Title

ASIC3 blockade inhibits durovascular and nitric oxide-mediated trigeminal pain

Running head

ASIC3 in migraine

Christopher M Holton. PhD[1*], Lauren C Strother. MSc[1*], Isaac Dripps. PhD[2] Amynah Pradhan. PhD[2], Peter J Goadsby. MD, PhD[1] and Philip R Holland. PhD[1]

*C.M.H and L.C.S. contributed equally to this work.

[1]Headache Group, Basic and Clinical Neuroscience, Institute of Psychiatry, Psychology and Neuroscience, King's College London UK.

[2]Department of Psychiatry, University of Illinois at Chicago, USA.

Correspondence:

Dr Philip R Holland
Department of Basic and Clinical Neuroscience, Institute of Psychiatry, Psychology & Neuroscience, King's College London, 125 Coldharbour Lane, London SE5 9NU. UK.
Telephone: +44 02078484222
Email: philip.holland@kcl.ac.uk

No of Characters in title: 78, No of Characters in running title: 17, No of words in the abstract: 250; introduction: 595; discussion: 415 and body of the manuscript: 2149.

No of Figures: 3, No of Colour Figures: 0, No of Tables: 0, No of References: 26.

Acknowledgments

This work was supported by the FP7 project EUROHEADPAIN (no. 602633), The Wellcome Trust (Synaptopathies; 104033), the Medical Research Council (MR/P006264/1), The Migraine Trust and the NIH (no. DA40688).

Abstract

Background and Purpose

There is a major unmet need to develop new therapies for migraine. We have previously demonstrated the therapeutic potential of the acid sensing ion channel (ASIC) blockade in migraine, via an ASIC1 mechanism. ASIC3 is expressed in the trigeminal ganglion and its response is potentiated by nitric oxide that can trigger migraine attacks in patients, and thus we sought to explore the potential therapeutic effect of ASIC3 blockade in migraine.

Experimental Approach

To investigate this, we utilised validated electrophysiological and behavioural rodent preclinical models. In rats, ASIC3 blockade via APETx2 (i.v. 50 or 100 μgkg^{-1}) was measured by using durovascular and nitric oxide-evoked trigeminal nociceptive responses and cortical spreading depression models. In mice, we sought to determine if periorbital mechanical sensitivity, induced by acute nitroglycerin (i.p. $10mgkg^{-1}$), was attenuated by APETx2 (i.p. $230\mu gkg^{-1}$), as well as latent sensitisation induced by bright light stress in a chronic nitroglycerin model.

Key Results

Here, we show that the ASIC3 blocker APETx2 inhibits durovascular evoked and nitric oxide-induced sensitisation of trigeminal nociceptive responses in rats. In agreement, acute and chronic periorbital mechanosensitivity induced in mice by nitroglycerin and subsequent bright light stress evoked latent sensitivity as a model of chronic migraine, are all reversed by APETx2.

Conclusion and Implications

These results support the development of specific ASIC3 or combined ASIC1/3 blockers for migraine-related pain and point to a potential role for ASIC-dependent nitric oxide mediated attack triggering. This has key implications for migraine, given the major unmet need for novel therapeutic targets.

Keywords:

Acid sensing ion channels, migraine, pain, headache.

Abbreviations

ASIC, acid sensing ion channel; CSD, cortical spreading depression; NO, nitric oxide; NTG, nitroglycerin; SNP, sodium nitroprusside; TNC, trigeminal nucleus caudalis

What is already known

- Acid sensing ion channels (ASIC) are a potential therapeutic target for migraine, likely via ASIC1.

What does this study add

- ASIC3 blockade with APETx2 inhibits durovascular-evoked trigeminal nociceptive processing.
- APETx2 inhibits trigeminal sensitization induced by nitric oxide donors that are known migraine triggers clinically.

What is the clinical significance

- ASIC3 or combined ASIC1/3 blockers represent potential therapies for migraine.
- Nitric oxide mediated migraine triggering may be in part ASIC-dependent.

Introduction

Migraine is a severe disabling brain disorder (Stovner, Nichols, Steiner, Abd-Allah, Abdelalim & Al-Raddadi, 2018) characterized by bouts of unilateral pain resulting from activation of trigeminal sensory neurons and sensitization of nociceptive processing (Goadsby, Holland, Martins-Oliveira, Hoffmann, Schankin & Akerman, 2017). Preclinically, nitric oxide (NO) donors induce a delayed cutaneous allodynia-like phenotype in rodents (Bates et al., 2010) in conjunction with increased trigeminal neuronal activity and hypersensitivity to intracranial and extracranial sensory stimulation (Akerman et al., 2019). Clinically, exposure to nitroglycerin (NTG) an 'NO donor' produces a transient headache in healthy controls (Ashina, Hansen, BO & Olesen, 2017) and the occurrence of migraine premonitory symptoms (Afridi, Kaube & Goadsby, 2004), delayed migraine-like attacks (Ashina, Hansen, BO & Olesen, 2017) and triptan responsive cranial allodynia (Akerman et al., 2019) in migraineurs. While vasodilation may contribute to the acute headache, alternate mechanisms are likely involved in the delayed migraine-like attacks (Marone et al., 2018); however, the mechanisms that lead to delayed hyperalgesia remain to be fully characterised. A greater understanding of which is required to help elucidate how individual migraine attacks are initiated and aid in the development of novel therapeutic targets.

Sensory neurons expressing acid sensing ion channels (ASICs) convey nociception during several pain states in response to decreased extracellular pH (Ugawa, Ueda, Ishida, Nishigaki, Shibata & Shimada, 2002; Yan, Edelmayer, Wei, De Felice, Porreca & Dussor, 2011; Yan, Wei, Bischoff, Edelmayer & Dussor, 2013). ASIC3 is the most sensitive ASIC to physiologically decreased pH (Deval et al., 2008) and as such, may play a critical role in the initial phases of trigeminal sensitisation. It is co-expressed with CGRP in the rat trigeminal ganglion (Ichikawa & Sugimoto, 2002), where decreased pH results in CGRP release (Durham & Masterson, 2013). Pharmacologically, the anti-migraine therapeutic agent sumatriptan

inhibits the activity of ASICs in the rat trigeminal ganglion (Guo, Zhao, Ming, Hong, Liu & Li, 2018), while an ASIC-sensitive proton-mediated mechanism for the release of CGRP has been demonstrated. Given the therapeutic utility of the triptans (Ong & De Felice, 2018) and targeted modulation of CGRP signalling (Goadsby et al., 2017), ASIC modulation may represent a novel interface with important translational implications.

Importantly, ASIC3 is potentiated by NO donors and NTG increases acid-evoked pain in humans (Cadiou et al., 2007). In agreement with a role for ASICs in migraine, we and others have previously identified the anti-migraine efficacy of targeting specific ASICs in several validated preclinical models (Holland, Akerman, Andreou, Karsan, Wemmie & Goadsby, 2012; Verkest et al., 2018; Wang et al., 2018; Yan, Edelmayer, Wei, De Felice, Porreca & Dussor, 2011).

Given the emerging role for ASICs in migraine (Karsan, Gonzales & Dussor, 2018), the expression of ASIC3 in the trigeminal ganglion (Ichikawa & Sugimoto, 2002) and trigeminal nucleus caudalis (TNC) (Wang et al., 2018), the enhancement of ASIC3 activity by NO donors (Cadiou et al., 2007), we sought to determine the role of ASIC3 in migraine and further determine if NO-induced hyperalgesia may be in part ASIC3-dependent. We report that durovascular-evoked and NO-induced sensitisation of trigeminal nociceptive responses in the TNC are inhibited by ASIC3 blockade. We further demonstrate that NTG-evoked delayed cutaneous allodynia in mice is attenuated by ASIC3 blockade. Finally, ASIC3 blockade reverses the delayed cutaneous allodynia evoked by environmental bright light stress in an NTG-mediated preclinical model of chronic migraine (Kopruszinski et al., 2017; Tipton, Tarash, McGuire, Charles & Pradhan, 2016). As such, ASIC3 may play a key role in migraine pathophysiology and NO-induced delayed migraine in patients, highlighting further the potential of targeted modulation of ASICs and the future development of mambalgins for migraine.

Methods

All procedures were conducted according to the Animals (Scientific Procedures) Act (1986), ethically approved by local animal welfare and ethical review bodies. Experimental protocols and design are in agreement with BJP and ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson & Altman, 2010). In total 43 male adult Sprague Dawley rats (280-315 g) and 36 adult male C57Bl6/J mice (20-30 g) (Charles River, UK) were included in the study. Animals were group housed in standard cages in climate-controlled rooms with a 12hr light/dark cycle (07:00 – 19:00) and food and water provided *ad libitum.*

General surgical setup

On the day of the surgery, rats (n = 43) were initially anesthetised with isoflurane (IsoFlo, 5%, Abbott, UK (RRID:SCR_010477)) and maintained with 1.5 - 2%. Following cannulation of the left femoral artery and both femoral veins the animals were switched to a continuous intravenous propofol infusion (Propoflo, 33 - 50 $mgkgh^{-1}$, Abbott, UK). The additionally cannulated vein and artery were then used for the administration of test substances and to continuously monitor blood pressure, respectively. Anaesthetic depth was confirmed by the lack of a withdrawal response or gross fluctuations in blood pressure to noxious pinching of the hind paw. Following cannulation, rats were tracheotomised and ventilated with oxygen enriched air, with end-tidal CO_2 continuously monitored and ventilation adjusted as required to maintain normal physiological paramaters (3.5 - 4.5%). Rectal temperature was maintained at 36.5 - 37°C via a rectal probe connected to a heating pad.

Following this initial surgery, rats were placed in a stereotaxic frame and underwent one of the following surgical preparations and experimental procedure as detailed below.

Neuronal recording in the trigeminal nucleus caudalis

To assess trigeminal nociceptive responses, the parietal bone was thinned to access the dura mater overlying the middle meningeal artery (MMA) and the area was covered in mineral oil to prevent drying. To access the trigeminal nucleus caudalis (TNC), a partial laminectomy of the first cervical vertebra was performed and the dura mater was opened to expose the caudal medulla. After completion of the surgery, animals were left to stabilize for at least 30 min before recording.

Stimulation of perivascular afferents of the trigeminal nerve was performed by placing a bipolar stimulating electrode on the dura mater adjacent to the MMA. Dural nociceptive neurons in the TNC were identified via electrical stimulation (8-15 V, 0.5 Hz, 0.3-0.5 ms, 20 square wave electrical pulses) of the dura mater. Tungsten microelectrodes (0.5–1 MΩ) were carefully lowered into the TNC and used to record extracellularly from neurons, activated by dural electrical stimulation and with cutaneous facial receptive fields in the ophthalmic dermatome. The signal was amplified, filtered and recorded as previously described (Vila-Pueyo, Strother, Kefel, Goadsby & Holland, 2019).

When a cluster of wide dynamic range neurons sensitive to stimulation of the ophthalmic dermatome of the trigeminal nerve was identified, it was tested for convergent input from the dura mater. Trains of 20 stimuli were delivered at 5 min intervals to assess the baseline response to dural electrical stimulation. Responses were analysed using post-stimulus histograms with a sweep length of 100 ms and a bin width of 1 ms. When stable baseline values of the stimulus-evoked responses were achieved (average of 3 stimulation series) responses were tested for up to 60 min following physiological intervention. For studies involving sodium nitroprusside (SNP) Facial receptive field characterization consisted of 10 brush strokes applied to the facial receptive field over 7 – 8 s for the innocuous response, and pinch with forceps for 5 s for the

noxious response, with the change in cell firing from baseline at 50 mins post intervention used for analysis. Spontaneous activity (spikes per second, Hz) was recorded throughout and measures for analysis consisted of 60 s epochs at baseline and then every 10 mins. Post and peri-stimulus time histograms of neural activity were displayed and analysed using Spike2 v8.

Following the establishment of stable baseline neuronal responses, rats (18 neuronal clusters from n = 16 rats) were administered either vehicle (0.5 ml normal saline) or APETx2 (Alomone Labs, Israel) at 50 or 100µgkg^{-1} (n = 6 neuronal clusters per group) and durovascular-evoked neuronal responses and spontaneous TNC neuronal activity recorded for 60 minutes.

To explore the impact of APETx2 on nitric oxide donor-induced trigeminal neuronal sensitisation rats (28 neuronal clusters from n = 22 rats) were assessed. Following the establishment of stable baseline neuronal responses, rats received one of 4 treatment protocols (n = 7 neuronal clusters per group): saline/saline; APETx2 100 µgkg^{-1}/saline; saline/SNP 60 µgkg^{-1} or APETx2 100 µgkg^{-1}/ SNP 60 µgkg^{-1}. The impact of APETx2 on noxious and non-noxious stimulation of the periorbital receptive field was conducted on a subset of the above rats (n = 5 per group).

Saline and APETx2 were administered as a slow intravenous bolus and SNP as a slow intravenous infusion (4 µgkgmin^{-1}) over 15 minutes that resulted in a transient sensitisation over the infusion period that returned to baseline within 10 minutes of cessation. SNP-induced neuronal sensitisation was recorded immediately following the cessation of acute SNP infusion, 50 mins post APETx2 or vehicle control.

Cortical spreading depression recording

In a separate cohort of rats to assess cortical spreading depression (CSD) induction, (n = 5) a cranial window of approximately 2 x 2 mm was drilled in the parietal bone using a saline-

cooled drill and the underlying dura mater carefully removed. This area was used for the insertion of a single needle 500 µm into the cortex to induce a CSD event. Posterior to bregma, a similar opening was drilled in the parietal bone. In this area, a glass pipette with a tip diameter of 10 µm filled with 3M NaCl was placed 500 µm below the cortical surface for cortical steady state potential recording (direct current (DC) shift). The pipette was connected to an Ag/AgCl pellet electrode and an Ag/AgCl reference electrode that was placed subcutaneously in the neck. The electrode was connected to a headstage and signal was amplified, filtered and displayed in a personal computer as previously described (Holland, Akerman, Andreou, Karsan, Wemmie & Goadsby, 2012). To ensure reliable CSD induction, 2 control CSDs were initiated, the second of which occurred 5 mins following the administration of vehicle control. Following an appropriate refractory period, rats were then administered APETx2 at 100µgkg^{-1} and subsequent CSD inductions conducted at 30 and 70 mins post APETx2.

Nitric Oxide induced periorbital mechanical hypersensitivity

To further assess the efficacy of ASIC3 blockade on NO donor induced trigeminal sensitisation we utilised two different models of nitroglycerin (NTG)-evoked periorbital mechanical hypersensitivity in mice. Facial mechanosensitivity was assessed via von-Frey filament application to the periorbital region of the face. To do so, animals were acclimatized to the testing apparatus at least one day before and again 1 hour prior to testing. The testing apparatus consisted of individual ventilated acrylic enclosures (6 x 6 cm) placed on a self-standing perforated metal platform (Ugo Basile). To facilitate facial sensory testing, mice were additionally habituated to a 4 oz paper cup, placed inside the acrylic enclosure. (Tipton, Tarash, McGuire, Charles & Pradhan, 2016). Facial mechanical sensitivity was then tested with the mouse freely moving within the up-right paper cup that permitted unhindered access to the

periorbital region, caudal to the eyes and near the midline. Graduated von Frey filaments were then applied to the periorbital region starting with the 0.4 g filament using the up-down method to calculate mechanical withdrawal thresholds (Chaplan, Bach, Pogrel, Chung & Yaksh, 1994).

In the acute study mice (n = 12) were tested at baseline and following 2 hours post NTG treatment (10mgkg^{-1}, i.p.). To minimize the number of mice and in keeping with the ARRIVE guidelines, mice were retested following one week. Mice received 2 doses of APETx2 (0.23 mgkg^{-1}, i.p.) and a single dose of NTG (10 mgkg^{-1} i.p.) and periorbital withdrawal thresholds were assessed 2 hours post NTG. Due to its short half-life, APETx2 was administered 30 mins before NTG treatment and again 30 mins before sensory threshold testing.

To assess the efficacy of APETx2 to block NO donor-induced trigeminal sensitisation in a more chronic paradigm we adapted a rodent model of triptan-induced latent sensitisation (Kopruszinski et al., 2017). In this model, rodents exposed to persistent sumatriptan exposure that is subsequently withdrawn develop a basal periorbital mechanical hypersensitivity that normalises following triptan withdrawal. However, rodents previously exposed to sumatriptan but not vehicle control demonstrate a "latent sensitisation" whereby exposure to a presumed migraine trigger (bright light stress) reinstates the periorbital mechanical hypersensitivity. Persistent exposure to NTG, like sumatriptan, is also known to induce a basal periorbital mechanical hypersensitivity (Tipton, Tarash, McGuire, Charles & Pradhan, 2016) and as such, we hypothesised that bright light stress- induced latent sensitisation was also possible following chronic NTG administration and subsequent recovery, and furthermore, that ASIC3 blockade could modulate this. To test this hypothesis, baseline periorbital mechanosensitivity was assessed in a separate cohort of mice (n = 24), which were then counterbalanced into three groups that were randomly assigned to receive chronic vehicle control followed by APETx2 prior to bright light stress, chronic NTG followed by acute administration of vehicle control prior to bright light stress or chronic NTG followed by acute administration of APETx2 prior

to bright light stress. After baseline assessment, one animal was excluded due to a consistently low mechanical sensitivity threshold and as such the final analysis was conducted on n = 7, 8 and 8 mice respectively. Vehicle (0.9% saline, i.p.) and NTG (10 $mgkg^{-1}$ NTG, i.p,) were administered on days 1, 3, 5, 7 and 9, with mechanosensitivity assessed on days 1, 5 and 9 both before (basal) and 2 hrs post administration (Tipton, Tarash, McGuire, Charles & Pradhan, 2016). After the establishment of a basal hyperalgesia (day 9), mice were allowed to recover for at least 1 week until basal mechanosensitivity returned to baseline levels. All mice were then exposed to 1 hr of bright light stress (3000-3500 lux) on day 17 and 18 as previously reported (Kopruszinski et al., 2017). On day 18, mice were treated with APETx2 (0.23$mgkg^{-1}$) or vehicle (0.9% saline, i.p.) 30 mins before and again 30 mins post bright light stress and periorbital mechanosensitivity was assessed 2 hrs post bright light stress as detailed in figure 3D.

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis, Ashton, Moon & Ahluwalia, 2018) and were performed using GraphPad (RRID:SCR_002798) or SPSS (RRID:SCR_002865), in agreement with BJP guidelines. All studies were designed to generate groups of equal size and experimental animals or groups (behavioural analysis) were randomly assigned to treatment groups before being analysed blind to the experimental group. For behavioural experiments this consisted of initially counterbalancing the mice into four equal groups to minimize baseline variances and the four groups were then randomly assigned to control or experimental compounds. One mouse was subsequently excluded from the behavioural analysis as it showed signs of uncontrollable distress and was immediately and humanely culled. Therefore, in figure 3B-C

group sizes are n = 7, 8 and 8 for control, NTG and NTG plus APETx2, respectively. All statistical analysis was conducted on group sizes of at least n = 5. For behavioural analysis the group size reported is the number of independent values (mice) and statistical analysis was conducted on these independent values. For *in-vivo* electrophysiological studies to minimise animal use and only where stable recordings existed following testing of a vehicle control, a small proportion of rats were then randomly assigned to receive one of the experimental compounds/doses. To ensure these were not technical replicates an entirely new neuronal cluster was identified and as such the results contain the following biological replicates. In figure 1A-B the results reported are from n = 12 neuronal clusters in n = 10 rats. While in figure 2A the results reported are from n = 28 neuronal clusters in 22 rats. Sample sizes are based on previous studies (Holland, Akerman, Andreou, Karsan, Wemmie & Goadsby, 2012; Tipton, Tarash, McGuire, Charles & Pradhan, 2016) and sample size calculations with an estimated effect size = 20-40%, probability = 0.05, and power = 0.8-0.9 calculated using G.power software.

For graphical purposes, data is presented as mean ± standard error and all statistical analysis was conducted on raw data tested for homogeneity of sample variance (where appropriate) using the Levene's test. A level of probability of $P \leq 0.05$ was defined as the threshold for statistical significance and where appropriate, post hoc tests were only conducted following a significant ANOVA to protect against type 1 errors. The impact of APETx2 on evoked-trigeminal-neuronal responses was assessed via a mixed model 2-way ANOVA with Sidak's multiple comparisons, compared to the vehicle control treated rats. The impact of APETx2 on spontaneous trigeminal-neuronal firing was assessed via a repeated measures (RM)-ANOVA, compared to baseline firing rates. The impact of APETx2 on SNP-evoked trigeminal sensitisation was assessed via one-way ANOVA with Sidaks multiple comparison test, comparing the change in cell firing from baseline at the 50 min time point. For behavioural

analysis, the impact of APETx2 on acute NTG-evoked mechanical sensitivity was assessed via RM-ANOVA with Sidaks multiple comparison test. Changes in basal mechanical sensitivity in response to chronic NTG were assessed via mixed model 2-way ANOVA with Sidaks multiple comparison test. Subsequent analysis of the effect of bright light stress and APETx2 on NTG-primed mice was assessed via one-way ANOVA with Sidaks multiple comparison test as detailed in table 1. Where appropriate, if Mauchly's test of sphericity was violated, appropriate corrections to degrees of freedom according to Greenhouse-Geisser were made.

Materials

APETx2 (Alomone Labs, Israel) was dissolved in water to 500 $\mu g ml^{-1}$ and diluted in 0.9% saline. For rat studies APETx2 was administered intravenously at 50 or 100 $\mu g kg^{-1}$, while for mice APETx2 was administered intraperitoneally at 0.23 $mg kg^{-1}$. Sodium nitroprusside (SNP) (Sigma, UK) was dissolved in 0.9% saline immediately prior to use, with 60 $\mu g kg^{-1}$ infused intravenously over 15 minutes at 0.5 $ml hour^{-1}$. Nitroglycerin (Hospira, UK) was dissolved in 0.9% saline immediately prior to intraperitoneal injection at a final dose of 10 $mg kg^{-1}$.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

Results

APETx2 reduces durovascular-evoked nociceptive responses in the TNC

Durovascular nociceptive-evoked TNC neuronal responses (Figure 1A) were significantly reduced following ASIC3 blockade with APETx2 100μgkg^{-1} (Figure 1B-C, n = 6 neuronal clusters per group in 10 rats) in the absence of any blood pressure effects when compared to vehicle control treated rats. APETx2 also significantly reduced spontaneous TNC neuronal activity (Figure 1D-F, n = 6 neuronal clusters per group in 10 rats). APETx2 at 50μgkg^{-1} had no effect on any parameter tested (data not shown, n = 6 rats).

APETx2 reduces NO-evoked nociceptive responses in the TNC

Intravenous infusion of the NO donor SNP resulted in a transient increase in evoked TNC neural activity (Figure 2A, n = 7 neuronal clusters per group in 22 rats). Pre-treatment with APETx2 50-mins before SNP-induced sensitisation inhibited SNP-induced durovascular-evoked firing (Figure 2A). Further, SNP resulted in a modest non-significant increase in noxious pinch-evoked responses from the periorbital region (Figure 2B, n = 5 neuronal clusters per group in 20 rats). Pretreatment with APETx2 significantly reduced the noxious pinch-evoked responses when compared to SNP treated rats (Figure 2B). There was no impact of SNP on non-noxious stimulation of the periorbital region and APETx2 alone had no significant impact on durovascular, noxious or non-noxious evoked responses in the TNC (data not shown).

APETx2 does not inhibit mechanically-induced CSD

We have previously identified a potential ASIC1-dependent inhibition of CSD (Holland, Akerman, Andreou, Karsan, Wemmie & Goadsby, 2012); however, APETx2 has limited blood

brain barrier penetration, suggesting a potential lack of CNS actions. In agreement, with a likely peripheral action of APETx2 on durovascular-evoked nociceptive responses, we did not observe any inhibition of mechanically-induced CSDs in all animals (data not shown, n = 5).

APETx2 reduces nitroglycerin-evoked periorbital mechanosensitivity in mice

Acute NTG administration resulted in a significantly increased periorbital mechanosensitivity in mice. Pre-treatment with APETx2 resulted in a significant decrease in this acute NTG-induced periorbital mechanosensitivity that did not fully recover to baseline (Figure 3A, n = 12 mice).

In addition, we adapted a model of medication overuse "latent sensitization" in mice (Kopruszinski et al., 2017), whereby rats systemically exposed to triptans demonstrate cutaneous allodynia that normalizes following withdrawal (Kopruszinski et al., 2017). Despite normalized facial mechanosensitivity, these mice maintain a heightened response to presumed migraine triggers such as exposure to bright light stress. Herein, we induced this latent sensitization via chronic NTG exposure and following 1 week of withdrawal exposed mice to bright light stress. In agreement, exposure of mice to NTG for 9 days generated a periorbital mechanosensitivity when compared to vehicle control treated mice that normalised after 1 week withdrawal (Figure 3B, n = 23 mice). Subsequent exposure to bright light stress resulted in increased periorbital mechanosensitivity in NTG-primed mice, but not NTG-naïve mice (Figure 3C, n = 8 and 7 mice, respectively as 1 mouse was excluded from the control group due to ill health). While NTG-primed mice pre-treated with APETx2 showed no periorbital mechanosensitivity (Figure 3C, n = 8 mice per group) when compared to NTG-primed mice exposed to vehicle control.

Discussion

Here we demonstrate a clear translational potential for ASIC3 blockade in migraine. The ASIC3 blocker APETx2 inhibits both durovascular and NO-evoked trigeminal nociception in rats. Further, APETx2 inhibits NTG and bright light stress-evoked cutaneous allodynia in NTG-naïve and NTG-primed mice respectively. Given the ability of NO-donors such as NTG to trigger migraine attacks reliably in patients (Ashina, Hansen, BO & Olesen, 2017) and cutaneous allodynia/trigeminal sensitisation in rodents (Akerman et al., 2019; Bates et al., 2010), our data suggests that this NO-mediated effect may be at least in part be ASIC3-dependent. ASIC3 is expressed on trigeminal ganglion neurons (Ichikawa & Sugimoto, 2002) and dural afferents (Yan, Edelmayer, Wei, De Felice, Porreca & Dussor, 2011) and its activation by decreased pH leads to elevated CGRP release (Durham & Masterson, 2013). Given that targeted CGRP therapies likely have a significant peripheral effect at the level of the trigeminal ganglion, and represent the current state of the art therapeutics for migraine (Ong, Wei & Goadsby, 2018), we propose herein that ASIC3 blockade may represent a potential adjunct target for reducing CGRP release. Importantly, we have previously demonstrated that amiloride, via an ASIC1 mechanism showed similar beneficial effects on durovascular-evoked trigeminal nociception (Holland, Akerman, Andreou, Karsan, Wemmie & Goadsby, 2012). Specific ASIC1 blockade further inhibited CSD propagation that we did not observe in the current study, likely due limited blood brain barrier penetrability of APETx2, suggesting that the ASIC3-mediated effects are likely peripheral at the level of the trigeminal afferents (Yan, Wei, Bischoff, Edelmayer & Dussor, 2013). Although specific central effects cannot be ruled out as ASIC3 is expressed centrally, including in the hypothalamus (Meng, Wang, Chen, Xu & Zhou, 2009) and TNC, where its expression is upregulated in a dural-inflammatory mediated preclinical model of migraine (Wang et al., 2018). In agreement with the potential efficacy of targeting ASIC signalling for migraine it has recently been

demonstrated that specific blockade of the ASIC1 subunit can inhibit NO-evoked cutaneous allodynia in rats (Verkest et al., 2018), that supports our data and suggests that NO-mediated attack triggering may have important central and peripheral actions on ASICs.

The preclinical models utilised herein have demonstrated clear translational validity (Holland, Akerman, Andreou, Karsan, Wemmie & Goadsby, 2012; Kopruszinski et al., 2017; Tipton, Tarash, McGuire, Charles & Pradhan, 2016), including predicting clinical trial failure (Goadsby, Hoskin & Knight, 1998). As such, we propose that an ASIC3 or combined ASIC1/3 blocker may prove beneficial for the treatment of migraine and that ASIC-dependent mechanisms may in part underlie the increased susceptibility of migraineurs to NO-donors.

Conflict of interest

The authors report no actual or potential conflicts of interest relating to this specific study.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

References

Afridi SK, Kaube H, & Goadsby PJ (2004). Glyceryl trinitrate triggers premonitory symptoms in migraineurs. Pain 110**:** 675-680.

Akerman S, Karsan N, Bose P, Hoffmann JR, Holland PR, Romero-Reyes M*, et al.* (2019). Nitroglycerine triggers triptan-responsive cranial allodynia and trigeminal neuronal hypersensitivity. Brain : a journal of neurology 142**:** 103-119.

Alexander SPH, Mathie A, Peters JA, Veale EL, Striessnig J, Kelly E*, et al.* (2019). THE CONCISE GUIDE TO PHARMACOLOGY 2019/20: Ion channels. Br J Pharmacol 176 Suppl 1**:** S142-S228.

Ashina M, Hansen JM, BO AD, & Olesen J (2017). Human models of migraine - short-term pain for long-term gain. Nat Rev Neurol 13**:** 713-724.

Bates EA, Nikai T, Brennan KC, Fu YH, Charles AC, Basbaum AI*, et al.* (2010). Sumatriptan alleviates nitroglycerin-induced mechanical and thermal allodynia in mice. Cephalalgia 30**:** 170-178.

Cadiou H, Studer M, Jones NG, Smith ES, Ballard A, McMahon SB*, et al.* (2007). Modulation of acid-sensing ion channel activity by nitric oxide. The Journal of neuroscience : the official journal of the Society for Neuroscience 27**:** 13251-13260.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, & Yaksh TL (1994). Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 53**:** 55-63.

Curtis MJ, Ashton JC, Moon LDF, & Ahluwalia A (2018). Clarification of the basis for the selection of requirements for publication in the British Journal of Pharmacology. Br J Pharmacol 175**:** 3633-3635.

Deval E, Noël J, Lay N, Alloui A, Diochot S, Friend V*, et al.* (2008). ASIC3, a sensor of acidic and primary inflammatory pain. The EMBO Journal 27**:** 3047-3055.

Durham PL, & Masterson CG (2013). Two mechanisms involved in trigeminal CGRP release: implications for migraine treatment. Headache 53**:** 67-80.

Goadsby PJ, Holland PR, Martins-Oliveira M, Hoffmann J, Schankin C, & Akerman S (2017). Pathophysiology of Migraine: A Disorder of Sensory Processing. Physiol Rev 97**:** 553-622.

Goadsby PJ, Hoskin KL, & Knight YE (1998). Substance P blockade with the potent and centrally acting antagonist GR205171 does not effect central trigeminal activity with superior sagittal sinus stimulation. Neuroscience 86**:** 337-343.

Goadsby PJ, Reuter U, Hallstrom Y, Broessner G, Bonner JH, Zhang F*, et al.* (2017). A Controlled Trial of Erenumab for Episodic Migraine. N Engl J Med 377**:** 2123-2132.

Guo L, Zhao L, Ming P, Hong L, Liu A, & Li R (2018). Sumatriptan inhibits the electrophysiological activity of ASICs in rat trigeminal ganglion neurons. Eur J Pharmacol 841**:** 98-103.

Harding SD, Sharman JL, Faccenda E, Southan C, Pawson AJ, Ireland S*, et al.* (2018). The IUPHAR/BPS Guide to PHARMACOLOGY in 2018: updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. Nucleic Acids Res 46**:** D1091-D1106.

Holland PR, Akerman S, Andreou AP, Karsan N, Wemmie JA, & Goadsby PJ (2012). Acid-sensing ion channel 1: a novel therapeutic target for migraine with aura. Ann Neurol 72**:** 559-563.

Ichikawa H, & Sugimoto T (2002). The co-expression of ASIC3 with calcitonin gene-related peptide and parvalbumin in the rat trigeminal ganglion. Brain Research 943**:** 287-291.

Karsan N, Gonzales EB, & Dussor G (2018). Targeted Acid-Sensing Ion Channel Therapies for Migraine. Neurotherapeutics 15**:** 402-414.

Kilkenny C, Browne W, Cuthill IC, Emerson M, & Altman DG (2010). Animal research: Reporting in vivo experiments: The ARRIVE guidelines. British Journal of Pharmacology 160**:** 1577-1579.

Kopruszinski CM, Xie JY, Eyde NM, Remeniuk B, Walter S, Stratton J*, et al.* (2017). Prevention of stress- or nitric oxide donor-induced medication overuse headache by a calcitonin gene-related peptide antibody in rodents. Cephalalgia 37**:** 560-570.

Marone IM, De Logu F, Nassini R, De Carvalho Goncalves M, Benemei S, Ferreira J*, et al.* (2018). TRPA1/NOX in the soma of trigeminal ganglion neurons mediates migraine-related pain of glyceryl trinitrate in mice. Brain 141**:** 2312-2328.

Meng QY, Wang W, Chen XN, Xu TL, & Zhou JN (2009). Distribution of acid-sensing ion channel 3 in the rat hypothalamus. Neuroscience 159**:** 1126-1134.

Ong JJY, & De Felice M (2018). Migraine Treatment: Current Acute Medications and Their Potential Mechanisms of Action (vol 15, pg 274, 2018). Neurotherapeutics 15**:** 525-526.

Ong JJY, Wei DY, & Goadsby PJ (2018). Recent Advances in Pharmacotherapy for Migraine Prevention: From Pathophysiology to New Drugs. Drugs 78**:** 411-437.

Stovner LJ, Nichols E, Steiner TJ, Abd-Allah F, Abdelalim A, & Al-Raddadi RM (2018). Global, regional, and national burden of migraine and tension-type headache, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Neurol 17**:** 954-976.

Tipton AF, Tarash I, McGuire B, Charles A, & Pradhan AA (2016). The effects of acute and preventive migraine therapies in a mouse model of chronic migraine. Cephalalgia : an international journal of headache 36**:** 1048-1056.

Ugawa S, Ueda T, Ishida Y, Nishigaki M, Shibata Y, & Shimada S (2002). Amiloride-blockable acid-sensing ion channels are leading acid sensors expressed in human nociceptors. The Journal of clinical investigation 110**:** 1185-1190.

Verkest C, Piquet E, Diochot S, Dauvois M, Lanteri-Minet M, Lingueglia E*, et al.* (2018). Effects of systemic inhibitors of acid-sensing ion channels 1 (ASIC1) against acute and chronic mechanical allodynia in a rodent model of migraine. Br J Pharmacol 175**:** 4154-4166.

Vila-Pueyo M, Strother LC, Kefel M, Goadsby PJ, & Holland PR (2019). Divergent influences of the locus coeruleus on migraine pathophysiology. Pain 160**:** 385-394.

Wang S, Wu BX, Liu CY, Qin GC, Yan WH, Zhou JY*, et al.* (2018). Expression of ASIC3 in the Trigeminal Nucleus Caudalis Plays a Role in a Rat Model of Recurrent Migraine. J Mol Neurosci 66**:** 44-52.

Yan J, Edelmayer RM, Wei X, De Felice M, Porreca F, & Dussor G (2011). Dural afferents express acid-sensing ion channels: a role for decreased meningeal pH in migraine headache. Pain 152**:** 106-113.

Yan J, Wei X, Bischoff C, Edelmayer RM, & Dussor G (2013). pH-Evoked Dural Afferent Signaling Is Mediated by ASIC3 and Is Sensitized by Mast Cell Mediators. Headache: The Journal of Head and Face Pain 53**:** 1250-1261.

Figure legends

Figure 1. APETx2 reduces durovascular nociceptive-evoked and spontaneous neuronal activity in the trigeminal nucleus caudalis (TNC). Experimental setup in the rat (A). Durovascular nociceptive afferents arising in the trigeminal ganglion (TG) are activated via stimulation of the dura-mater surrounding the middle meningeal artery (MMA). Durovascular-evoked responses are then recorded in the TNC. TNC durovascular nociceptive-evoked neuronal responses are significantly reduced following APETx2 (B), starting from 45 mins post infusion and remain significantly reduced at 1 hr (C). Examples of TNC spontaneous neuronal activity following control (D) and APETx2 infusion (E) that was significantly decreased over the 1-hour recording window in APETx2 (F), but not vehicle control treated rats compared to baseline. *$P < 0.05$, n = 6 neuronal clusters per group from n = 5 rats per group.

Figure 2. APETx2 blocks nitric oxide (NO)-induced sensitisation to noxious stimuli

There was a significant overall difference in durovascular-evoked trigeminal nucleus caudalis neuronal activation across all groups. Sodium nitropruside (SNP) induced increased durovascular-evoked responses (A) when compared to vehicle control treated rats. Pretreatment with APETx2 significantly reduced the SNP-induced increase in durovascular-evoked responses (A). There was a significant overall difference in noxious pinch-evoked trigeminal nucleus caudalis neuronal activation (B) across all groups. Sodium nitropruside induced a non-significant modest increase in noxious pinch-evoked responses (B) when compared to vehicle control treated rats. Pretreatment with APETx2 significantly reduced the noxious pinch-evoked responses when compared to SNP treated rats (B). * $P < 0.05$; n = 7/5 neuronal clusters per group from 22 and 20 rats for A and B, respectively.

Figure 3: APETx2 inhibits nitroglycerin-evoked periorbital mechanosensitivity in mice.

There was a significant overall difference in periorbital mechanosensitivity across all groups (A). Acute nitroglycerin (NTG) induced a decreased periorbital mechanosensitivity in mice**,** that was partially reversed by pretreatment with APETx2**.** Chronic NTG administration in mice (NTG-primed) produced a basal periorbital mechanosensitivity (B) that reached significance from day 5 when compared to vehicle control mice. Following withdrawal of NTG periorbital mechanosensitivity returned to that of the non-sensitised vehicle control group. Subsequent exposure to bright light stress (BLS) resulted in a significant overall difference in periorbital mechanosensitivity (C). NTG-primed mice demonstrated an increased periorbital mechanosensitivity when compared to vehicle control and APETx2 treated mice. The periorbital mechanosensitivity evoked in response to BLS was blocked by pretreatment with APETx2, returning to that of vehicle control and APETx2 treated mice. Timeline of latent sensitisation to BLS protocol (D). Animals were sensitized with chronic administration of NTG (10 $mgkg^{-1}$ i.p.) every 2^{nd} day for 9 days. Periorbital mechanical sensitivity was assessed with the von Frey assay (black circles) before and 2 hrs post NTG treatment on days 1, 5 and 9. Animals were recovered for 1 week, then on day 17, mechanical sensitivity was reassessed prior to 1 hr of BLS. On Day 18, animals were treated with either APETx2 (0.23 $mgkg^{-1}$) or vehicle control 30 mins prior to and again 30 mins post BLS. Mechanical sensitivity was then assessed 2 hrs post BLS. $*P < 0.05$; n = 12 mice for A and n = 7/8/8 mice per group for B and C. For B: NTG 1 and NTG 2 represent NTG-primed mice that subsequently received vehicle control or APETx2, respectively in C. One mouse was excluded from the control group due to ill health.