Published in final edited form as:

Headache. 2013 September ; 53(8): 1250–1261. doi:10.1111/head.12152.

pH-evoked dural afferent signaling is mediated by ASIC3 and is sensitized by mast cell mediators

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Abstract

Background—Prior studies have shown that decreased meningeal pH activates dural afferents via opening of acid-sensing ion channels (ASICs) suggesting one pathophysiological mechanism for the generation of headaches. The studies described here further examined the ASIC subtype mediating pH-induced dural-afferent activation and examined whether sensitization influences pH responses.

Objective—Given the potential importance of meningeal mast cells to headache, the goal of this study was to evaluate dural afferent responses to pH following sensitization with mast cell mediators.

Methods—Cutaneous allodynia was measured in rats following stimulation of the dura with decreased pH alone or in combination with mast cell mediators. Trigeminal ganglion neurons retrogradely-labeled from the dura were stained with an ASIC3 antibody using immunohistochemistry. Currents and action potentials evoked by changes in pH alone or in combination with mast cell mediators were measured in retrogradely-labeled dural afferents using patch-clamp electrophysiology.

Results—pH-sensitive dural afferents generated currents in response to the ASIC3 activator 2-guanidine-4-methylquinazoline (GMQ), approximately 80% of these neurons express ASIC3 protein, and pH-evoked behavioral responses were inhibited by the ASIC3 blocker APETx2. Following exposure to mast cell mediators, dural afferents exhibited increased pH-evoked excitability and cutaneous allodynia was observed at higher pH than with pH stimuli alone.

Conclusion—These data indicate that the predominant ASIC subtype responding to decreased meningeal pH is ASIC3. Additionally, they demonstrate that in the presence of inflammation, dural afferents respond to even smaller decreases in pH providing further support for the ability of small pH changes within the meninges to initiate afferent input leading to headache.

Keywords

Dural afferents; ASIC3; Meninges

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Conflict of interest: The authors report no conflicts of interest

Introduction

Migraine is a painful and debilitating neurological disorder accompanied by a variety of symptoms, including aura, nausea, vomiting, photophobia and phonophobia.¹ It is not yet clear how afferent pain signaling is initiated during a migraine attack but previous studies in our laboratory demonstrated that decreased extracellular pH excites dural afferents and causes migraine-related pain behavior via activation of acid-sensing ion channels (ASICs).²

Acid sensing ion channels (ASICs) are voltage-insensitive cationic channels activated by decreased extracellular pH.³ The ASIC family consists of 4 members ASIC1-ASIC4, with several splice variants.⁴ ASIC1-3 subunits exist as homomeric or heteromeric channels and are sensitive to different ranges of pH (ASIC4 is not pH sensitive). ASIC3 homomers and heteromers exhibit extreme sensitivity to pH change, with p*H*₅₀'s of 6.2-6.8.^{4, 5} The ASIC3 subtype, which is expressed in sensory neurons innervating visceral organs including the colon and heart as well as skeletal muscle, has been proposed to modulate pain conditions associated with these tissues, including angina, postoperative pain, various GI disorders and muscle pain.⁶⁻¹¹ Our prior work has suggested a role for ASIC3 in migraine pathophysiology but this was not examined pharmacologically or histologically.² Here we determined whether ASIC3 is responsible for dural afferent pH-evoked current and pH-induced migraine-related pain behavior using specific activators and inhibitors of ASIC3 channels and immunohistochemistry of dural afferents.

Migraine is hypothesized to be an inflammatory disorder based on the established, albeit limited efficacy of NSAIDs in migraine therapy as well as increased intracranial levels of inflammatory mediators during migraine attacks.¹² One potential endogenous source for inflammatory mediators is a group of immune cells called mast cells, which are known to reside within the dura.^{13, 14} Mast cells have established roles in modulating a variety of inflammatory diseases, possibly including migraine.¹⁵ Upon activation, dural mast cells can release a host of cytokines, vasoactive and proinflammatory mediators.¹⁵ Dural mast cells are located near blood vessels and in close proximity to meningeal nociceptive fibers,¹⁶ and thus mast cell degranulation can directly activate and sensitize primary afferent neurons.¹⁷ Since a drop in pH is likely not the sole pathological change in the meninges during migraine attacks, the purpose of these studies was to determine whether exposure of dural afferents both in vitro and in vivo to mast cell mediators increases pH-evoked firing of action potentials and raises the threshold for pH-induced cutaneous allodynia.

Materials and Methods

Animals

Adult male Sprague Dawley rats (175-200g) were maintained in a climate-controlled room on a 12 hr light/dark cycle with food and water *ad libitum*. All procedures were performed in accordance with the policies and recommendations of the International Association for the Study of Pain, the National Institutes of Health guidelines for the handling and use of laboratory animals, and were approved by the Institutional Animal Care and Use Committee of the University of Arizona.

Surgical preparation

Tracer injection—Dural afferents were identified as previously described using a retrograde labeling protocol where the fluorescent tracer fluorogold is applied to the dura 7 days before animals are sacrificed and trigeminal ganglia are removed for culture.^{2, 18} Importantly, undamaged dura at the injection sites was evaluated under a microscope at the

time the animals were sacrificed and only animals with intact dura and no signs of damage, e.g. holes in the dura due to drilling, were used for further experiments.

Immunohistochemistry—Seven days following the retrograde labeling procedure described above, rats were deeply anesthetized with ketamine and xylazine (80 mg/kg and 10 mg/kg, respectively), transcardially perfused with 200 ml PBS (pH 7.4) and then fixed with 500 ml of 2% formalin/12.5% picric acid in PBS (pH 7.4). Trigeminal ganglia were then removed and immersed in a 30% sucrose solution for 2 days (or until they sank to the bottom of the solution). Next, they were mounted in O.C.T compound (Tissue-Tek) and frozen. Ganglia were sliced using a cryostat/microtome (Microm, Richard-Allen Scientific) at 20 μ m thickness and mounted on slides. Trigeminal ganglion sections were then subjected to the following immunohistochemistry protocol. Sections were washed with PBS 3 times; permeabilized with PBS + 10% normal goat serum (NGS) + 0.2% Triton $\times$ 100 and blocked with PBS + 10% NGS + 0.01% Na-azide. Sections were incubated overnight with a primary rabbit ASIC3 antibody (1:400; Alomone) followed by a 1 hour incubation in an anti-rabbit secondary antibody conjugated to AlexaFluor 555 (Invitrogen), mounted in Fluoro-Gel with Tris Buffer (Electron Microscopy Sciences) and coverslipped. Sections were visualized using an Olympus BX51 upright microscope and acquired using HC image software. Backlabeled trigeminal ganglion neurons were present in 3-4 sections per ganglion and only 1 section per ganglion was used for analyzing colocalization.

Dural cannulation—Dural cannulae were implanted as previously described.^{2, 18-20} Animals were anesthetized with a combination of ketamine and xylazine (80 mg/kg and 12 mg/kg; Sigma-Aldrich). A 2cm incision was made to expose the skull. A 1 mm hole was made in the skull (above the transverse sinus; 2 mm left of the sagittal suture and 2 mm anterior to the lambdoid suture) with a hand drill (DH-0 Pin Vise; Plastics One, Roanoke, VA) to carefully expose the dura. A guide cannula (22 GA, #C313G; Plastics One), designed to extend 0.5 mm from the pedestal to avoid irritation of the dural tissue, 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 (#C313DC; Plastics One) was inserted to ensure patency of the guide cannula. Immediately postoperatively, animals received a single subcutaneous injection of gentamicin (8mg/kg) to minimize infection. Rats were housed separately and allowed 6 to 8 days of recovery.

Cell culture—Seven days following fluorogold application, rats were sacrificed, trigeminal ganglia were removed, enzymatically treated, and mechanically dissociated as previously described.^{2, 18} Rats were anesthetized with isoflurane (Phoenix Pharmaceuticals) and sacrificed by decapitation. The trigeminal ganglion (TG) were removed and placed in ice-cold Hanks balanced-salt solution (divalent free). Ganglia were cut into small pieces and incubated for 25 mins in 20 U/ml Papain (Worthington) followed by 25 mins in 3 mg/ml Collagenase Type II (Worthington). Ganglia were then triturated through fire-polished pasteur pipettes and plated on poly-D-lysine (Becton Dickinson) and laminin (Sigma)-coated plates. After several hours at room temperature to allow adhesion, cells were cultured in a room-temperature, humidified chamber in Liebovitz L-15 medium supplemented with 10% FBS, 10 mM glucose, 10 mM HEPES and 50 U/ml penicillin/streptomycin. Cells were used within 24 h post plating.

Electrophysiology—Whole cell patch-clamp experiments were performed on isolated rat TG using a MultiClamp 700B (Axon Instruments) patch-clamp amplifier and pClamp 10 acquisition software (Axon Instruments). Recordings were sampled at 2 kHz and filtered at 1 kHz (Digidata 1322A, Axon Instruments). 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 M resistance using a microforge (MF-83, Narishige). Series resistance was typically < 7 M and was compensated 80%. All recordings were performed at room temperature. A Nikon TE2000-S Microscope equipped with a mercury arc lamp (X-Cite® 120) was used to identify FG-labeled dural afferents. Data were analyzed using Clampfit 10 (Molecular Devices) and Origin 8 (OriginLab). Cell sizes were not significantly different among groups. Labeled neurons fall in a range of capacitance values from 15 to 80 pF (average of 57.47 ± 5.47). Pipette solution contained (in mM) 140 KCl, 11 EGTA, 2 MgCl₂, 10 NaCl, 10 HEPES, 1CaCl₂ 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.

Behavioral testing—Cutaneous allodynia measurements following stimulation of the dura was performed as described previously.^{2, 18, 19} Rats were acclimated to suspended Plexiglas chambers (30cm long × 15cm wide × 20cm high) with a wire mesh bottom (1cm²). Ten µl of vehicle or testing solution was injected through an injection cannula (28GA, #C313I; Plastics One) cut to fit the guide cannula. Withdrawal thresholds to probing the face and hind-paws were determined at 1-hour intervals after administration. A behavioral response to calibrated von Frey filaments applied to the midline of the forehead, at the level of the eyes, was indicated by a sharp withdrawal of the head. Paw withdrawal (PW) thresholds were determined by applying von Frey filaments to the plantar aspect of the hind-paws, and a response was indicated by a withdrawal of the paw. The withdrawal thresholds were determined by the Dixon up-down method.²¹ Maximum filament strengths were 8 and 15 gm for the face and hind-paws, respectively. Experiments were conducted with the investigator blinded to the experimental conditions.

Data analysis—All data are presented as means ± SEM unless otherwise noted. Statistical evaluation was performed by Student's t-test or one-way ANOVA followed by post hoc Dunnett's test. For behavioral experiments, data were converted to area over the time-effect curve to allow for multiple comparisons.

Chemicals—Fluorogold, Amiloride and AMG-9810 were prepared as previously described.² 2-guanidine-4-methylquinazoline (GMQ, Sigma) was dissolved in bath solutions to the desired concentration. rAPETx2 (Alomone Labs) was diluted in SIF (synthetic interstitial fluid) solutions with designated pH to the final concentration of 20 µM. The SIF consisted of 10 mM Hepes, 5 mM KCl, 1 mM MgCl₂, 5 mM CaCl₂, and 135 mM NaCl, pH 7.3. Stock mast cell mediators were composed of 100 mM Histamine in distilled water (Sigma), 100 mM serotonin in distilled water (Sigma), 10 mM AC55541, a PAR2 agonist,²² in DMSO (Tocris) and 13.8 mM Iloprost in 0.5% in methyl acetate (Caymen Chemical). Stock mast cell mediators were diluted to the desired concentrations in bath solutions (electrophysiology) or SIF with different pH values (behavior).

Results

Cutaneous allodynia following acidic stimulation of the dura is mediated by ASIC3

A preclinical in vivo migraine model was used to evaluate the effect of a drop in meningeal pH on mechanical withdrawal thresholds both to the face and hindpaws.¹⁹ Our previous findings showed that dural application of pH 5.0 SIF results in significant facial and hindpaw mechanical allodynia in rats.² Consistent with this observation, pH 6.0 SIF dural application also produced significant ($p < 0.0001$) time dependent and reversible reductions in withdrawal thresholds to tactile stimuli applied to the face or the hindpaws (Fig. 1A and B) compared with pH 7.4 SIF application. Maximal effects occurred 2 hrs after pH 6.0

application (consistent with prior studies from our laboratory with multiple stimuli),^{2, 18, 19, 23} and facial and hindpaw responses approached baseline by 5 hrs (Fig. 1A and B). Cutaneous allodynia following pH 6.0 application was significantly (** $p < 0.01$, * $p < 0.05$) blocked by co-application with 100 nmol amiloride, a non-specific ASIC antagonist, but not with 10 nmol AMG-9810, a TRPV1 antagonist, suggesting that pH 6.0-evoked responses were also mediated through activation of ASICs (Fig. 1C).

In our previous study, we suggested (but did not systematically evaluate) that dural afferent pH evoked responses were mediated through ASIC3 subtype-containing channels.² This was based on the observation that the kinetics of pH-evoked currents in dural afferents were mostly consistent with that of recombinant ASIC3 channels, but not ASIC1 channels.² Here we tested the effects of pharmacological inhibition of pH evoked mechanical allodynia by a selective ASIC3 inhibitory peptide APETx2. APETx2, a sea anemone peptide, blocks ASIC3 homomeric and heteromeric channels both in transfected cells and rat primary sensory neuron cultures.²⁴ This peptide also blocks ASIC3-evoked sustained window current in DRG neurons.²⁵ Co-application of APETx2 (200 pmol) with pH 6.0 SIF solution significantly blocked the development of tactile allodynia of the face and the hindpaw (Fig. 1D). This dose of APETx2 was suggested to be selective for ASIC3 in a prior study that examined the role of this channel in postoperative pain.⁸ Application of APETx2 in pH 7.4 SIF produced no change compared to pH 7.4 SIF alone (data not shown). These data indicate that pH-evoked migraine-related pain behaviors are mediated by ASIC3 subtype-containing channels.

Dural afferents express ASIC3 protein

In order to evaluate whether ASIC3 is expressed by dural afferents, trigeminal ganglion neurons retrogradely labeled from the dura were immunolabeled with an ASIC3-specific antibody. Trigeminal ganglia were removed seven days after application of the retrograde tracer fluorogold to the dura and were subjected to an immunohistochemistry protocol. Overlap was examined between immunolabeling for ASIC3 and the presence of fluorogold to determine whether and what percentage of identified dural afferents express ASIC3 protein. A representative section is shown in Figure 2 with ASIC3 immunolabeling, fluorogold expression, and an overlay of the two images showing co-localization. Of the trigeminal neurons expressing fluorogold (i.e. identified dural afferents), approximately 80% (100 of 138) expressed immunolabeling for ASIC3 indicating the presence of ASIC3 in the majority of dural afferents. These data are consistent with our prior report that showed 80% of dural afferents *in vitro* responded to pH 6.0 application with ASIC-like currents.²

pH-evoked currents in dural afferents are mediated by ASIC3-containing channels—To further confirm the contribution of the ASIC3 subunit in dural afferent pH evoked responses, we tested whether pH-responsive dural afferents also responded to a specific ASIC3 agonist *in vitro*. Recently, a non-proton ligand was identified for ASIC3 channels.²⁶ At normal pH (i.e. 7.4), 2-guanidine-4-methylquinazoline (GMQ) caused persistent activation of ASIC3 channels, but not of ASIC1 or ASIC2 channels.²⁶ Moreover, injection of GMQ into the paw has been shown to induce allodynia mediated by activation of ASIC3.²⁶ These findings indicate that this ligand is selective for ASIC3-containing channels and can be used as a tool to probe the contribution of ASIC3 to currents in dural afferents. Here, we found that 15 out of 16 pH-responsive dural afferents also exhibited currents ranging from 100 pA to 1400 pA in response to 3 mM GMQ application (Figure 3). Thus, almost all neurons that respond to pH 6.0 also respond to GMQ. On the other hand, for 6 pH-insensitive neurons, 0 responded to 3 mM GMQ application at the cutoff amplitude (50 pA). No particular size of neuron was selected for these studies (i.e. fluorescent neurons were selected regardless of size, see Methods for cell size range) and pH-sensitive neurons

expressed GMQ current regardless of size. Consistent with the behavioral and histological findings described above, these data indicate that pH 6.0-evoked currents in dural afferents are mediated by ASIC3-containing channels.

Dural application of low pH solutions dose-dependently elicits cutaneous allodynia

We next determined the pH threshold for evoking mechanical allodynia following dural application of SIF solutions with different pH values. Application of pH 6.4 SIF solution to the dura produced significant ($*p < 0.05$) time-dependent and reversible reductions in both facial and hindpaw withdrawal thresholds compared with SIF pH 7.4 application (Figure 4). Dural application of a slightly higher pH of 6.6 caused a significant ($*p < 0.05$) decrease in hindpaw withdrawal threshold, but not facial withdrawal threshold, likely due to assay sensitivity differences between the face and hindpaw (i.e. a more accurate reading is generally obtained in the hindpaws since the animals cannot see the tester approaching them) rather than mechanistic differences contributing to the behavior. Tactile allodynia of either the face or hindpaw was not observed following pH 6.8 application ($p > 0.05$) (Figure 4B). These findings demonstrate that pH dose-dependently causes facial and hindpaw allodynia with thresholds at pH 6.4 and pH 6.6 for the face and hindpaws, respectively.

Sensitization of pH-evoked responses by mast cell mediators

A decrease in pH is likely not the sole pathological event happening within the meninges during migraine attacks. As a result, allodynia might be produced at higher pH in the presence of other factors. One event that may lead to the presence of decreased pH as well as an increase in inflammatory mediators is the degranulation of meningeal mast cells. Mast cell mediators released following degranulation have been shown to sensitize trigeminal meningeal nociceptors^{17, 27} suggesting that mast-cell derived substances²⁷ might also enhance pH-induced tactile allodynia. Electrophysiological single-unit recording experiments have shown that mast cell mediators, including histamine, serotonin, and prostacyclin directly sensitize meningeal nociceptors in anesthetized animals.²⁷ Along with these mediators, tryptase is the most abundant secretory granule-derived serine proteinase contained in mast cells.²⁸ Tryptase has been shown to act on dural afferents, through cleavage and activation of the PAR2 receptor.²⁹ Therefore, in this study we used mast cell mediators including histamine, serotonin, AC55541 (a PAR2 agonist) and iloprost (a stable prostacyclin analogue). First, we identified a combination of mast cell mediators (200 μ M Histamine, 200 μ M Serotonin, 10 μ M AC55541 and 20 μ M Iloprost) that did not cause allodynia when administered alone (10 μ l, Black Bar, $n = 9$, Figure 5A and 5B). Next, we co-applied the sub-threshold dose of mast cell mediators with pH 6.6, both of which do not cause significant facial allodynia when applied alone. The combination of mast cell mediators and pH 6.6 significantly ($**p < 0.01$, red bar, $n = 12$, Figure 5A) decreased facial withdrawal threshold compared with pH 7.4 SIF application (white bar, $n = 11$, Figure 5A), whereas pH 6.6 by itself did not (red bar, $n = 13$, Figure 5A). Similarly, the pH threshold for evoking paw allodynia now shifted to pH 6.8 following co-application with sub-threshold mast cell mediators (red bar, $n = 12$, Figure 5B). Consistent with the facial and hindpaw allodynia evoked by low pH alone, coapplication of mast cell mediators with low pH produced maximal effects 2 hrs after application and facial and hindpaw responses approached baseline by 5 hrs (time course data not shown). These data indicate that in the presence of mast cell mediators, the pH necessary to induce cutaneous allodynia of both the face and hindpaw is shifted to more neutral values.

Since co-application with mast cell mediators shifted pH thresholds towards neutral pH *in vivo*, we predicted that the ability of dural afferents to generate pH-induced action potential firing would increase following sensitization *in vitro*. The percentage of dural afferents firing action potentials was calculated before and after a 5 min application of mast cell

mediators (100 μ M Histamine, 10 μ M Serotonin, 10 μ M AC55541, 1 μ M Iloprost). These concentrations of mast cell mediators have been shown previously to produce sensitization of dural afferents following mechanical stimuli.^{27, 29} Consistent with this observation, here we demonstrated that the percentage of neurons firing action potentials was increased for each pH tested (Figure 6A). A short burst of action potentials was rapidly evoked in 48%, 32% and 16% of dural afferents following application of pH 6.8, 6.9 and 7.0, respectively, while following mast cell mediators, pH 6.8, 6.9 and 7.0 stimulation was able to evoke action potentials in 64%, 51% and 41% of dural afferents (n=23). In addition, the number of spikes was counted from the same dural afferent before and after application of mast cell mediators. Dural afferents exhibited significantly more spikes following application of mast cell mediators (Figure 6B, paired t-test, $**p < 0.01$ for pH 6.9, $*p < 0.05$ for pH 6.8 and pH 7.0). As shown in the raw traces of Figures 6C and 6D, application of mast cell mediators can lead to longer-duration firing at pH 6.9 and to firing at a previously ineffective pH of 7.0. Although the exact mechanisms responsible for this sensitization are not fully known, recruitment of additional pH-sensitive channels likely does not occur as the kinetics of desensitization in pH-evoked currents were the same before and after application of mast cell mediators (303.06 ± 15.44 ms vs. 306.39 ± 17.09 ms, data not shown) and other pH-sensitive channels such as TRPV1 have substantially slower desensitization kinetics. Resting membrane potentials were significantly depolarized ($*p < 0.05$, paired t-test) following application of mast cell mediators (-63.13 ± 1.485 mV, n = 44) compared with baseline levels (-69.31 ± 1.255 mV, n = 44). This change in resting membrane potential was not sufficient to excite dural afferents without a pH change since application of mast cell mediators evoked spontaneous firing in only 1 out of 31 dural afferents examined but may contribute to the sensitization. Taken together, these data indicate that dural afferents exhibit an increased sensitivity to pH changes within the dura following application of mast cell mediators.

Discussion

Understanding the molecular events mediating activation and sensitization of dural afferents will not only help elucidate the pathophysiology of headache but also accelerate the development of new therapeutics. Continuing our previous work, we demonstrate here that pH-evoked dural afferent activation both *in vitro* and *in vivo* is mediated through activation of ASIC3-containing channels. We also show that sensitization of dural afferents by mast cell mediators results in increased sensitivity to pH changes *in vitro* and *in vivo*. These data suggest an important role of the ASIC3 subunit in detecting pH changes within the dura and in the subsequent initiation of dural afferent signaling. Additionally, they further support a role for dural afferent activation and ultimately headache following a small change in meningeal pH, particularly under the setting of inflammation following mast cell degranulation.

Signaling from the dura following a decrease in pH can occur via activation of either ASICs or TRPV1 channels. Consistent with our previous work,² the contribution of TRPV1 was ruled out here since the specific TRPV1 antagonist AMG-9810 failed to block the pH 5 (previously demonstrated)² and pH 6-evoked allodynia (shown here). Although these experiments did not examine the ability of AMG-9810 to block allodynia at pH values higher than 6.0, it is unlikely that TRPV1 would play a role at these higher pH values since less than 10% of TRPV1 channels open and generate currents at pH values above 6.0.³⁰ In contrast, the non-specific ASIC antagonist amiloride blocked pH-evoked dural afferent activation and allodynia, further suggesting a role of ASICs in pH evoked signaling from the dura in a higher pH range than previously demonstrated.

Among the ASIC subtypes, ASIC3-containing channels are the most suitable candidates for detecting pH changes within the dura and initiating afferent signaling. This argument is based on their activation threshold at higher pH values compared with other ASIC subtypes, their high expression level in sensory neurons, and that ASIC3 is expressed in most trigeminal neurons.³¹ Our previous data implicated ASIC3 as the predominant ASIC subtype contributing to dural afferent signaling since the decay time constants of most dural afferent pH-evoked currents were within the range of ASIC3-, but not ASIC1-containing channels.⁵ Additionally, most dural afferents exhibited sustained currents at moderate pH, which is a property exclusive to ASIC3-containing channels.^{2, 7} Combined with our previous data, stimulation of the dura with pH values above 6.0 *in vitro* and *in vivo* as shown here is also consistent with a role for ASIC3-containing channels in dural afferent activation. Our experimental setting minimized the contributions of ASIC4, which is pH insensitive and ASIC2, which has a half maximum activating pH of between 4 to 5.⁴ Regardless, the use of tools selective for ASIC3 can more conclusively demonstrate a role for this channel subtype in signaling from the dura. We now show that the ASIC3 antagonist APETx2 blocks pH 6.0-evoked allodynia in awake animals further supporting the hypothesis that ASIC3 is the predominant ASIC contributing to dural afferent signaling. Our results also demonstrated the contribution of ASIC3 in dural afferents by showing that almost all pH-sensitive dural afferents can be activated by the specific ASIC3 activator, GMQ.³² Additionally, the immunohistochemical study described here also confirmed that approximately 80% of dural afferents express ASIC3 protein, consistent with the prior observation that a similar percentage of neurons express ASIC3-like currents at pH 6.0.² Taken together, these data support the conclusion that dural afferent pH responses are mediated predominantly through ASIC3-containing channels both *in vitro* and *in vivo*. This finding suggests that ASIC3 is a novel target for developing migraine therapies.

Although the current study implicates ASIC3 as the predominant acid sensor in dural afferents, our conclusions are somewhat limited by the pharmacological tools available for ASIC3. Recent studies indicate that APETx2 partially blocks Nav1.8 currents at an IC₅₀ value higher than that which inhibits ASIC3 channels,^{33,34} therefore it remains possible that blockade of Nav1.8 by APETx2 in dural afferents could also contribute to the reversal of the effects on allodynia seen here. However, inhibition of ASIC3 by 20 μ M APETx2 led to significant reduction of postoperative pain, similar to *in vivo* knockdown of ASIC3 by small interfering RNA, indicating that this dose is specific for ASIC3.⁸ Additionally, a role for ASIC1a in dural afferents cannot be excluded here. The potential for the contribution of ASIC1 in a heteromer with ASIC3 is high as the current kinetics observed in our prior work are consistent with heteromeric channels. Our present findings with GMQ do not necessarily differentiate between ASIC3 homomers and heteromers so GMQ responses observed here could be mediated by ASIC1-containing channels. Recent studies, additionally, have demonstrated the importance of ASIC1a in cortical spreading depression,³⁵ but its role in dural afferents was not clear from these studies. Consequently, our results are consistent with a major role for ASIC3 in sensing pH within the dura, but they cannot exclude a role for other mechanisms.

In humans, pain due to acidic stimulation of the skin is augmented under sensitized conditions,³⁶ suggesting that pH-evoked responses from the meninges could also be enhanced by sensitization. Mast cells have been implicated in migraine pathophysiology³⁷ and are a likely source of sensitizing mediators within the dura. A cohort study conducted by the Mayo Clinic (Rochester, MN) showed a high prevalence of headache in patients with mastocytosis, a disorder characterized by an increased number of tissue mast cells, further supporting a role for mast cells in headaches such as migraine.³⁸ Following migraine-related triggers such as stress, CGRP, or nitroglycerin, mast cells can degranulate and release a host of inflammatory mediators.³⁹ Additionally, the internal contents of mast cell granules are

acidic.⁴⁰ Thus, mast cell degranulation may acidify the environment immediately surrounding dural afferent endings while the mediators released from mast cells can lead to further sensitization and prolonged activation. It should also be noted that ASIC3-expressing trigeminal ganglion neurons contribute to low pH-evoked CGRP release.³¹ Following a decrease in pH, ASIC3-expressing dural afferents may release CGRP contributing to further degranulation of mast cells. Consequently, there could be a cycle occurring within the meninges where mast-cells degranulate, the pH then drops leading to afferent activation, CGRP is released from afferents causing more mast cells to degranulate leading to further pH changes. The exact sequence of events that occurs is not clear but any of these components could feed into the cycle amplifying the inflammatory cascade and promoting activation/sensitization of dural afferents. Our results nonetheless demonstrated that mast cell mediators were able to sensitize dural afferents in response to pH stimuli. These experiments showed that the magnitude of the pH change within the dura that leads to activation of afferent signaling (and likely headache) can be smaller than previously proposed when the pH change is combined with other factors in the dura such as mediators released following mast cell degranulation. These data further highlight the importance of even small changes in pH in inducing afferent nociceptive signaling from the dura. Eliminating the ability of dural afferents to respond to small changes in pH, even during inflammation, may allow drugs blocking ASIC3 to have therapeutic efficacy for headache.

The exact mechanism of sensitization is not clear from these studies. Activation of the 5-HT₂ receptor potentiates ASIC currents and shifts the pH-dependence of activation curve upwards and to slightly more neutral pH while 5HT was shown recently to directly potentiate ASIC3.^{41, 42} Thus, it is possible that sensitization of ASICs by 5HT released from mast cells could lead to the enhanced sensitivity to pH change in dural afferents. It is also possible that 5HT could open the 5HT₃ channel and ASIC3 together, creating an additive effect that leads to afferent activation. Alternatively, many of the mast cell mediators can signal to ion channels through various second-messenger systems leading to sensitization of dural afferents. In this study, we observed a depolarization of the resting membrane potential in vitro with mast cell mediators, suggesting sensitization of voltage-gated channels, which likely contribute to the effects observed here. Further, in the presence of sodium channel sensitization, blockade of sodium channels by APETx2 may become more prominent and may contribute to the effects observed here with this toxin. Our data are not consistent with mast cell mediators recruiting additional pH-sensitive channels as the kinetics of pH-evoked currents are similar before and after sensitization, indicating that there is no significant change of channel composition in response to pH following sensitization. TRPV1 channels desensitize much more slowly than ASICs and an increase in TRPV1 current in dural afferents would have increased the desensitization time constant significantly. Further study is necessary to uncover the exact mechanism leading to increased sensitivity of dural afferents to pH in the presence of mast cell mediators.

In conclusion, this study identified ASIC3 as a key element in mediating pH evoked dural afferent activation and migraine-related pain behavior, particularly in the presence of mast-cell degranulation. Following sensitization, changes in meningeal pH are likely to become even more important contributors to activation of dural afferent signaling, suggesting that inhibitors of ASIC3 may represent new candidates for migraine therapy.

Acknowledgments

This work was supported by funds from The American Pain Society (GD), The National Headache Foundation (GD) and NIH NS072204 (GD).

Abbreviations

ASIC	Acid sensing ion channels (ASICs)
GMQ	2-guanidine-4-methylquinazoline

References

1. Goadsby PJ, Lipton RB, Ferrari MD. Migraine--current understanding and treatment. *N Engl J Med*. 2002; 346:257–270. [PubMed: 11807151]
2. Yan J, Edelmayer RM, Wei X, Felice MD, Porreca F, Dussor G. Dural afferents express acid-sensing ion channels: A role for decreased meningeal pH in migraine headache. *Pain*. 2011; 152:106–113. [PubMed: 20971560]
3. Wemmie JA, Price MP, Welsh MJ. Acid-sensing ion channels: advances, questions and therapeutic opportunities. *Trends Neurosci*. 2006; 29:578–586. [PubMed: 16891000]
4. Lingueglia E. Acid-sensing ion channels in sensory perception. *J Biol Chem*. 2007; 282:17325–17329. [PubMed: 17430882]
5. Benson CJ, Xie J, Wemmie JA, et al. Heteromultimers of DEG/ENaC subunits form H⁺-gated channels in mouse sensory neurons. *PNAS*. 2002; 99:2338–2343. [PubMed: 11854527]
6. Page AJ, Brierley SM, Martin CM, et al. Different contributions of ASIC channels 1a, 2, and 3 in gastrointestinal mechanosensory function. *Gut*. 2005; 54:1408–1415. [PubMed: 15987792]
7. Yagi J, Wenk HN, Naves LA, McCleskey EW. Sustained currents through ASIC3 ion channels at the modest pH changes that occur during myocardial ischemia. *Circ Res*. 2006; 99:501–509. [PubMed: 16873722]
8. Deval E, Noel J, Gasull X, et al. Acid-sensing ion channels in postoperative pain. *J Neurosci*. 2011; 31:6059–6066. [PubMed: 21508231]
9. Sluka KA, Radhakrishnan R, Benson CJ, et al. ASIC3 in muscle mediates mechanical, but not heat, hyperalgesia associated with muscle inflammation. *Pain*. 2007; 129:102–112. [PubMed: 17134831]
10. Jones RC 3rd, Xu L, Gebhart GF. The mechanosensitivity of mouse colon afferent fibers and their sensitization by inflammatory mediators require transient receptor potential vanilloid 1 and acid-sensing ion channel 3. *J Neurosci*. 2005; 25:10981–10989. [PubMed: 16306411]
11. Sutherland SP, Benson CJ, Adelman JP, McCleskey EW. Acid-sensing ion channel 3 matches the acid-gated current in cardiac ischemia-sensing neurons. *Proc Natl Acad Sci U S A*. 2001; 98:711–716. [PubMed: 11120882]
12. Waeber C, Moskowitz MA. Migraine as an inflammatory disorder. *Neurology*. 2005; 64:S9–15. [PubMed: 15911785]
13. Dimlich RV, Keller JT, Strauss TA, Fritts MJ. Linear arrays of homogeneous mast cells in the dura mater of the rat. *J Neurocytol*. 1991; 20:485–503. [PubMed: 1869885]
14. Strassman AM, Weissner W, Williams M, Ali S, Levy D. Axon diameters and intradural trajectories of the dural innervation in the rat. *J Comp Neurol*. 2004; 473:364–376. [PubMed: 15116396]
15. Theoharides TC, Kalogeromitros D. The critical role of mast cells in allergy and inflammation. *Ann N Y Acad Sci*. 2006; 1088:78–99. [PubMed: 17192558]
16. Rozniecki JJ, Dimitriadou V, Lambracht-Hall M, Pang X, Theoharides TC. Morphological and functional demonstration of rat dura mater mast cell-neuron interactions in vitro and in vivo. *Brain Res*. 1999; 849:1–15. [PubMed: 10592282]
17. Levy D, Burstein R, Kainz V, Jakubowski M, Strassman AM. Mast cell degranulation activates a pain pathway underlying migraine headache. *Pain*. 2007; 130:166–176. [PubMed: 17459586]
18. Wei X, Edelmayer RM, Yan J, Dussor G. Activation of TRPV4 on dural afferents produces headache-related behavior in a preclinical rat model. *Cephalalgia*. 2011
19. Edelmayer RM, Vanderah TW, Majuta L, et al. Medullary pain facilitating neurons mediate allodynia in headache-related pain. *Ann Neurol*. 2009; 65:184–193. [PubMed: 19259966]
20. Edelmayer RM, Le LN, Yan J, et al. Activation of TRPA1 on dural afferents: a potential mechanism of headache pain. *Pain*. 2012; 153:1949–1958. [PubMed: 22809691]

21. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods*. 1994; 53:55–63. [PubMed: 7990513]
22. Gardell LR, Ma JN, Seitzberg JG, et al. Identification and characterization of novel small-molecule protease-activated receptor 2 agonists. *J Pharmacol Exp Ther*. 2008; 327:799–808. [PubMed: 18768780]
23. Yan J, Melemedjian OK, Price TJ, Dussor G. Sensitization of dural afferents underlies migraine-related behavior following meningeal application of interleukin-6 (IL-6). *Mol Pain*. 2012; 8:6. [PubMed: 22273495]
24. Diochot S, Baron A, Rash LD, et al. A new sea anemone peptide, APETx2, inhibits ASIC3, a major acid-sensitive channel in sensory neurons. *Embo J*. 2004; 23:1516–1525. [PubMed: 15044953]
25. Deval E, Noel J, Gasull X, et al. Acid-sensing ion channels in postoperative pain. *J Neurosci*. 31:6059–6066. [PubMed: 21508231]
26. Yu Y, Chen Z, Li WG, et al. A nonproton ligand sensor in the acid-sensing ion channel. *Neuron*. 68:61–72. [PubMed: 20920791]
27. Zhang XC, Strassman AM, Burstein R, Levy D. Sensitization and activation of intracranial meningeal nociceptors by mast cell mediators. *J Pharmacol Exp Ther*. 2007; 322:806–812. [PubMed: 17483291]
28. Vanderslice P, Ballinger SM, Tam EK, Goldstein SM, Craik CS, Caughey GH. Human mast cell tryptase: multiple cDNAs and genes reveal a multigene serine protease family. *Proc Natl Acad Sci U S A*. 1990; 87:3811–3815. [PubMed: 2187193]
29. Zhang XC, Levy D. Modulation of meningeal nociceptors mechanosensitivity by peripheral proteinase-activated receptor-2: the role of mast cells. *Cephalalgia*. 2008; 28:276–284. [PubMed: 18254896]
30. Neelands TR, Jarvis MF, Han P, Faltynek CR, Surowy CS. Acidification of rat TRPV1 alters the kinetics of capsaicin responses. *Mol Pain*. 2005; 1:28. [PubMed: 16191202]
31. Durham PL, Masterson CG. Two mechanisms involved in trigeminal CGRP release: implications for migraine treatment. *Headache*. 2013; 53:67–80. [PubMed: 23095108]
32. Yu Y, Chen Z, Li WG, et al. A nonproton ligand sensor in the acid-sensing ion channel. *Neuron*. 2010; 68:61–72. [PubMed: 20920791]
33. Blanchard MG, Rash LD, Kellenberger S. Inhibition of voltage-gated Na(+) currents in sensory neurons by the sea anemone toxin APETx2. *Br J Pharmacol*.
34. Peigneur S, Beress L, Moller C, Mari F, Forssmann WG, Tytgat J. A natural point mutation changes both target selectivity and mechanism of action of sea anemone toxins. *FASEB J*. 2012; 26:5141–5151. [PubMed: 22972919]
35. Holland PR, Akerman S, Andreou AP, Karsan N, Wemmie JA, Goadsby PJ. Acid-sensing ion channel 1: a novel therapeutic target for migraine with aura. *Ann Neurol*. 2012; 72:559–563. [PubMed: 23109150]
36. Jones NG, Slater R, Cadiou H, McNaughton P, McMahon SB. Acid-Induced Pain and Its Modulation in Humans. *J Neurosci*. 2004; 24:10974–10979. [PubMed: 15574747]
37. Theoharides TC, Donelan J, Kandere-Grzybowska K, Konstantinidou A. The role of mast cells in migraine pathophysiology. *Brain Res Brain Res Rev*. 2005; 49:65–76. [PubMed: 15960987]
38. Smith JH, Butterfield JH, Pardanani A, Deluca GC, Cutrer FM. Neurologic symptoms and diagnosis in adults with mast cell disease. *Clin Neurol Neurosurg*. 113:570–574. [PubMed: 21664760]
39. Levy D. Migraine pain, meningeal inflammation, and mast cells. *Curr Pain Headache Rep*. 2009; 13:237–240. [PubMed: 19457286]
40. De Young MB, Nemeth EF, Scarpa A. Measurement of the internal pH of mast cell granules using microvolumetric fluorescence and isotopic techniques. *Arch Biochem Biophys*. 1987; 254:222–233. [PubMed: 3579299]
41. Qiu F, Qiu CY, Liu YQ, Wu D, Li JD, Hu WP. Potentiation of acid-sensing ion channel activity by the activation of 5-HT(2) receptors in rat dorsal root ganglion neurons. *Neuropharmacology*. 2012; 63:494–500. [PubMed: 22580376]

42. Wang X, Li WG, Yu Y, et al. Serotonin facilitates peripheral pain sensitivity in a manner that depends on the nonproton ligand sensing domain of ASIC3 channel. *J Neurosci.* 2013; 33:4265–4279. [PubMed: 23467344]

Figure 1. Application of pH 6.0 SIF solution to the dura elicited cutaneous allodynia via activation of ASIC3 subunit

Withdrawal thresholds to tactile stimuli applied to the face (A) and the hindpaws (B) were measured in rats before and immediately after dural application of pH 6.0 SIF ($n = 11$) or pH 7.4 SIF ($n = 13$). (C) Application of pH 6.0 solution was given with either vehicle (1% DMSO, white bar, $n = 13$), amiloride (100 nmol, grey bar, $n = 12$) or AMG-9810 (10 nmol, black bar, $n = 10$). Withdrawal thresholds to tactile stimuli were measured for 5 hrs and data were converted to area over the time-effect curve and normalized as a percentage of the pH 6.0 – treated group to allow for multiple comparisons. Comparisons among several treatment groups were performed by one-way ANOVA followed by post hoc Dunnett's test.

Coapplication of amiloride significantly abolished behavioral signs of tactile allodynia of the face and hindpaw (* $p < 0.05$, ** $p < 0.01$). Cotreatment with AMG-9810 failed to prevent development of behavioral signs of tactile allodynia of the face or hindpaw. (D) Application of pH 6.0 solution was given alone (white bar, $n = 13$) or with the selective ASIC3 antagonist APETx2 (200 pmol, grey bar, $n = 13$). Coapplication of APETx2 (200 pmol) significantly abolished behavioral signs of tactile allodynia of the face and hindpaw (* $p < 0.05$, ** $p < 0.01$).

Figure 2. Retrogradely-labeled dural afferents express ASIC3 immunoreactivity

Images are representative sections of trigeminal ganglia taken from rats in which fluorogold was applied to the dura 7 days prior. Immunolabeling with an ASIC3-specific primary antibody and Alexa Fluor 555-conjugated secondary antibody is shown in red in (A). Retrogradely-labeled dural afferents (fluorogold-positive neurons) are shown in blue in (B). An overlay of (A) and (B) is shown in (C). Neurons that appear purple are dural afferents that express ASIC3 immunoreactivity.

Figure 3. pH-evoked responses were mediated through ASIC3 subunits in vitro
GMQ-evoked currents in pH-sensitive dural afferents. Representative traces illustrating acid-(pH 6.0) and GMQ-induced currents in a dural afferent.

Figure 4. Application of pH to the dura dose dependently elicited cutaneous allodynia
 (A) Withdrawal thresholds to tactile stimuli applied to the face and the hindpaws were measured in rats before and immediately after dural application of pH 6.4, pH 6.6, pH 6.8, and pH 7.4 solutions. (B) Data were converted to area over the time-effect curve to allow for multiple comparisons. pH dose-dependently decreased the withdrawal threshold both in the face and the hindpaws. Significant (* $p < 0.05$) differences among means for each group were determined by one-way ANOVA followed by Dunnett's post hoc test.

Figure 5. Animals exhibited enhanced withdrawal responses to pH changes following coapplication of sub-threshold mast cell mediators

M indicates sub-threshold mast cell mediators, which were composed of Histamine (200 μ M), Serotonin (200 μ M), AC55541 (10 μ M) and Iloprost (20 μ M). Data were converted to area over the time-effect curve to allow for multiple comparisons. Significant (** $p < 0.01$) differences among means for each group were determined by one-way ANOVA followed by Dunnett's post hoc test. Application of sub-threshold mast cell mediators (black bar, $n = 9$) by itself did not cause significant changes in facial and hindpaw withdrawal thresholds compared with SIF administration (white bar, $n = 11$). (A) Coapplication of sub-threshold mast cell mediators and pH 6.6 solution caused a significant decrease in facial withdrawal threshold (red bar, $n = 12$) compared with pH 7.4 application, whereas application of pH 6.6 solution by itself did not (red bar, $n = 13$). (B) Coapplication of sub-threshold mast cell mediators and pH 6.8 solution caused a significant decrease in hindpaw withdrawal threshold (blue bar, $n = 14$) compared with pH 7.4 application, whereas application of pH 6.8 solution by itself did not (blue bar, $n = 14$).

Figure 6. Dural afferents exhibited enhanced sensitivity to pH-induced action potential firing following application of mast cell mediators

Mast cell mediators were composed of Histamine (100 μ M), Serotonin (10 μ M), AC55541 (10 μ M), Ilprost (1 μ M). (A) The percentage of neurons firing action potentials was calculated before and after a 5 min application of mast cell mediators. For each pH tested, the percentage of neurons firing action potentials increased following application of mast cell mediators. $n = 23$. (B) Number of spikes was counted from the same dural afferent before and after 5 mins application of mast cell mediators. Application of mast cell mediators significantly increased the number of spikes for each pH tested (paired t-test, $**p < 0.01$ for pH 6.9, $*p < 0.05$ for pH 6.8 and pH 7.0) (C) Application of mast cell mediators led to the transient and persistent firing of action potential at pH 7.0 and 6.9 in two representative dural afferents.