 min after injection. Application of 30% UMB, however significantly decreased the number of vertical entries (* $p = 0.002$) and the time spent rearing (* $p = 0.001$) in comparison to mineral oil treated animals (Fig. 4A and B). The highest concentration of UMB (100%) caused the most significant decrease in rearing behavior in comparison to mineral oil; number of vertical entries (* $p < 0.001$) and time spent rearing (* $p < 0.001$) (Fig. 4A and B). Based on these data, 30% UMB was chosen as the dose for all future activity studies. Similar to MO, the decrease in rearing following dural UMB application occurred primarily within the first 15 min (data not shown).

In order to ensure that the decrease in rearing behavior was unrelated to an overall dysfunction in coordination, motor coordination was monitored on the rotarod following dural MO and UMB. Separate groups of rats were treated with mineral oil, 10% MO, or 30% UMB and tested on the rotarod 15 min after dural injection. All rats completed the 180 sec rotarod test following dural injection and there was no difference from baseline measurements (data not shown).

Pretreatment with p.o. HC-030031 (100 mg/kg) 2 hrs prior to application of 10% MO on the dura significantly prevented the decrease in MO-induced rearing behavior. HC-030031 prevented the decrease in the number of vertical entries (* $p = 0.007$) and the time spent rearing (* $p = 0.015$) caused by 10% MO in comparison to animals that were pretreated with vehicle before 10% MO (Fig. 5A and B). Pretreatment with p.o. HC-030031 (100 mg/kg) 2 hrs prior to application of 30% UMB on the dura significantly prevented the decrease in UMB-induced rearing behavior. HC-030031 prevented the decrease in the number of vertical entries (* $p = 0.027$) and the time spent rearing (* $p = 0.022$) caused by 30% UMB in

comparison to animals that were pretreated with vehicle before 30% UMB (Fig. 5A and B). The antagonist caused no significant behavioral differences from the oral vehicle control group in mineral oil treated animals (Fig. 5). Importantly, HC-030031 has previously been shown to have no effect on locomotor coordination at the dose and route of administration employed in these studies [22].

Pretreatment with the anti-migraine therapeutic sumatriptan (0.6 mg/kg, s.c.) 1 hr prior to dural application of 10% MO also prevented the decrease in MO-induced rearing behavior. Sumatriptan partially attenuated the decrease in the vertical entries (* $p = 0.044$) and the time spent rearing (* $p = 0.043$) caused by 10% MO in comparison to animals treated with s.c. saline before MO treatment (Fig. 6A and B). Sumatriptan and s.c. saline pretreatment did not cause behavioral changes in the mineral oil treated animals (Fig. 6). As sumatriptan is a medication which is generally only effective for migraine headache pain, and not other types of pain these data suggest that MO-induced activation of dural-afferent TRPA1 channels produces a change in rearing behavior that may be related to headache pain.

4. Discussion

Although migraine affects 10% of the world's population and is a leading cause of health-related disability, there is poor understanding of the underlying complex pathogenetic mechanisms. Sensitization of peripheral trigeminovascular afferents has been implicated as the event that initiates headache pain, while sensitization of central pathways has been proposed as the mechanism of cephalic and extracephalic allodynia that occur during migraine attacks [10,21]. However, the mechanisms that induce dural-afferent activation and therefore the headache pain associated with the attack are still unclear. Here, analysis of *in vitro* patch clamp recordings from TG neurons revealed that a substantial fraction of dural afferents likely express TRPA1. The possibility that TRPA1 contributes to headache pain was evaluated behaviorally using an established model of cutaneous allodynia, a feature of the migraine syndrome commonly observed clinically, and a novel measure of non-reflexive rearing behavior, being reported here for the first time, that may reflect the movement-induced exacerbation of headache pain that is one of the most sensitive diagnostic features of migraine. Taken together, the findings of the current work suggest that TRPA1-mediated dural afferent activation is a plausible mechanism that could contribute to headache pain.

Preclinically, cutaneous allodynia of the face and hindpaw has previously been reported as a consequence of dural afferent activation using various mediators [21,24,43,56–58]. Such delayed and generalized hypersensitivity may occur as a result of trigeminal-mediated central sensitization within the medullary dorsal horn, and additionally due to neuronal activation within brainstem pain modulatory centers (e.g. the rostroventromedial medulla, RVM) following peripheral afferent input [21]. A second possibility is that MO or UMB penetrates the dura, is widely distributed throughout the CSF, and produces delayed and generalized allodynia due to activation of afferent nerve endings at locations away from the primary injection site. Regardless of the exact mechanism, hypersensitivity was observed following stimulation of the dura specifically with MO or UMB, compounds that are known to activate TRPA1. Generalized allodynia was not detected with vehicle application to dural afferents, and the allodynia was prevented using pretreatment with the TRPA1 selective antagonist HC-030031, indicating a TRPA1-specific effect resulting from MO and UMB application to the dura mater. Additional non-neuronal actions, including potentially non-neurogenic inflammatory responses produced by MO [3] or UMB cannot be ruled out by these *in vivo* studies. However, our behavioral observations are consistent with MO- and UMB-induced activation of TRPA1 channels on identified dural afferents using whole cell patch-clamp leading to direct initiation of afferent signaling. Thus, TRPA1 activation is a

mechanism that can activate dural nociceptors and lead to central sensitization and cutaneous allodynia suggesting a role for TRPA1 in headache pain.

As the majority of patients develop cephalic and extracephalic cutaneous allodynia during a migraine attack, preclinical evaluation of this evoked response following activation of dural afferents may be useful as a translational endpoint to study mechanisms contributing to migraine headache. However, the use of other behavioral endpoints, especially those that do not employ reflexive threshold measures, may provide additional confidence that activation of dural afferents produces a headache-like state. Recent studies have established that spontaneous exploratory activity in rats is decreased following induction of non-cephalic pain states including neuropathic [55] and joint inflammation [36], as well as following chronic administration of inflammatory mediators to the dura [49]. These studies support the concept that decreases in exploratory activity may be objective non-reflexive measures of pain-like states including headache.

The behavioral paradigm used here exploits the natural instinct of rodents to explore when exposed to a novel environment. We hypothesized that animals experiencing a headache-like state would spend less time exploring the environment than those that are pain free. Activation of dural-afferent TRPA1 receptors with MO or UMB did not consistently affect horizontal exploratory activity, nor did it have an effect on motor coordination. However, dural MO and UMB significantly decreased the exploratory rearing behavior of the rats. This change in behavior was prevented by pre-treating animals with the TRPA1 antagonist HC-030031 and in the case of MO, with sumatriptan, a gold-standard acute migraine specific therapy. The efficacy of sumatriptan in the activity model correlates to previous studies demonstrating that sumatriptan attenuates facial and hindpaw allodynia following dural inflammation [21]. Sumatriptan is not known to interact with the TRPA1 receptor, thus its efficacy in this activity model may be related to an attenuation of dural afferent signaling through a reduction in neurogenic inflammation, or alternatively to a centrally mediated effect by blocking facilitation of headache pain. The decrease in time spent rearing and in the number of rearing episodes is suggestive of a behavioral state that is consistent with “headache” pain due to activation of TRPA1 on meningeal nerve endings. The headache-like state overrides the competing motivation to explore the vertical aspects of the novel environment. This response is similar to the decrease in and avoidance of activity seen in patients during a migraine attack. Exacerbation of migraine headache during movement, particularly moving from a horizontal to vertical position, is a diagnostic criterion and clinically the most sensitive and specific feature of migraine headache [25,48]. This phenomenon, suspected to be secondary to sensitization of trigeminovascular peripheral afferents and worsening of pain, occurs with even small changes in intracranial pressure [12,13]. Although not directly tested here, it is tempting to speculate that changes in intracranial pressure, induced by changes in posture, especially assuming an upright position, lead to an increase in the headache-like state experienced by these rats resulting in a decreased desire to explore the vertical plane. Nonetheless, the data from this behavioral model is consistent with a possible role for TRPA1 in migraine headache pathogenesis and potentially other types of primary headache disorders.

Prior evidence from the literature suggests a physiological role for TRPA1 activity during headache. Notably, in anesthetized rats, dural application of MO decreases dural mechanical thresholds, increases the cutaneous mechanoreceptive field, and induces sensitization of second order neurons receiving convergent trigeminal and cervical nociceptive input [5]. The TRPA1 agonist UMB has also been shown to increase meningeal blood flow and trigeminal release of CGRP [33,42] (a peptide known to be pivotal in migraine pathogenesis and to induce neurogenic vasodilation) and these effects can be blocked with pretreatment of HC-030031 [33]. These latter studies suggest that meningeal TRPA1 may mediate migraine

headache in response to environmental irritants, one of the most common triggers for migraine headache [25] much like TRPA1 channel activation in the upper and lower airways can initiate noxious responses to irritating agents [9]. Our studies using MO and the environmental irritant UMB now provide direct support that TRPA1 activation in dural afferents produces evoked and spontaneous behavioral responses in animals consistent with a headache-like state.

Although these functional studies support a potential role for TRPA1 in migraine headache, the endogenous mechanisms leading to TRPA1 activation within the meninges remain unclear. Evidence from other pain models suggests that stimulation of TRPA1 can occur through several mechanisms. This channel can be activated downstream of bradykinin receptor activation [2], and by exposure to oxidative and nitrative stress byproducts [16,35,51,52], or mechanical stimuli [29,34], any of which could be present within the dura before or during a migraine attack. TRPA1 has also been shown recently to be sensitive to changes in oxygen levels [50] and thus it is possible that hyper- or hypoxia within the meninges contributes to channel activation and afferent signaling. Ultimately, more studies are necessary to determine whether these or other as yet unidentified mechanisms contribute to TRPA1 activation within the meninges.

In summary, the present findings support a mechanism by which activation of TRPA1 on dural afferents contributes to the induction of both spontaneous behaviors relevant to headache pain and evoked behaviors that likely reflect the presence of central sensitization. While the mechanisms that drive TRPA1 activation within the dura are still unknown, TRPA1 activity in the *in vitro* and *in vivo* models used here adds to the accumulating scientific and anecdotal evidence arguing for a causal relationship between TRPA1-mediated irritant exposure and headache development [8,26,33]. Ultimately, these findings may lead to new therapeutics targeting TRPA1 that have efficacy and/or tolerability advantages over currently available anti-migraine agents.

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Fig. 1. Dural afferents express TRPA1-like currents in response to mustard oil and umbellulone oil

(A) An example of mustard oil (MO; 100 μ M) evoked current from a representative dural afferent following a 2 min step from extracellular bath to MO. (B) MO evoked action potential firing recorded in a representative dural afferent following a 2 min step from extracellular bath to 100 μ M MO. (C) Examples of MO (100 μ M) and umbellulone oil (UMB; 100 μ M) evoked currents from the same representative dural afferent following a 2 min step from extracellular bath to TRPA1 agonist solution. The two traces are on the same vertical and horizontal scales.

Fig. 2. Mustard oil (MO) and umbellulone oil (UMB) application on the dura induces significant facial and hindpaw allodynia

(A and B) MO (10%) produces generalized allodynia (N = 8) in comparison to Mineral oil (100%) application (N = 10). (C and D) UMB (10%) produces facial and hindpaw allodynia (N = 9) in comparison to Mineral oil (100%) application (N = 10). Dural injection took place immediately after baseline and animals were evaluated using von Frey filaments for 5 hrs at 1 hr intervals following dural injection. A decrease in withdrawal threshold (y-axis) from baseline (BL) is indicative of allodynia development. Data were analyzed among groups and across time (baseline to 5 hr) using two-factor ANOVA. Significance was set at $p < 0.05$. Note: error bars for the vehicle treated groups are too small for visualization in these graphs.

Fig. 3. The TRPA1 antagonist HC-030031 (HC; 100 mg/kg) prevents allodynia stimulated by mustard oil (MO; 10%) and umbellulone oil (10%) application to the dura

Animals are pretreated systemically (p.o.) 1 hr before dural application and evaluated using von Frey filaments for 5 hrs at 1 hr intervals following dura injection. Antagonist (N = 6) or vehicle (Veh) (N = 6) oral pretreatment did not produce significant allodynia in Mineral oil (Min oil) treated animals. MO (N = 7) and UMB (N = 9) produce generalized allodynia in animals with Veh pretreatment. HC-030031 significantly prevents MO induced facial (A) and hindpaw (B) allodynia (* $p < 0.05$) (N = 7) in comparison to Veh + MO treated animals. HC-030031 significantly prevents UMB induced facial (A) and hindpaw (B) allodynia (* $p < 0.05$) (N = 7) in comparison to Veh + UMB treated animals. Data are presented as area

over the time-effect curve (AOC; baseline to 5 hr). A decrease in the AOC indicates a decrease in the presence of allodynia following dural inflammation. Data were analyzed among groups and across time (baseline to 5 hr) using two-factor ANOVA. Significance was set at $p < 0.05$.

Fig. 4. Activation of meningeal TRPA1 produces decreases in exploratory rearing behavior

Exploratory rearing activity, measured using a photobeam tracking system, was decreased in animals receiving dural application of TRPA1 agonist. Behavior was evaluated for 30 min immediately following dural application. Dural Mustard oil (MO; 10%; N = 16) caused a significant decrease in (A) the number of times the animal reared (# vertical entries) as well as the (B) time spent rearing within the vertical plane of lasers in comparison to Mineral oil (Min oil; N = 19) injected animals (* $p < 0.05$). Dural umbellulone (UMB; 10%; N = 21) did not produce a change in rearing but UMB 30% (N = 10) and 100% (N = 19) caused a significant decrease in (A) number of vertical entries as well as the (B) time spent rearing in comparison to mineral oil (Min oil; N = 19) injected animals (* $p < 0.05$). Naïve (N = 14) and Surgery (N = 19; dura cannulated rats without dura injection) groups did not have significantly decreased rearing behavior in comparison to Min oil treated rats (A and B). The decrease in the rearing activity caused by MO application was most apparent during the first 15 minutes following dura injection when data are analyzed in 5 min intervals for the # vertical entries (C) and time spent rearing (D). Activity data were evaluated using either an unpaired t-test for continuous data (time analysis of vertical rearing behavior) or the Mann-Whitney test for non-parametric ordinal data (counts of vertical entries). Significance was set at $p < 0.05$.

Fig. 5. The TRPA1 antagonist HC-030031 (HC; 100 mg/kg) blocks decreased rearing behavior stimulated by mustard oil (MO; 10%) and umbellulone (UMB; 30%) applied to the dura Animals were pretreated systemically (p.o.) 2 hrs before TRPA1 agonist application and behavior was evaluated for 30 min following dura injection. Among Mineral oil (Min oil) treated animals, there was no significant difference in behavior produced between rats pretreated with antagonist (N = 12) or vehicle (Veh; N = 11) (A and B). HC-030031 significantly prevents the MO stimulated (N = 9) decrease in # of vertical entries (A) and the time spent rearing (B) (* $p < 0.05$) in comparison to animals treated with Veh + MO (N = 10). HC-030031 significantly prevents the UMB stimulated (N = 9) decrease in # of vertical entries (A) and the time spent rearing (B) (* $p < 0.05$) in comparison to animals treated with Veh + UMB (N = 13). Pilot studies indicated that a 1 hr pretreatment with 3 ml of Veh or dH₂O produced decreased rearing behavior that is unrelated to the test compound (data not shown). This effect was less robust with a 2 hr pre-treatment; therefore this time course was used for activity testing requiring p.o. dosing. Activity data were evaluated using either an

unpaired t-test for continuous data (time analysis of vertical rearing behavior) or the Mann-Whitney test for non-parametric ordinal data (counts of vertical entries). Significance was set at $p < 0.05$.

Fig. 6. The anti-migraine agent sumatriptan attenuates decreased rearing behavior stimulated by mustard oil (MO; 10%) applied to the dura

Animals were pretreated systemically (s.c.) 1 hr before TRPA1 agonist application and behavior was evaluated for 30 min following dura injection. Among Mineral oil (Min oil) treated animals, there was no significant difference in behavior produced between rats pretreated with sumatriptan (Suma; N = 21; 0.6 mg/kg) or vehicle (Veh; N = 19) (A and B). Sumatriptan (N = 19) significantly prevents the MO stimulated decrease in # of vertical entries (A) and the time spent rearing (B) (* $p < 0.05$) in comparison to animals pretreated with Veh + MO (N = 18). Activity data were evaluated using either an unpaired t-test for continuous data (time analysis of vertical rearing behavior) or the Mann-Whitney test for non-parametric ordinal data (counts of vertical entries). Significance was set at $p < 0.05$.

Table 1
Quantification of currents produced from dural afferents following application of mustard oil (MO) or umbellulone oil (UMB) or both TRPA1 agonists

Whole cell patch-clamp experiments were performed on identified dural afferent rat trigeminal ganglion cells. Currents were only observed when solutions were switched from external bath to MO or UMB containing solution using our drug application system. A minimum of a 4 minute washout period was used for recovery between applications of TRPA1 agonist. A cutoff of 20 pA was selected as a minimum amplitude for response for all the experiments based on preliminary studies. Dural afferents were sampled from a total of 22 animals labeled with fluorogold tracer. Out of 119 cells, 42.02% exhibited TRPA1-like current in response to MO. Out of 52 cells sampled with UMB, 38.46% exhibited TRPA1-like current. In experiments where UMB and MO were both applied to a single cell, 85–90% of cells exhibited TRPA1-like current in response to both TRPA1 agonists.

	MO Responders	UMB Responders	% UMB That Also Respond To MO	% MO That Also Respond To UMB
% Cells	42.02	38.46	90.00	85.71
Number of Cells	119	52	20	21