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Migraine-provoking substances evoke periorbital allodynia in mice

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Abstract

Background: Administration of endogenous mediators or exogenous chemicals in migraine patients provoke early headaches and delayed migraine-like attacks. Although migraine provoking substances are normally vasodilators, dilation of arterial vessels does not seem to be the sole contributing factor, and the underlying mechanisms of the delayed migraine pain are mostly unknown. Sustained mechanical allodynia is a common response associated with the local administration of various proalgesic substances in experimental animals and humans. Here, we investigated the ability of a series of endogenous mediators which provoke or do not provoke migraine in patients, to cause or not cause mechanical allodynia upon their injection in the mouse periorbital area.

Methods: Mechanical allodynia was assessed with the von Frey filament assay. Stimuli were given by subcutaneous injection in the periorbital area of C57BL/6J mice; antagonists were administered by local and systemic injections.

Results: Calcitonin gene related peptide (CGRP), but not adrenomedullin and amylin, pituitary adenylyl cyclase activating peptide (PACAP), but not vasoactive intestinal polypeptide (VIP), histamine, prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2), but not $PGF_{2\alpha}$, evoked a dose-dependent periorbital mechanical allodynia. The painful responses were attenuated by systemic or local (periorbital) administration of antagonists for CGRP (CLR/RAMP1), PACAP (PAC-1), histamine H_1, PGE_2 (EP_4), and PGI_2 (IP) receptors, respectively.

Conclusions: The correspondence between substances that provoke (CGRP; PACAP, histamine, PGE_2, PGI_2), or do not provoke (VIP and $PGF_{2\alpha}$), migraine-like attacks in patients and periorbital allodynia in mice suggests that the study of allodynia in mice may provide information on the proalgesic mechanisms of migraine-provoking agents in humans. Results underline the ability of migraine-provoking substances to initiate mechanical allodynia by acting on peripheral terminals of trigeminal afferents.

Keywords: Migraine, calcitonin gene related peptide, pituitary adenylyl cyclase activating peptide, prostaglandin, histamine, vasoactive intestinal polypeptide, allodynia

Background

Migraine is a pain disorder that affects about 15% of the adult population worldwide. Thus, the burden of migraine is enormous in terms of suffering, disability, healthcare, and social and economic costs [1]. For these reasons, migraine is ranked among the most disabling medical conditions [2]. Although considerable progress has been made in the development of new treatment options [3, 4], our current understanding of the mechanisms underlying migraine pain is incomplete. Migraine attacks are elicited by a variety of provoking agents [5], and this peculiar feature provides an opportunity to explore disease mechanisms by endogenous mediators or exogenous chemicals that provoke migraine-like attacks in patients [6].

A prototypical example of a migraine-provoking agent is glyceryl trinitrate (GTN). Occupational exposure to, or treatment with, organic nitrates has long been known to provoke headaches [7–10]. Typically, GTN causes an early, mild and short-lived headache minutes after administration, followed by a remarkably delayed migraine-like

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attack hours later [9, 10]. The ability of GTN to provoke the mild/early headache is temporally associated with the short-lived (<10 min) release of nitric oxide (NO) [11] and ensuing vasodilation [12]. However, the prolonged migraine-like attacks typically begin with a remarkable delay of hours, underlying the temporal dissociation between the early vasomotor response and the delayed proalgesic effect [6, 13, 14]. Thus, the vascular response can hardly explain the delayed migraine-like attack, which, therefore, implicates additional mechanisms. Recently, we reported that GTN administration in mice evokes an early and short-lived (10 minutes) vasodilatation due to a direct vascular action of NO, and a delayed and prolonged (8 hours) periorbital mechanical allodynia (PMA) that is independent from vascular changes and is due to the activation of an oxidative stress-mediated pathway in the soma of trigeminal primary sensory neurons [15]. We also observed that GTN-evoked PMA in mice exhibits a temporal pattern [15] similar to the migraine-like attacks in patients, which are characterized by delayed onset and prolonged duration [6].

In the last three decades, rigorous studies with randomized, double blind and crossover designs have been undertaken, resulting in a systematic investigation of the ability of a series of endogenous mediators or exogenous chemicals to provoke early headaches and delayed migraine-like attacks [6]. Vasodilatation has been proposed as the underlying mechanism of migraine headaches [16]. Notably, both intra and extracranial artery vasodilatation or only intracranial artery vasodilatation have been reported in association with spontaneous migraine attacks [17–19]. Although vasodilatation is elicited by a majority of the migraine provoking agents [6, 14, 20], the vascular response does not seem essential for generating delayed migraine attacks, as robust vasodilators, such as the vasoactive intestinal polypeptide (VIP) or adrenomedullin, do not induce migraine [21, 22]. Thus, an experimental animal model that explores the correspondence between the pain-producing ability of mediators that provoke migraine might be useful for a better understanding of the pro-migraine action of such mediators.

Here, we have investigated whether a series of endogenous mediators, which have been found to provoke or not provoke migraine-like attacks in patients, elicit or do not elicit delayed and prolonged PMA after their injection in the periorbital skin of mice. Provocation tests in humans are usually performed by systemic administration of the stimulus [6]. However, in the present study in mice the local administration was purposely chosen to investigate the interaction between the various mediators and the peripheral terminals of trigeminal nociceptors. These mediators include calcitonin gene-related peptide (CGRP), adrenomedullin, amylin, pituitary adenylyl cyclase activating peptide (PACAP), VIP, histamine,

prostaglandin E_2 (PGE$_2$), prostacyclin (PGI$_2$) and prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$). The receptor type implicated in the PMA evoked by each mediator was also studied. A close correspondence was found between agents that provoke/ not provoke delayed migraine in patients and PMA in mice. Thus, the study of PMA in mice may provide information on the proalgesic mechanisms that, in humans, result in the development of migraine-like attacks provoked by endogenous mediators and exogenous chemicals.

Methods
Animals
In vivo experiments were carried out according to the European Union (EU) guidelines and Italian legislation (DLgs 26/2014, EU Directive application 2010/63/EU) for animal care (research permit #194/2015-PR). C57BL/ 6J mice (male, 20-22 g, 6-7 weeks old; Envigo, Milan, Italy) were used. Animals were housed in a temperature (20-24°C)- and relative humidity (45-65%) -controlled *vivarium*, maintained on a 12-hour dark/light cycle (light off from 7.00 PM to 7.00 AM), and with free access to food and water. Animal studies were reported in compliance with the ARRIVE guidelines [23]. The total number of C57BL/6J mice used was 486. Group size of n=6 animals for behavioural experiments were determined using G*Power (v3.1) [24] to detect a minimum difference between paired means of 1.4 standard deviations (or 1.8 standard deviations between groups) in post-hoc tests with type 1 and 2 error rates of 5 and 20%, respectively [15]. Allocation concealment was performed using a randomization procedure (http:// www.randomizer.org/). Experiments were done in a quiet, temperature-controlled (20-24°C) room between 9.00 AM and 5.00 PM and were performed by an operator blinded to drug treatment. At the end of each experiment, mice were euthanized with inhaled CO_2 plus 10-50% O_2.

Reagents
CGRP, amylin, PACAP, VIP, PGF$_{2\alpha}$, olcegepant, astemizole, ER819762 and Ro1138452 were from Tocris Bioscience (Bristol, UK); adrenomedullin, PACAP6-38, PGE$_2$, PGI$_2$ and histamine were from Sigma Aldrich (Milan, Italy); the mouse monoclonal anti-CGRP antibody (clone [4901]) and the inactive immunoglobulin (mouse monoclonal IgG2a) were from Abcam (Cambridge, UK)

Behavioural experiments
Treatment protocols
C57BL/6J mice were injected subcutaneously in the periorbital area (p.orb., 10 μl/site) with CGRP, adrenomedullin, amylin, PACAP, VIP, histamine, PGE$_2$, PGI$_2$ and PGF$_{2\alpha}$ (0.15, 1.5 and 15 nmol) or their vehicles (0.9%

NaCl). The subcutaneous injection was performed unilaterally on the right side of the periorbital area. The mouse was restrained by the double handed manual restraint method [25]. Briefly, the mouse was lifted by the base of the tail and placed on a solid surface with one hand and the tail was pulled back. Then, it was quickly and firmly picked up by the scruff of the neck behind the ears with the thumb and index finger of the other hand. In this way, the mouse face was constrained, and the operator was able to inject the tested compound. Injection was performed as quickly as possible by a single operator, with only minimal animal restraint.

Some mice were pre-treated (30 minutes before the stimuli) with intraperitoneal (i.p., 10 ml/kg) olcegepant (1 μmol/kg corresponding to 0.869 mg/kg), astemizole (4 μmol/kg corresponding to 1.8 mg/kg), ER819762 (60 μmol/kg corresponding to 29.4 mg/kg) or their vehicle (4% dimethyl sulfoxide, DMSO, and 4% tween 80 in 0.9% NaCl) and intravenous (i.v., 1 ml/kg) PACAP6-38 (12 nmol/kg corresponding to 48 μg/kg) and Ro1138452 (30 μmol/kg corresponding to 10.4 mg/kg), or their vehicle (0.9% NaCl). Other mice received locally (p.orb., 10 μl, 30 minutes before the stimuli) olcegepant (4 nmol/site), astemizole (10 nmol/site), ER819762 (10 nmol/site), or their vehicle (4% DMSO and 4% tween 80 in 0.9% NaCl) and PACAP6-38 (240 pmol/site) and Ro1138452 (10 nmol/site), or their vehicle (0.9% NaCl), or a mouse monoclonal anti-CGRP antibody or, as a control, a mouse monoclonal IgG2a (both, 60 pmol/site). In another set of experiments, C57BL/6J mice received intraplantar (i.pl., 20 μl, 30 minutes before the stimuli) injections of olcegepant (4 nmol/site), astemizole (10 nmol/site), ER819762 (10 nmol/site), or their vehicle (4% DMSO and 4% tween 80 in 0.9% NaCl), or PACAP6-38 (240 pmol/site) and Ro1138452 (10 nmol/site), or their vehicle (0.9% NaCl).

Acute nociceptive test
Immediately after the p.orb. injections, mice were placed inside a plexiglass chamber and spontaneous nociception was assessed by measuring the time (seconds) that the animal spent face rubbing the injected area with its paws [26] over the next 10 minutes. The p.orb. injection with vehicles produced nociceptive behaviour for a maximum of 3 seconds.
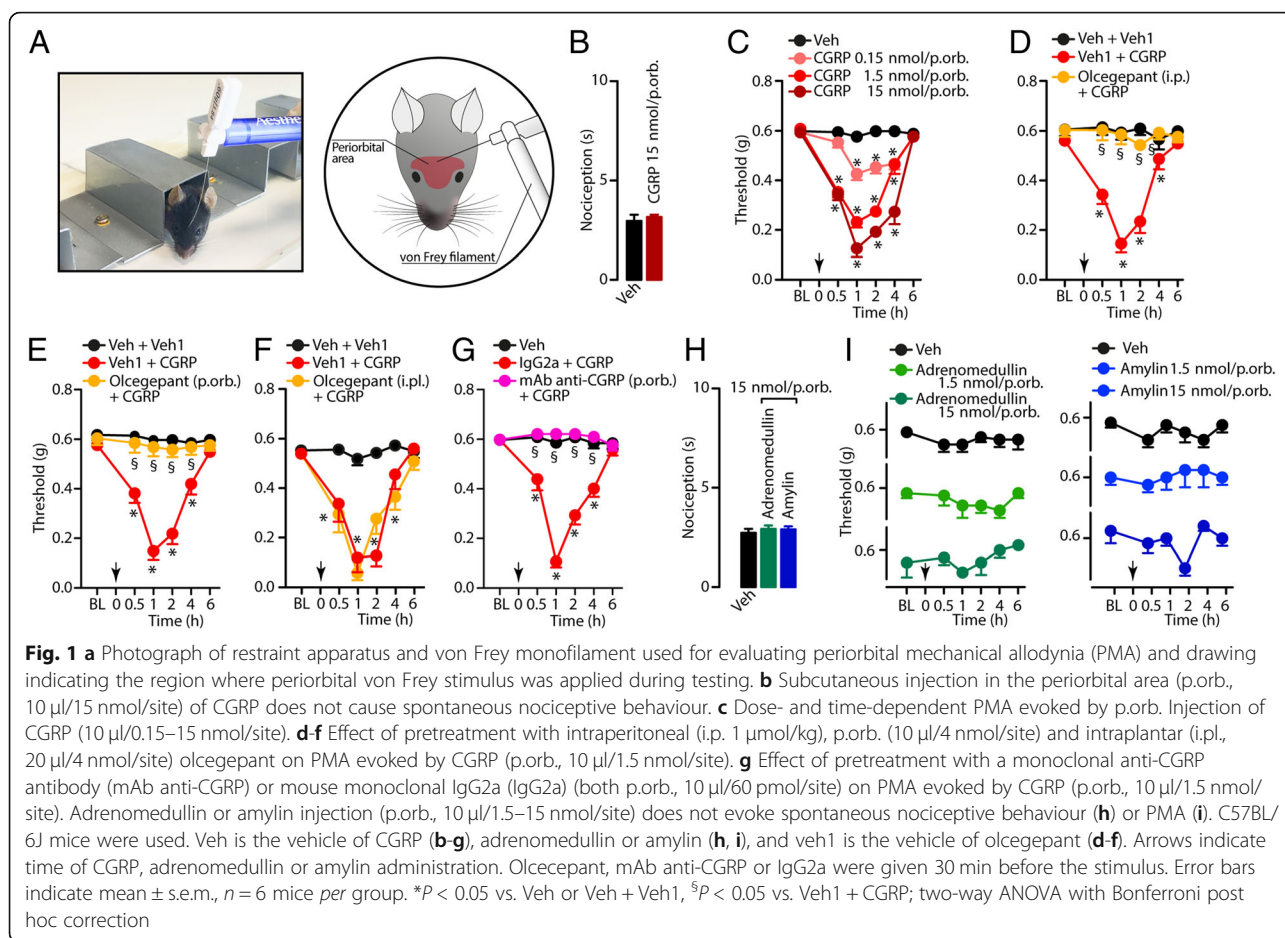
Periorbital mechanical allodynia
The measurement of PMA was performed by using the up-and-down paradigm as described previously [27, 28] in the same mice in which acute nociceptive responses were monitored for 10 minutes after the stimulus. Animals were allocated in a restraint apparatus designed for the evaluation of periorbital mechanical thresholds. The apparatus consists in an individual clear three-walled

plexiglass box (4 H × 4 W x 10 L cm) with an opening for the tail and one for the head and front paws, located on a platform to allow the operator to access to the periorbital area. The box size allowed for head and forepaw movements but prevented the animal from turning around inside it (Fig 1A). One day before the first behavioural observations, mice were habituated to the apparatus. PMA was evaluated in the periorbital region over the rostral portion of the eye (*i.e.*, the area of the periorbital region facing the sphenoidal rostrum) of the mice [29] (Fig 1a), before (basal threshold) and after (0.5, 1, 2, 4, 6, 8 hours) treatment.

The day of the experiment, after 20 minutes of adaptation inside the chamber, a series of 7 Von Frey filaments in logarithmic increments of force (0.02, 0.04, 0.07, 0.16, 0.4, 1.0 and 1.4 g) were applied to the periorbital area perpendicular to the skin, with sufficient force to cause slight buckling, and held for approximately 5 seconds to elicit a positive response. The response was considered positive by the following criteria: mouse vigorously stroked its face with the forepaw, head withdrawal from the stimulus, or head shaking. The stimulation initiated with the 0.16 g filament. Absence of response after 5 seconds led to the use of a filament with increased weight, whereas a positive response led to the use of a weaker (*i.e.* lighter) filament. Six measurements were collected for each mouse or until four consecutive positive or negative responses occurred. The 50% mechanical withdrawal threshold (expressed in g) was then calculated from these scores by using a δ value of 0.205, previously determined.

Statistical Analysis
All data were expressed as mean ± s.e.m. Statistical analysis was performed by the unpaired two-tailed Student's t-test for comparisons between two groups. Group means for single factor experiments were analysed with a one-way ANOVA, while behavioural experiments with repeated measures employed a two-way mixed model ANOVA, first to determine the presence of an interaction effect, and then to compare the control and treated groups of mice at each time point tested. In both cases, post-hoc comparisons employed the Bonferroni criterion to maintain the experiment-wise error rate at 5%. To avoid uncertainties that would follow from the use of these parametric methods on data that may not attain an interval level of measurement, as well as the potential violation of other ANOVA assumptions, including that of normal sampling distribution, analyses were repeated using non-parametric methods. Both methods led to similar conclusions, and we presented only the parametric analyses, which maintain the original, and more intuitive, units of measure. Statistical analyses were performed on raw data using Prism 5

Fig. 1 a Photograph of restraint apparatus and von Frey monofilament used for evaluating periorbital mechanical allodynia (PMA) and drawing indicating the region where periorbital von Frey stimulus was applied during testing. **b** Subcutaneous injection in the periorbital area (p.orb., 10 μl/15 nmol/site) of CGRP does not cause spontaneous nociceptive behaviour. **c** Dose- and time-dependent PMA evoked by p.orb. Injection of CGRP (10 μl/0.15–15 nmol/site). **d-f** Effect of pretreatment with intraperitoneal (i.p. 1 μmol/kg), p.orb. (10 μl/4 nmol/site) and intraplantar (i.pl., 20 μl/4 nmol/site) olcegepant on PMA evoked by CGRP (p.orb., 10 μl/1.5 nmol/site). **g** Effect of pretreatment with a monoclonal anti-CGRP antibody (mAb anti-CGRP) or mouse monoclonal IgG2a (IgG2a) (both p.orb., 10 μl/60 pmol/site) on PMA evoked by CGRP (p.orb., 10 μl/1.5 nmol/site). Adrenomedullin or amylin injection (p.orb., 10 μl/1.5–15 nmol/site) does not evoke spontaneous nociceptive behaviour (**h**) or PMA (**i**). C57BL/6J mice were used. Veh is the vehicle of CGRP (**b-g**), adrenomedullin or amylin (**h, i**), and veh1 is the vehicle of olcegepant (**d-f**). Arrows indicate time of CGRP, adrenomedullin or amylin administration. Olcecepant, mAb anti-CGRP or IgG2a were given 30 min before the stimulus. Error bars indicate mean ± s.e.m., *n* = 6 mice *per* group. *$P < 0.05$ vs. Veh or Veh + Veh1, §$P < 0.05$ vs. Veh1 + CGRP; two-way ANOVA with Bonferroni post hoc correction

GraphPad software (GraphPad Software Inc., San Diego, CA, USA), as well as IBM SPSS (v.25, IBM Corp., Armonk, NY, USA). P<0.05 was considered statistically significant.

Results

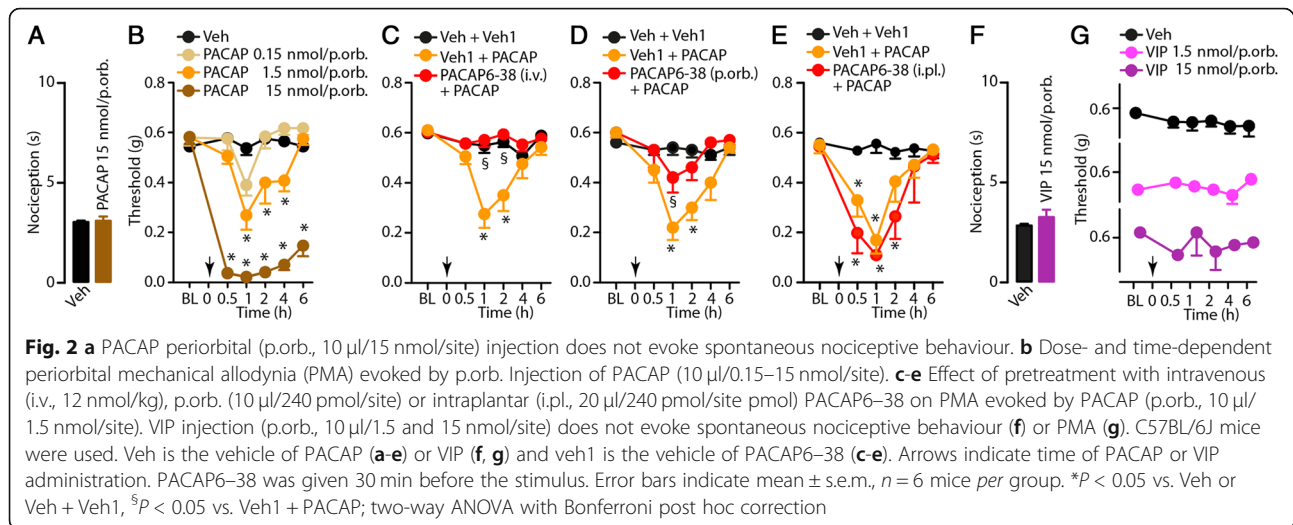### CGRP, adrenomedullin, amylin

CGRP, amylin and adrenomedullin belong to the larger calcitonin family of peptides, which activate, with different potencies, a series of receptors resulting from the multiple combinations of the 3 forms of the calcitonin (CT, further divided into the a, b and δ(1-47)b subtypes) receptor and the CT receptor-like receptor (CLR) with the 3 forms of receptor-activity-modifying proteins (RAMPs) [30]. Although CGRP can bind to all these receptor complexes, it exhibits a higher affinity for the RAMP1/CLR [30]. Adrenomedullin binds with higher potency to the RAMP2-3/CLR and amylin to the RAMP1/CT(a) and the RAMP1-2/CT(b) [30]. Whereas periorbital (p.orb., 10 μl/site) injection of CGRP (0.15, 1.5 and 15 nmol/site), even at the highest dose, did not evoke an acute spontaneous nociceptive response (Fig. 1b), it did cause a robust, dose-dependent and sustained PMA (Fig. 1c). The prolonged PMA was present at

0.5 hour, peaked at 2 hours and declined, to return to baseline values, 6 hours after CGRP injection. Systemic intraperitoneal (i.p., 1 μmol/kg) or local (p.orb., 4 nmol/site), but not intraplantar (i.pl., 20 μl, 4 nmol/site) injection (30 minutes before CGRP) of the CGRP receptor antagonist, olcegepant, prevented PMA (Fig. 1d-f). Furthermore, p.orb. (10 μl) pretreatment (30 min before) with a monoclonal anti-CGRP antibody (60 pmol/site), but not with the inactive mouse monoclonal IgG2a, also prevented the development of PMA induced by p.orb. CGRP (Fig. 1g).

Local (p.orb., 10 μl) administration of adrenomedullin or amylin at the same pro-allodynic dose of CGRP (1.5 or 15 nmol/site), was unable to produce any measurable acute nociceptive response, even at the highest dose. Adrenomedullin or amylin also failed to produce PMA over the entire period of observation (6 hours) (Fig. 1h, i).

PACAP and VIP

The members of the family of the PACAP and VIP vasoactive peptides act on VPAC-1 and VPAC-2 receptors with comparable affinity, whereas the PAC-1 receptor isoform has 100-fold higher affinity for PACAP [31, 32]. Local (p.orb., 10 μl) injection of PACAP (0.15, 1.5 and 15 nmol/

Fig. 2 a PACAP periorbital (p.orb., 10 μl/15 nmol/site) injection does not evoke spontaneous nociceptive behaviour. **b** Dose- and time-dependent periorbital mechanical allodynia (PMA) evoked by p.orb. Injection of PACAP (10 μl/0.15–15 nmol/site). **c-e** Effect of pretreatment with intravenous (i.v., 12 nmol/kg), p.orb. (10 μl/240 pmol/site) or intraplantar (i.pl., 20 μl/240 pmol/site pmol) PACAP6–38 on PMA evoked by PACAP (p.orb., 10 μl/ 1.5 nmol/site). VIP injection (p.orb., 10 μl/1.5 and 15 nmol/site) does not evoke spontaneous nociceptive behaviour (**f**) or PMA (**g**). C57BL/6J mice were used. Veh is the vehicle of PACAP (**a-e**) or VIP (**f, g**) and veh1 is the vehicle of PACAP6–38 (**c-e**). Arrows indicate time of PACAP or VIP administration. PACAP6–38 was given 30 min before the stimulus. Error bars indicate mean ± s.e.m., $n = 6$ mice *per* group. *$P < 0.05$ vs. Veh or Veh + Veh1, §$P < 0.05$ vs. Veh1 + PACAP; two-way ANOVA with Bonferroni post hoc correction

site), which did not provoke any detectable spontaneous nociceptive behaviour even at the highest dose, induced a marked, dose-dependent and sustained (1-6 hours) PMA (Fig. 2a, b). Intravenous (i.v., 1 ml/kg, 12 nmol/kg) or p.orb. (10 μl, 240 pmol/site), but not i.pl. (20 μl, 240 pmol/site), pretreatment with the selective PACAP receptor antagonist, PACAP6-38, prevented PACAP-induced PMA (Fig. 2c, e). VIP (1.5 or 15 nmol/site, p.orb.) was unable to produce either acute nociception or PMA (Fig. 2f, g).

Histamine
Histamine is a ubiquitous mediator released from mast cells, enterochromaffin-like cells and neurons, implicated in pathophysiological responses such as arousal state, allergy, inflammation, itch and pain [33–35]. Its actions are mediated by four distinct receptors, the H_1, H_2, H_3 and H_4 receptors [36]. Local injection (p.orb., 10μl) of histamine (0.15, 1.5 and 15 nmol/site) was unable to produce any spontaneous acute nociception, even at the highest dose administered, but induced a dose-dependent and sustained (4-6 hours) PMA (Fig. 3a, b). Systemic (i.p., 10 ml/kg, 4 μmol/kg) or p.orb. (10 μl, 10 nmol/site), but not i.pl. (20 μl, 10 nmol/site) pretreatment with the histamine H_1 receptor antagonist, astemizole, prevented histamine-induced PMA (Fig. 3c-e).
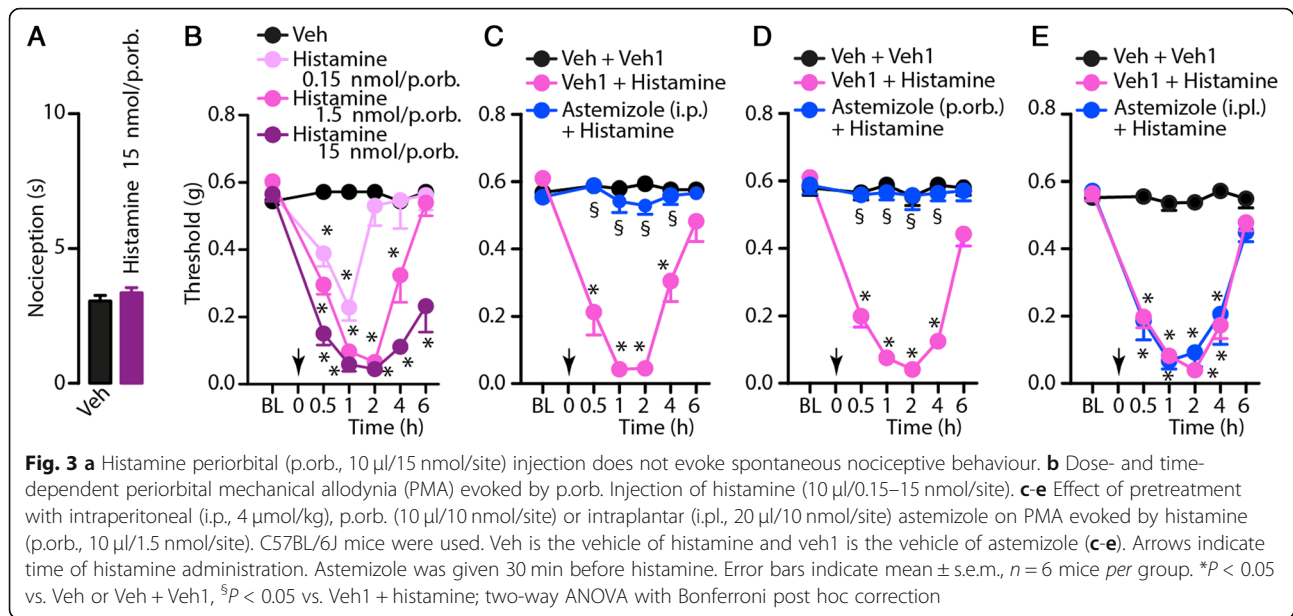
PGE₂, PGI₂, PGF₂α
Prostanoids are ubiquitous mediators which play a major role in a large variety of physiological responses and pathological process, including inflammation and pain [37]. Cyclooxygenase inhibition by non-steroidal anti-inflammatory drugs (NSAIDs), which prevents the transformation of arachidonic acid into the inactive prostaglandin precursor, prostaglandin H_2 (PGH_2), is a mainstay of the acute migraine attack, thus implicating prostaglandins in migraine pain [38, 39]. PGE_2

administration in the mouse paw is known to evoke spontaneous acute nociception [40]. Accordingly, we found that PGE_2 (0.15, 1.5 and 15 nmol/site), but not PGI_2 (0.15, 1.5 and 15 nmol/site) or $PGF_{2\alpha}$ (1.5-15 nmol/site) injection into the mouse periorbital skin elicited a marked spontaneous nociceptive response (Fig. 4a, f, k). Furthermore, injection of both PGE_2 and PGI_2, but not $PGF_{2\alpha}$, evoked a dose-dependent sustained (0.5-6 hours) PMA (Fig. 4b, g, k). Pretreatment with i.p. (10 ml/kg, 60 μmol/kg) and p.orb. (10 μl, 10 nmol/site), but not i.pl. (20 μl, 10 nmol/site) prostaglandin receptor 4 (EP_4) antagonist, ER819762, prevented PGE_2-induced spontaneous nociception and PMA (Fig. 4a, c-e). Pretreatment with i.v. (1 ml/kg, 30 μmol/kg) and p.orb., (10 μl, 10 nmol/site), but not i.pl. (20 μl, 10 nmol/site) antagonist for the prostacyclin receptor (IP), Ro1138452, prevented PGI_2-induced PMA (Fig. 4h-j). Conversely, Ro1138452 (i.v., 30 μmol/kg) did not affect spontaneous nociception and PMA evoked by PGE_2 and ER819762 (i.p., 60 μmol/kg) did not affect PMA evoked by PGI_2 (Fig. 4a, c, h).

Discussion
The members of the calcitonin family of peptides activate a variety of receptors deriving from the dimerization of CLR or CL with RAMP proteins. Adrenomedullin, which stimulates the combinations of the CLR with RAMP2 or RAMP3 with a potency higher than CGRP (AM_1 and AM_2 receptor, respectively), and amylin, which is equipotent to CGRP on the receptor combinations formed by the three CT subtypes with RAMP1, RAMP2 or RAMP3, failed to evoke allodynia. A possible effect of amylin and adrenomedullin on the RAMP1/CLR, or of CGRP on the different combinations of receptors for amylin and adrenomedullin has been claimed to contribute to the pro-migraine action of

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Fig. 3 **a** Histamine periorbital (p.orb., 10 μl/15 nmol/site) injection does not evoke spontaneous nociceptive behaviour. **b** Dose- and time-dependent periorbital mechanical allodynia (PMA) evoked by p.orb. Injection of histamine (10 μl/0.15–15 nmol/site). **c-e** Effect of pretreatment with intraperitoneal (i.p., 4 μmol/kg), p.orb. (10 μl/10 nmol/site) or intraplantar (i.pl., 20 μl/10 nmol/site) astemizole on PMA evoked by histamine (p.orb., 10 μl/1.5 nmol/site). C57BL/6J mice were used. Veh is the vehicle of histamine and veh1 is the vehicle of astemizole (**c-e**). Arrows indicate time of histamine administration. Astemizole was given 30 min before histamine. Error bars indicate mean ± s.e.m., $n = 6$ mice *per* group. *$P < 0.05$ vs. Veh or Veh + Veh1, $^§P < 0.05$ vs. Veh1 + histamine; two-way ANOVA with Bonferroni post hoc correction
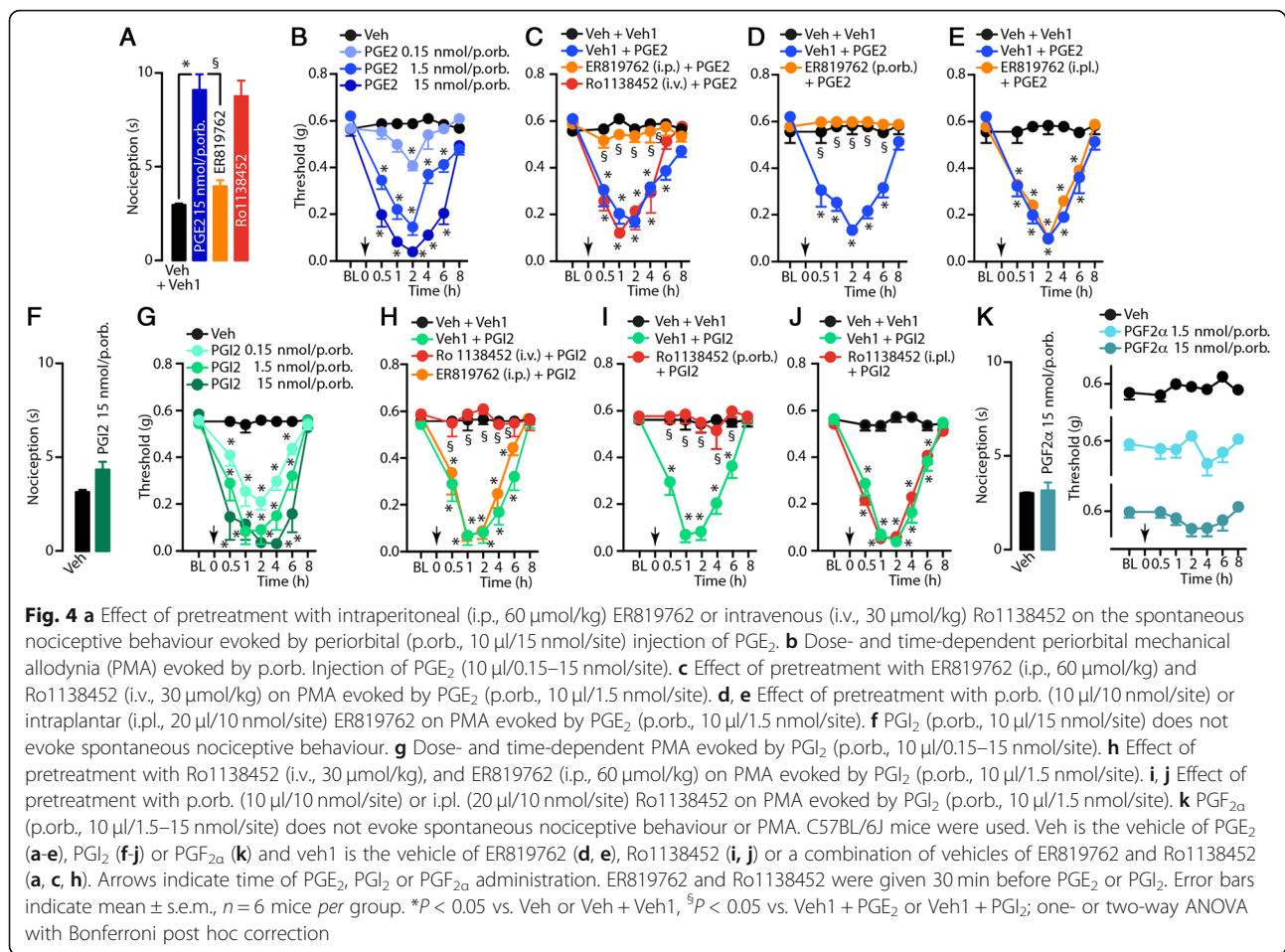
CGRP or its receptor [30]. As previously reported in the mouse hindpaw [41] and periorbital area [15], we confirm that CGRP causes a robust and sustained mechanical allodynia, which is attenuated by both systemic and local administration of the selective RAMP1/CLR (CGRP receptor) antagonist, olcegepant. The observation that, under the same experimental conditions neither adrenomedullin nor amylin evoked PMA negates the implication of their preferred receptors in CGRP-mediated pain-like responses. Furthermore, the present results do not support the hypothesis that amylin or adrenomedullin act on RAMP1/CLR to evoke pain-like responses. Previous results that CGRP administration to migraineurs induced delayed migraine-like attacks [42], whereas adrenomedullin was found to be inactive [22], strengthened and excluded the role in migraine mechanism of CGRP and adrenomedullin, respectively. The present findings on the calcitonin related peptides, recapitulating human results, support the predictive value of mouse PMA in investigating pain mechanisms of migraine.

Clinical trials with anti CGRP or anti RAMP1/CLR monoclonal antibodies, while showing excellent efficacy and safety profile, also indicate that a subset of migraine patients either do not respond or have a partial benefit [4, 43, 44]. This observation suggests that additional mechanisms and mediators contribute to migraine pain, thus prompting the study of substances other than CGRP. PGE_2 and PGI_2, two prostaglandins that induce headaches and migraine-like attacks in humans [45–48], elicited PMA in mice. In contrast, $PGF_{2\alpha}$, a prostaglandin, which was unable to evoke migraine-like attack in patients [49], failed to elicit PMA in mice. The use of selective antagonists for the

EP_4 and IP receptors showed that PGE_2 and PGI_2 caused allodynia exclusively by activating the respective preferred receptor. This conclusion suggests that in humans PGE_2 and PGI_2 elicit migraine-like attacks by acting on EP_4 and IP receptors, respectively. As reported previously, in the mouse hindpaw [40], PGE_2 was the sole compound among all the presently investigated substances that evoked an early spontaneous nociceptive response, which, similarly to allodynia, was abated by EP4 receptor antagonism. However, given that only one of the migraine-provoking substances elicited spontaneous nociception, the significance of such early non-evoked pain-like responses for migraine pain mechanism remains unclear.

Histamine, a key proinflammatory and allergic mediator with a proalgesic role provokes migraine-like attacks in patients [50–52]. Furthermore, anecdotical reports and clinical investigations [53] have proposed the use of increasing doses of histamine to desensitize the pain-producing mechanism in migraine patients. Present data show that, by targeting the H_1 receptor subtype, histamine evokes PMA in mice and provides indirect support to the contribution of the H_1 receptor, rather than H_2 receptors [51], in provoking migraine [52], and to the desensitization process that is supposed to ameliorate migraine [53]. It should be underlined that, despite the ability of histamine to sensitise nociceptors *via* H_1 receptor activation, the H_1-antagonists were not always effective in reducing migraine [54].

VIP and PACAP, which belong to the glucagon/secretin family of regulatory peptides, stimulate three distinct receptors: the PAC-1, selectively activated by PACAP, and the VPAC-1 and VPCA-2, which are equipotently activated by both PACAP and VIP. The observation that PACAP, but not VIP, elicited allodynia, suggests that the

Fig. 4 a Effect of pretreatment with intraperitoneal (i.p., 60 μmol/kg) ER819762 or intravenous (i.v., 30 μmol/kg) Ro1138452 on the spontaneous nociceptive behaviour evoked by periorbital (p.orb., 10 μl/15 nmol/site) injection of PGE_2. **b** Dose- and time-dependent periorbital mechanical allodynia (PMA) evoked by p.orb. Injection of PGE_2 (10 μl/0.15–15 nmol/site). **c** Effect of pretreatment with ER819762 (i.p., 60 μmol/kg) and Ro1138452 (i.v., 30 μmol/kg) on PMA evoked by PGE_2 (p.orb., 10 μl/1.5 nmol/site). **d, e** Effect of pretreatment with p.orb. (10 μl/10 nmol/site) or intraplantar (i.pl., 20 μl/10 nmol/site) ER819762 on PMA evoked by PGE_2 (p.orb., 10 μl/1.5 nmol/site). **f** PGI_2 (p.orb., 10 μl/15 nmol/site) does not evoke spontaneous nociceptive behaviour. **g** Dose- and time-dependent PMA evoked by PGI_2 (p.orb., 10 μl/0.15–15 nmol/site). **h** Effect of pretreatment with Ro1138452 (i.v., 30 μmol/kg), and ER819762 (i.p., 60 μmol/kg) on PMA evoked by PGI_2 (p.orb., 10 μl/1.5 nmol/site). **i, j** Effect of pretreatment with p.orb. (10 μl/10 nmol/site) or i.pl. (20 μl/10 nmol/site) Ro1138452 on PMA evoked by PGI_2 (p.orb., 10 μl/1.5 nmol/site). **k** $PGF_{2\alpha}$ (p.orb., 10 μl/1.5–15 nmol/site) does not evoke spontaneous nociceptive behaviour or PMA. C57BL/6J mice were used. Veh is the vehicle of PGE_2 (**a-e**), PGI_2 (**f-j**) or $PGF_{2\alpha}$ (**k**) and veh1 is the vehicle of ER819762 (**d, e**), Ro1138452 (**i, j**) or a combination of vehicles of ER819762 and Ro1138452 (**a, c, h**). Arrows indicate time of PGE_2, PGI_2 or $PGF_{2\alpha}$ administration. ER819762 and Ro1138452 were given 30 min before PGE_2 or PGI_2. Error bars indicate mean ± s.e.m., $n = 6$ mice per group. *$P < 0.05$ vs. Veh or Veh + Veh1, §$P < 0.05$ vs. Veh1 + PGE_2 or Veh1 + PGI_2; one- or two-way ANOVA with Bonferroni post hoc correction

PACAP/PAC-1 is the sole pathway implicated in generating pain-like responses. PACAP and VIP are both vasodilator substances [55, 56]. The ability of PACAP, and not VIP, to cause allodynia in mice and migraine-like attacks in humans [21, 57], supports the hypothesis that vasodilatation is not *per se* a major factor contributing to allodynia in mice and migraine pain in humans. These findings are in line with previous observations that PACAP, but not VIP, causes delayed activation and sensitization of central trigeminovascular neurons *via* activation of the PAC1 receptor [58]. The implication of mast cells has been proposed in the pathway activated by PACAP to elicit pain. Mast cells may release PACAP [59], and PACAP, *via* a hitherto uncharacterized receptor, degranulates mast cells [60]. The present model could be used to further explore local mechanisms that, activated by PACAP and implicating mast cells, result in pain responses.

The underlying mechanism that promotes migraine attacks is unclear. Clinical investigation with small molecule CGRP receptor antagonists underscores the key role of CGRP in the genesis of migraine pain [4, 61]. However, the specific site(s) of the proalgesic action of CGRP in migraine pain remains elusive. Recent clinical trials with monoclonal antibodies that block CGRP or its receptor [43, 44], underline the hypothesis that the pain produced by CGRP during migraine attacks originates at a peripheral site, outside the blood brain barrier. However, the precise location of such a peripheral site is uncertain. The observation that PMA was attenuated only if antagonists were given locally, close to (p.orb.), but not at distance from (i.pl.) site where the respective agonists were injected, indicates that the anatomical site where pain hypersensitivity initially originates is the terminal region of peripheral trigeminal fibres.

Differences may exist between the trigeminal fibres of the skin and those innervating meningeal blood vessels [62, 63] that are possibly implicated in migraine pathogenesis. Nevertheless, the local subcutaneous injection of stimuli was adopted purposively to selectively investigate the interaction between pro-migraine mediators and peripheral terminals of trigeminal nociceptors and to minimize confounding factors, deriving from the systemic administration or the surgical procedures required for dural application of the stimuli. The old dispute regarding the contribution of the peripheral or central nervous system to allodynia and

hyperalgesia [64–67] has not yet been completely resolved. The present investigation reports a condition of hypersensitivity that originates peripherally in the periorbital area of mice, but by no means implies that central neural pathways do not contribute to sustain allodynia. However, pathways and mechanisms regulating mechanical hypersensitivity in the central nervous system are not the object of the present study. Clinical investigation shows that, while blockade of the CGRP system provides benefit in a large proportion of patients, a subset of migraineurs appears to be resistant [43, 44], thus suggesting that additional mediators and mechanisms contribute to migraine pain. The ability to evoke PMA in mice adds support to the role of additional migraine provoking mediators in spontaneous pain attacks.

Conclusions

The major finding of the present study is the strict correspondence between mediators that provoke migraine in patients and evoke periorbital allodynia in mice. The same correspondence was observed between mediators that do not provoke migraine in patients and do not evoke allodynia in mice. An additional relevant finding is that, although most of the pro-allodynic substances tested in the present study are vasodilators, two robust vasodilators, VIP and adrenomedullin, did not evoke allodynia, thus indicating that vascular activity is not *per se* sufficient to elicit pain. Cutaneous allodynia is frequently reported by migraine patients during the attack [68, 69]. However, it should be considered that migraine-like attacks induced by provoking substances are characterized by delayed and prolonged spontaneous, non-evoked pain. Therefore, mechanical allodynia cannot recapitulate the complete spectrum of the pain modalities experienced by migraineurs during their attacks. Nevertheless, disclosing the mechanisms used by the different mediators, and particularly CGRP, to evoke delayed and sustained mechanical allodynia in mice may provide insights for a better understanding of the mechanisms by which the same substances generate migraine pain in patients.

Abbreviations

ANOVA: Analysis of variance; CGRP: Calcitonin gene related peptide; CLR: CT receptor-like receptor; CT: Calcitonin; DMSO: Dimethyl sulfoxide; EP_4: Prostaglandin receptor 4; GTN: Glyceryl trinitrate; i.p.: Intraperitoneal; i.pl.: Intraplantar; i.v.: Intravenous; IP: Prostacyclin receptor; NSAIDs: Non-steroidal anti-inflammatory drugs; p.orb.: Periorbital; PACAP: Pituitary adenylyl cyclase activating peptide; PG: Prostaglandin; PGI_2: Prostacyclin; PMA: Periorbital mechanical allodynia; RAMPs: Receptor-activity-modifying proteins; VIP: Vasoactive intestinal polypeptide; VPAC: VIP receptor

Authors' contributions
FDL, LL, SLP, RN performed experiments, analysed and interpreted data. FDL, RN, PG, FDC and MNJ interpreted data and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. *In vivo* experiments were in accordance to European Union (EU) guidelines and Italian legislation (DLgs 26/2014, EU Directive application 2010/63/EU) for animal care procedures, and under the University of Florence research permit #194/2015-PR.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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References
1. Olesen J, Gustavsson A, Svensson M, Wittchen HU, Jonsson B (2012) The economic cost of brain disorders in Europe. Eur J Neurol 19(1):155–162
2. Murray CJ, Vos T, Lozano R, Naghavi M, Flaxman AD, Michaud C et al (2012) Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 380(9859):2197–2223
3. Negro A, Martelletti P (2011) Chronic migraine plus medication overuse headache: two entities or not? J Headache Pain 12(6):593–601
4. Edvinsson L, Haanes KA, Warfvinge K, Krause DN (2018) CGRP as the target of new migraine therapies - successful translation from bench to clinic. Nat Rev Neurol 14(6):338–350
5. Kelman L (2007) The triggers or precipitants of the acute migraine attack. Cephalalgia 27(5):394–402
6. Ashina M, Hansen JM, AD BO, Olesen J (2017) Human models of migraine - short-term pain for long-term gain. Nat Rev Neurol 13(12):713–724
7. Thadani U, Rodgers T (2006) Side effects of using nitrates to treat angina. Expert Opin Drug Saf 5(5):667–674
8. Trainor DC, Jones RC (1966) Headaches in explosive magazine workers. Arch Environ Health 12(2):231–234
9. Iversen HK, Olesen J, Tfelt-Hansen P (1989) Intravenous nitroglycerin as an experimental model of vascular headache. Basic characteristics Pain 38(1):17–24
10. Sicuteri F, Del Bene E, Poggioni M, Bonazzi A (1987) Unmasking latent dysnociception in healthy subjects. Headache 27(4):180–185
11. Persson MG, Agvald P, Gustafsson LE (1994) Detection of nitric oxide in exhaled air during administration of nitroglycerin in vivo. Br J Pharmacol 111(3):825–828
12. Iversen HK, Olesen J (1996) Headache induced by a nitric oxide donor (nitroglycerin) responds to sumatriptan. A human model for development of migraine drugs. Cephalalgia 16(6):412–418
13. Olesen J, Iversen HK, Thomsen LL (1993) Nitric oxide supersensitivity: a possible molecular mechanism of migraine pain. Neuroreport 4(8):1027–1030
14. Thomsen LL, Kruuse C, Iversen HK, Olesen J (1994) A nitric oxide donor (nitroglycerin) triggers genuine migraine attacks. Eur J Neurol 1(1):73–80

15. Marone IM, De Logu F, Nassini R, De Carvalho GM, 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 6(5050181)

16. Graham JR, Wolf HG (1938) Mechanism of migraine headache and action of ergotamine tartrate. Arch NeurPsych 39(4):26

17. Asghar MS, Hansen AE, Amin FM, van der Geest RJ, Koning P, Larsson HB et al (2011) Evidence for a vascular factor in migraine. Ann Neurol 69(4):635–645

18. Amin FM, Asghar MS, Hougaard A, Hansen AE, Larsen VA, de Koning PJ et al (2013) Magnetic resonance angiography of intracranial and extracranial arteries in patients with spontaneous migraine without aura: a cross-sectional study. Lancet Neurol 12(5):454–461

19. Thomsen LL, Iversen HK, Olesen J (1995) Cerebral blood flow velocities are reduced during attacks of unilateral migraine without aura. Cephalalgia 15(2):109–116

20. Asghar MS, Hansen AE, Kapijimpanga T, van der Geest RJ, van der Koning P, Larsson HB et al (2010) Dilation by CGRP of middle meningeal artery and reversal by sumatriptan in normal volunteers. Neurology 75(17):1520–1526

21. Rahmann A, Wienecke T, Hansen JM, Fahrenkrug J, Olesen J, Ashina M (2008) Vasoactive intestinal peptide causes marked cephalic vasodilation, but does not induce migraine. Cephalalgia 28(3):226–236

22. Petersen KA, Birk S, Kitamura K, Olesen J (2009) Effect of adrenomedullin on the cerebral circulation: relevance to primary headache disorders. Cephalalgia 29(1):23–30

23. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. J Pharmacol Pharmacother 1(2):94–99

24. Faul F, Erdfelder E, Buchner A, Lang AG (2009) Statistical power analyses using G*Power 3.1: tests for correlation and regression analyses. Behav Res Methods 41(4):1149–1160

25. Danneman PJ, Suckow MA, Brayton C. The Laboratory Mouse. Edition n, editor: CRC Press Boca Raton; 2012

26. Luccarini P, Childeric A, Gaydier AM, Voisin D, Dallel R (2006) The orofacial formalin test in the mouse: a behavioral model for studying physiology and modulation of trigeminal nociception. J Pain 7(12):908–914

27. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 53(1):55–63

28. Dixon WJ (1980) Efficient analysis of experimental observations. Annu Rev Pharmacol Toxicol 20:441–462

29. Elliott MB, Oshinsky ML, Amenta PS, Awe OO, Jallo JI (2012) Nociceptive neuropeptide increases and periorbital allodynia in a model of traumatic brain injury. Headache 52(6):966–984

30. Walker CS, Hay DL (2013) CGRP in the trigeminovascular system: a role for CGRP, adrenomedullin and amylin receptors? Br J Pharmacol 170(7):1293–1307

31. Harmar AJ, Fahrenkrug J, Gozes I, Laburthe M, May V, Pisegna JR et al (2012) Pharmacology and functions of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide: IUPHAR review 1. Br J Pharmacol 166(1):4–17

32. Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR et al (1998) International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Pharmacol Rev 50(2):265–270

33. Thangam EB, Jemima EA, Singh H, Baig MS, Khan M, Mathias CB et al (2018) The Role of Histamine and Histamine Receptors in Mast Cell-Mediated Allergy and Inflammation: The Hunt for New Therapeutic Targets. Front Immunol 9(1873):1873

34. Brown RE, Stevens DR, Haas HL (2001) The physiology of brain histamine. Prog Neurobiol 63(6):637–672

35. Shim WS, Oh U (2008) Histamine-induced itch and its relationship with pain. Mol Pain 4(29):29

36. Panula P, Chazot PL, Cowart M, Gutzmer R, Leurs R, Liu WL et al (2015) International Union of Basic and Clinical Pharmacology. XCVIII Histamine Receptors Pharmacol Rev 67(3):601–655

37. Ricciotti E, FitzGerald GA (2011) Prostaglandins and inflammation. Arterioscler Thromb Vasc Biol 31(5):986–1000

38. Silberstein SD, Stirpe JC (2014) COX inhibitors for the treatment of migraine. Expert Opin Pharmacother 15(13):1863–1874

39. Antonova M, Wienecke T, Olesen J, Ashina M (2013) Prostaglandins in migraine: update. Curr Opin Neurol 26(3):269–275

40. Materazzi S, Nassini R, Andre E, Campi B, Amadesi S, Trevisani M et al (2008) Cox-dependent fatty acid metabolites cause pain through activation of the irritant receptor TRPA1. Proc Natl Acad Sci U S A 105(33):12045–12050

41. Shi X, Wang L, Li X, Sahbaie P, Kingery WS, Clark JD (2011) Neuropeptides contribute to peripheral nociceptive sensitization by regulating interleukin-1beta production in keratinocytes. Anesth Analg 113(1):175–183

42. Lassen LH, Haderslev PA, Jacobsen VB, Iversen HK, Sperling B, Olesen J (2002) CGRP may play a causative role in migraine. Cephalalgia 22(1):54–61

43. 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(22):2123–2132

44. Silberstein SD, Dodick DW, Bigal ME, Yeung PP, Goadsby PJ, Blankenbiller T et al (2017) Fremanezumab for the Preventive Treatment of Chronic Migraine. N Engl J Med 377(22):2113–2122

45. Wienecke T, Olesen J, Oturai PS, Ashina M (2009) Prostaglandin E2(PGE2) induces headache in healthy subjects. Cephalalgia 29(5):509–519

46. Wienecke T, Olesen J, Oturai PS, Ashina M (2008) Prostacyclin (epoprostenol) induces headache in healthy subjects. Pain 139(1):106–116

47. Wienecke T, Olesen J, Ashina M (2010) Prostaglandin I2 (epoprostenol) triggers migraine-like attacks in migraineurs. Cephalalgia 30(2):179–190

48. Antonova M, Wienecke T, Olesen J, Ashina M (2012) Prostaglandin E(2) induces immediate migraine-like attack in migraine patients without aura. Cephalalgia 32(11):822–833

49. Antonova M, Wienecke T, Olesen J, Ashina M (2011) Pro-inflammatory and vasoconstricting prostanoid PGF2alpha causes no headache in man. Cephalalgia 31(15):1532–1541

50. Sicuteri F, Ricci M, Monfardini R, Ficini M (1957) Experimental headache with endogenous histamine; first results obtained by 48/80, a histamine-liberator drug, in the cephalic and peripheral circulatory systems of man. Acta Allergol 11(3):188–192

51. Krabbe AA, Olesen J (1980) Headache provocation by continuous intravenous infusion of histamine. Clinical results and receptor mechanisms. Pain 8(2):253–259

52. Lassen LH, Thomsen LL, Olesen J (1995) Histamine induces migraine via the H1-receptor. Support for the NO hypothesis of migraine. Neuroreport 6(11):1475–1479

53. Millan-Guerrero RO, Isais-Millan S, Barreto-Vizcaino S, Rivera-Castano L, Rios-Madariaga C (2009) Subcutaneous histamine versus botulinum toxin type A in migraine prophylaxis: a randomized, double-blind study. Eur J Neurol 16(1):88–94

54. Yuan H, Silberstein SD (2018) Histamine and Migraine. Headache 58(1):184–193

55. Chan KY, Baun M, de Vries R, van den Bogaerdt AJ, Dirven CM, Danser AH et al (2011) Pharmacological characterization of VIP and PACAP receptors in the human meningeal and coronary artery. Cephalalgia 31(2):181–189

56. Dickson L, Finlayson K (2009) VPAC and PAC receptors: From ligands to function. Pharmacol Ther 121(3):294–316

57. Guo S, Vollesen AL, Hansen YB, Frandsen E, Andersen MR, Amin FM et al (2017) Part II: Biochemical changes after pituitary adenylate cyclase-activating polypeptide-38 infusion in migraine patients. Cephalalgia 37(2):136–147

58. Akerman S, Goadsby PJ. Neuronal PAC1 receptors mediate delayed activation and sensitization of trigeminocervical neurons: Relevance to migraine. Sci Transl Med. 2015;7(308):308ra157

59. Okragly AJ, Morin SM, DeRosa D, Martin AP, Johnson KW, Johnson MP et al (2018) Human mast cells release the migraine-inducing factor pituitary adenylate cyclase-activating polypeptide (PACAP). Cephalalgia 38(9):1564–1574

60. Jansen-Olesen I, Hougaard PS (2018) PACAP and its receptors in cranial arteries and mast cells. J Headache Pain. 19(1):16

61. Holland PR, Goadsby PJ (2018) Targeted CGRP Small Molecule Antagonists for Acute Migraine Therapy. Neurotherapeutics 15(2):304–312

62. O'Connor TP, van der Kooy D (1988) Enrichment of a vasoactive neuropeptide (calcitonin gene related peptide) in the trigeminal sensory projection to the intracranial arteries. J Neurosci 8(7):2468–2476

63. Horgan K, van der Kooy D (1992) Visceral targets specify calcitonin gene-related peptide and substance P enrichment in trigeminal afferent projections. J Neurosci 12(4):1135–1143

64. Treede RD, Meyer RA, Raja SN, Campbell JN (1992) Peripheral and central mechanisms of cutaneous hyperalgesia. Prog Neurobiol 38(4):397–421

65. LaMotte RH, Shain CN, Simone DA, Tsai EF (1991) Neurogenic hyperalgesia: psychophysical studies of underlying mechanisms. J Neurophysiol 66(1):190–211

66. Lewis T (1936) Experiments relating to cutaneous hyperalgesia and its spread through somatic nerves. Clin Sci 2

67. Hardy JD, Wolff HG, Goodell H (1950) Experimental evidence on the nature of cutaneous hyperalgesia. J Clin Invest 29(1):115–140

68. Lipton RB, Bigal ME, Ashina S, Burstein R, Silberstein S, Reed ML et al (2008) Cutaneous allodynia in the migraine population. Ann Neurol 63(2):148–158

69. Burstein R, Yarnitsky D, Goor-Aryeh I, Ransil BJ, Bajwa ZH (2000) An association between migraine and cutaneous allodynia. Ann Neurol 47(5):614–624