



Aromatase inhibitors evoke periorbital allodynia in mice via calcitonin gene-related peptide and its receptors in Schwann cells

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ABSTRACT

Background: Treatment with the currently recommended aromatase inhibitors (AIs) for adjuvant endocrine treatment of estrogen receptor-positive breast cancer is associated with debilitating musculoskeletal pain symptoms (AIMS) and headache. Recent evidence suggests that the proalgesic channel transient receptor potential ankyrin 1 (TRPA1) is implicated in AIMS. Here, we investigated the cellular and molecular mechanisms, including TRPA1, implicated in periorbital mechanical allodynia (PMA), a surrogate of headache-like pain, evoked by AIs in mice.

Methods: C57BL6/J mice were treated with intragastric letrozole (0.05-0.5 mg/kg), exemestane (1-5 mg/kg) or anastrozole (0.02-0.2 mg/kg) and were evaluated by applying von Frey filaments to the periorbital region over the rostral portion of the eye. Some mice were pretreated (subcutaneous in the periorbital area) with receptor, channel, or enzyme inhibitors. PMA was also investigated in mice with selective silencing of *Trpa1* and receptor activity modifying protein 1 [*Ramp1*, the component of calcitonin gene related peptide (CGRP) receptor required for its functioning] in Schwann cells (*Plp-Cre⁺-Trpa1^{fl/fl}* and *Plp-Cre⁺-Ramp1^{fl/fl}* mice, respectively) or trigeminal neurons (*Adv-Cre⁺-Trpa1^{fl/fl}* and *Adv-Cre⁺-Ramp1^{fl/fl}* mice, respectively).

Results: Letrozole dose-dependently produced PMA that was attenuated by a TRPA1 antagonist (A967079) or a CGRP receptor antagonist (olcegepant), whereas indomethacin was ineffective. Selective silencing of *Trpa1* in both Schwann cells and trigeminal neurons reduced letrozole-evoked PMA. Silencing of *Ramp1* in Schwann cells, but not in trigeminal neurons, attenuated PMA. Inhibition of the intracellular pathway known to promote PMA by CGRP action in Schwann cells, including adenylyl cyclase (SQ-22536), nitric oxide synthase (L-NG-Nitro arginine methyl ester), and oxidative stress (N-tert-butyl-a-phenylnitron) inhibitors reduced letrozole-evoked PMA. PMA evoked by exemestane (1, 5, 10 mg/kg i.g.) or anastrozole (0.02, 0.1, 0.2 mg/kg i.g.) was also markedly reduced in mice with selective silencing of TRPA1 in Schwann cells and nociceptors.

Conclusions: Data indicate that letrozole, targeting TRPA1 in peptidergic nerve terminals, releases CGRP that engages its receptor in adjacent Schwann cells to trigger a complex intracellular pathway that results in TRPA1 activation and the ensuing ROS release to sustain PMA. Should these mechanisms be present in patients, their inhibition may ameliorate cephalic mechanical allodynia associated with aromatase inhibitors-induced headaches.

Key words: Schwann cells, TRPA1, CGRP, aromatase inhibitors, headache.

Introduction

The use of aromatase inhibitors (AIs) for adjuvant endocrine treatment of estrogen receptor (ER)-positive breast cancer is associated in about 40% of the patients with a series of debilitating musculoskeletal pain symptoms (AIMS), which include typical inflammatory signs, such as morning stiffness and pain in the hands, knees, hips, lower back, and shoulders (1-3), although neuropathic and mixed pain may be also present (4, 5). Headache has been described as an important side effect reported by patients undergoing aromatase inhibitor (AI) treatment (6). These variable and widespread pain symptoms can cause discontinuation of AI treatment (7). In about 20% of patients who discontinued AI treatment, the major reason (46%) was arthralgia, although for a significant proportion of patients (9%), the cause was headache (8). The mechanism underlying the exacerbation of AIMS and headache is poorly understood, and patients are undertreated.

The three currently used AIs, exemestane, letrozole, and anastrozole (9), possess common chemical features, leading to the hypothesis that they target the proalgesic transient receptor potential ankyrin 1 (TRPA1) channel (10, 11). Steroidal exemestane includes a system of highly electrophilic conjugated Michael acceptor groups, which might react with the thiol groups of reactive cysteine residues of the TRPA1 (10, 12).

Anastrozole and letrozole, which are non-steroidal, contain aliphatic and aromatic moieties that, like tear gas 2-chlorobenzylidene malononitrile nitriles, react with cysteine residues of TRPA1 to activate the channel (10, 13).

A previous study reported that, *in vitro*, the three AIs directly target TRPA1 expressed by primary sensory neurons (10), thereby increasing intracellular calcium, and releasing the pro-migraine neuropeptide, calcitonin gene related peptide (CGRP) (10). In addition, AIs produced mechanical allodynia *in vivo* in mouse hind paw and decreased grip strength in the forelimb, all effects attenuated by pharmacological inhibition or global genetic deletion of *Trpa1* (10). More recently, we reported (14) that the activation of trigeminal nerve fibers of the mouse periorbital area by the TRP vanilloid 1 (TRPV1) agonist, capsaicin, causes acute and transient spontaneous nociceptive responses most likely mediated by direct TRPV1 stimulation and the ensuing afferent impulse. These responses were followed by sustained mechanical allodynia encoded by CGRP released from peptidergic nerve terminals *via* a hitherto unrecognized complex pathway in adjacent Schwann cells (scs). The binding of CGRP to its receptor (calcitonin-like receptor and receptor activity modifying protein 1, CLR-RAMP1) in scs promotes the internalization in endosomes associated with a sustained increase in cyclic AMP. The subsequent protein kinase A activation stimulates an endothelial nitric oxide synthase (enos) to

release NO that targets SC TRPA1. The SC TRPA1-dependent prolonged reactive oxygen species (ROS) generation sustains mechanical allodynia by sensitizing neuronal TRPA1 to innocuous mechanical stimuli (14, 15).

Here, we sought to understand whether AIs produce periorbital mechanical allodynia (PMA) in mice by targeting TRPA1 in trigeminal neurons that, by releasing CGRP, promote the SC-dependent proalgesic pathway. We used the currently most prescribed AI, letrozole, pharmacological antagonists, and selective silencing in scs and trigeminal neurons of TRPA1 and RAMP1. Exemestane and anastrozole were also used in some experiments. We found that letrozole and the other two AIs, by targeting neuronal TRPA1, elicit CGRP release and the ensuing SC-dependent pathway that sustains PMA.

Results

Neuronal TRPA1 and CGRP release are implicated in letrozole-induced periorbital mechanical allodynia. We initially investigated whether systemic administration of letrozole induces PMA. Intragastric letrozole elicited C57BL/6J mice in a dose-dependent manner (Figure 1a) and, consistently with previous reports, a concurrent hind paw mechanical allodynia (HMA) (Supplementary Figure 1). To assess the involvement of TRPA1 in letrozole-induced PMA, mice were treated subcutaneously in the periorbital area with the selective TRPA1 antagonist, A967079, which attenuated PMA (Figure 1b). Local administration of the CLR-RAMP1 antagonist, olcegepant, prevented PMA and HMA evoked by letrozole (Figure 1c and Supplementary Figure 1). The cyclooxygenase (COX) inhibitor, indomethacin, failed to reduce letrozole-evoked PMA (Figure 1d). Mice with selective *Trpa1* silencing in sensory neurons (*Adv-Cre⁺;Trpa1^{fl/fl}*) showed reduced PMA evoked by letrozole compared to control mice (Figure 1e). Overall, these data suggest that letrozole-evoked PMA and HMA are dependent on neuronal TRPA1 activation and the ensuing CGRP release.

Schwann cell RAMP1, intracellular mediators activated by CGRP and Schwann cell TRPA1, are implicated in letrozole-induced periorbital mechanical allodynia. We recently reported that exogenous or endogenous released CGRP provokes PMA through Schwann cell CLR/RAMP1 activation (14). Our present

observations indicate that PMA and HMA were reduced in mice with selective silencing of the *Ramp1* subunit in scs (*Plp-CreERT⁺;Ramp1^{fl/fl}* mice) (Figure 2a). In contrast, letrozole produced a similar PMA in mice with selective deletion of *Ramp1* in primary sensory neurons (*Adv-Cre⁺;Ramp1^{fl/fl}*) and control mice (Figure 2b), suggesting a critical and exclusive role of CLR/RAMP1 expressed in scs surrounding cutaneous terminals of trigeminal fibers.

SC CLR/RAMP1 activation was shown (14) to initiate a cascade of intracellular events, including camp increase, protein kinase A (PKA) activation, endothelial nitric oxide synthase (enos) phosphorylation, and subsequent nitric oxide (NO) release. Notably, subcutaneous (periorbital area) administration of selective inhibitors of adenylyl cyclase (SQ-22536) or nitric oxide synthase (L-NG-Nitro arginine methyl ester, L-NAME) inhibited PMA induced by letrozole (Figure 2c,d). Moreover, nitric oxide (NO), by targeting the oxidant-sensitive channel TRPA1 in scs, elicits persistent ROS generation (15). Local administration of a ROS scavenger (N-tert-butyl-a-phenylnitron, PBN) attenuated PMA induced by letrozole (Figure 2e). Lastly, PMA induced by letrozole was reduced in mice with selective silencing of *Trpa1* (*Plp-CreERT⁺;Trpa1^{fl/fl}*) in scs (Figure 2f).

Furthermore, considering that patients in clinical settings undergo daily treatment with AIs for up to 5 years, the ability of prolonged letrozole administrations to induce PMA dependent on SC TRPA1 and RAMP1 was tested. In control mice, repeated (once a day for 15 consecutive days) systemic letrozole produced a sustained PMA that was attenuated in both *Plp-CreERT⁺;Trpa1^{fl/fl}* and *Plp-CreERT⁺;Ramp1^{fl/fl}* mice (Figure 2g,h).

Exemestane and anastrozole-induced periorbital mechanical allodynia is mediated by Schwann cell RAMP1 and TRPA1. AIs, which include both the steroidal exemestane and non-steroidal azole derivatives, such as anastrozole (9), have been found to directly activate the TRPA1 channel, similar to letrozole (10). Our study demonstrated that both exemestane and anastrozole induce dose-related and sustained PMA (Figure 3a,b). Consistent with our findings with letrozole, PMA induced by exemestane and anastrozole was also reduced in mice with SC-specific deletion of either *Ramp1* (*Plp-CreERT⁺;Ramp1^{fl/fl}*) or *Trpa1* (*Plp-CreERT⁺;Trpa1^{fl/fl}*) (Figure 3c-f). These results collectively support the view that PMA associated with AI treatment relies on sustained activation of CGRP and TRPA1 receptors on Schwann cells.

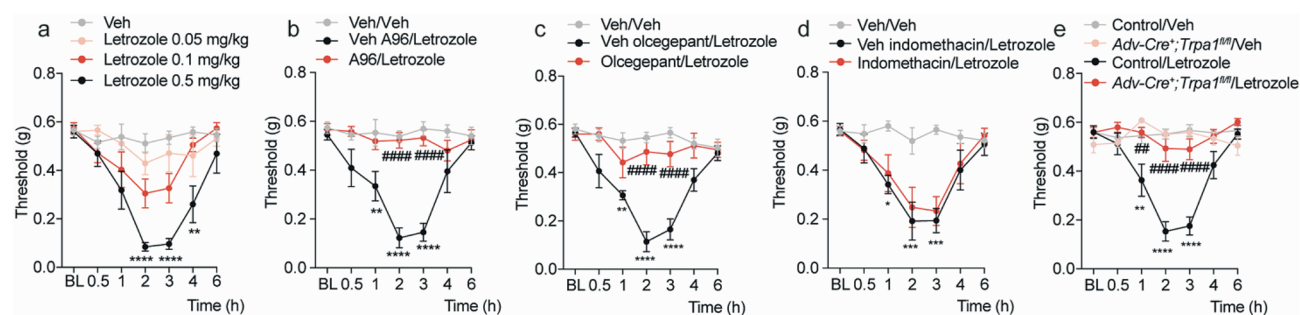


Figure 1. Neuronal TRPA1 and CGRP mediates letrozole-evoked periorbital mechanical allodynia (PMA). a) Dose- and time-dependent PMA evoked by letrozole (0.05, 0.1, 0.5 mg/kg, intragastric, i.g.) Or vehicle (veh) in C57BL/6J mice. b) PMA evoked by letrozole (0.5 mg/kg, i.g.) Or veh in C57BL/6J mice pretreated (30 min before) with A967079 (A96, 300 nmol, periorbital, p.orb.) Or veh. c) PMA evoked by letrozole (0.5 mg/kg, i.g.) Or veh in C57BL/6J mice pretreated (30 min before) with olcegepant (1 nmol, p.orb.) Or veh. d) PMA evoked by letrozole (0.5 mg/kg, i.g.) Or veh in C57BL/6J mice pretreated (30 min before) with indomethacin (280 nmol, p.orb.) Or veh. e) PMA evoked by letrozole (0.5 mg/kg, i.g.) Or vehicle in *Adv-Cre⁺;Trpa1^{fl/fl}* and Control mice. N=6 mice per group. Data are mean \pm s.e.m. 2-way ANOVA and Bonferroni correction. * $p < 0.05$ *** $p < 0.001$ **** $p < 0.0001$ vs. Veh, Veh/Veh, Control/Veh. ## $p < 0.01$, ### $p < 0.0001$ vs. Veh A96/Letrozole, Veh Olcegepant/Letrozole, Control/Letrozole.

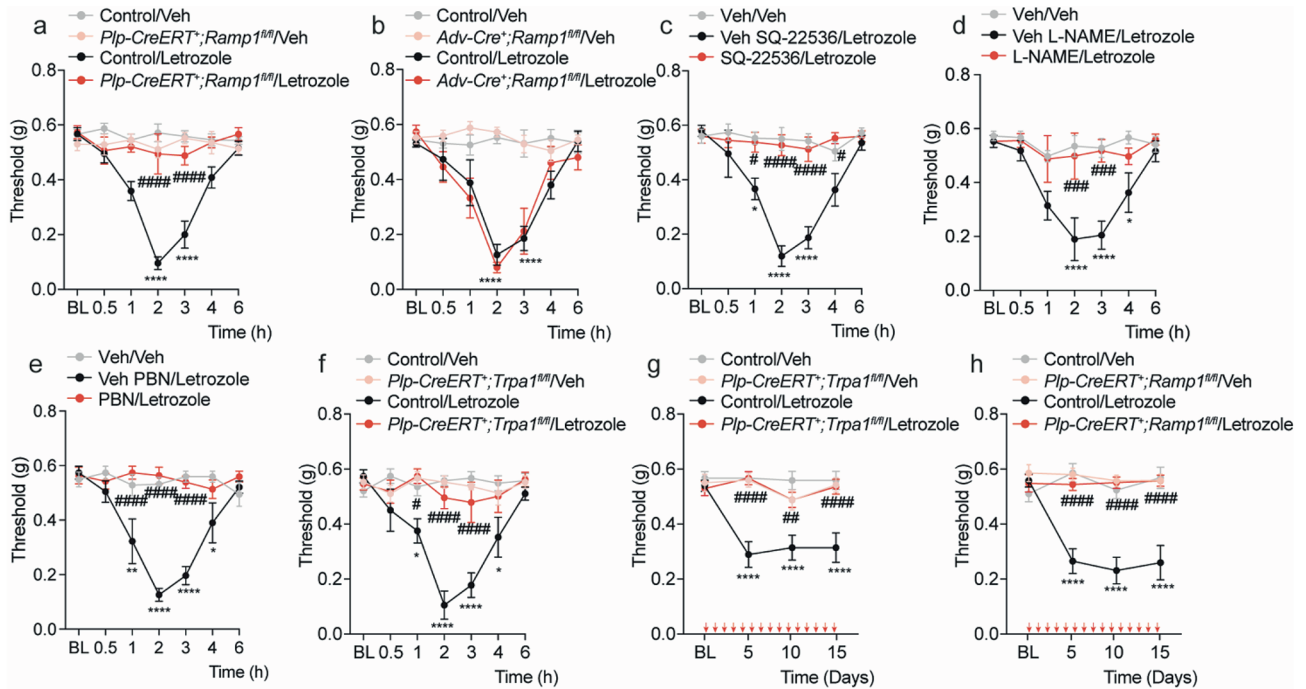


Figure 2. Schwann cell RAMP1 and TRPA1 activation sustains letrozole-evoked periorbital mechanical allodynia (PMA). a) PMA evoked by letrozole (0.5 mg/kg, intragastric, i.g.) Or vehicle (veh) in *Plp-CreERT⁺;Ramp1^{fl/fl}* and Control mice. b) PMA evoked by letrozole (0.5 mg/kg, i.g.) Or veh in *Adv-Cre⁺;Ramp1^{fl/fl}* and Control mice. c,d,e) PMA evoked by letrozole (0.5 mg/kg, i.g.) Or veh in C57BL/6J mice pre-treated (30 min before) with SQ-22536 (25 nmol, periorbital, p.orb.), L-NAME (1 μ mol, p.orb.) PBN (670 nmol, p.orb.) Or veh. f) PMA evoked by letrozole (0.5 mg/kg, i.g.) Or veh in *Plp-CreERT⁺;Trpa1^{fl/fl}* and Control mice. PMA evoked by letrozole (0.5 mg/kg, i.g.), once a day for 15 consecutive days, red arrow) or veh in (g) *Plp-CreERT⁺;Trpa1^{fl/fl}* and Control and (h) *Plp-CreERT⁺;Ramp1^{fl/fl}* and Control mice. N=6 mice per group. Data are mean \pm s.e.m. 2-way ANOVA and Bonferroni correction. * $p < 0.05$ ** $p < 0.01$ **** $p < 0.0001$ vs. Control/Veh, Veh/Veh. # $p < 0.05$ #### $p < 0.0001$ vs. Control/Letrozole, Veh SQ-22536/Letrozole, Veh L-NAME/Letrozole, Veh PBN/Letrozole.

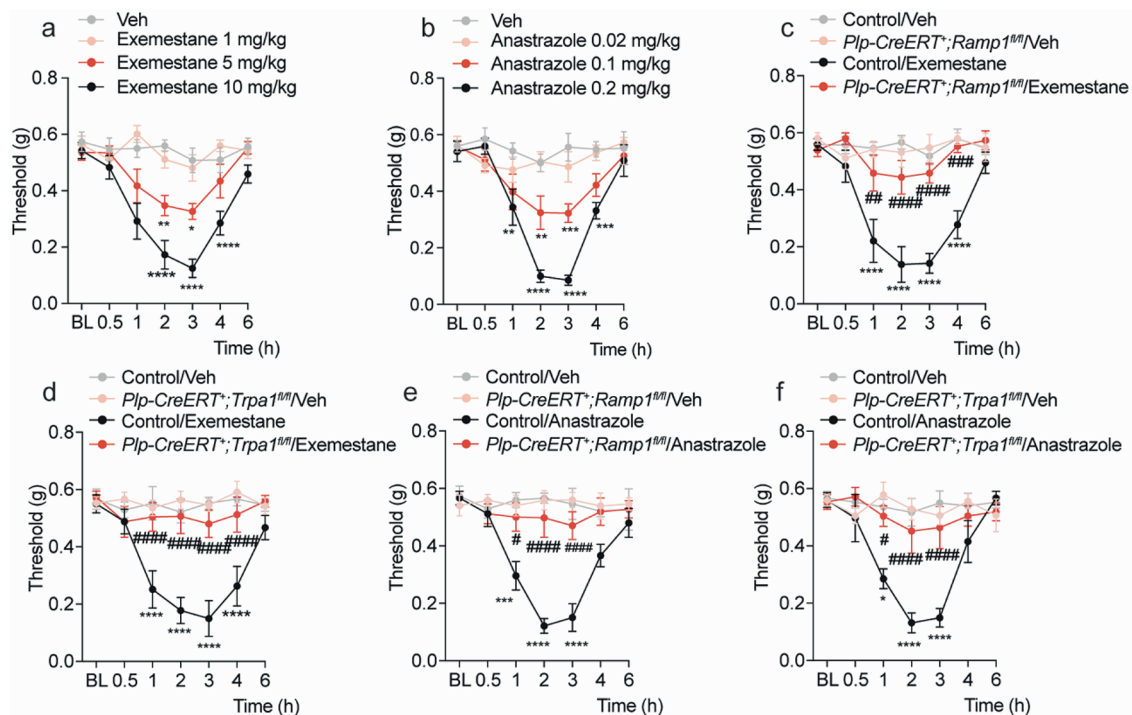


Figure 3. Schwann cell RAMP1 and TRPA1 sustains exemestane- and anastrozole-evoked periorbital mechanical allodynia (PMA). a) Dose- and time-dependent PMA evoked by exemestane (1, 5, 10 mg/kg, intragastric, i.g.) Or vehicle (veh) in C57BL/6J mice. b) Dose- and time-dependent PMA evoked by anastrozole (0.02, 0.1, 0.2 mg/kg, i.g.) Or veh in C57BL/6J mice. c,d) PMA evoked by exemestane (10 mg/kg, i.g.) Or veh in *Plp-CreERT⁺;Ramp1^{fl/fl}*, *Plp-CreERT⁺;Trpa1^{fl/fl}* and Control mice. e,f) PMA evoked by anastrozole (0.2 mg/kg, i.g.) Or veh in *Plp-CreERT⁺;Ramp1^{fl/fl}*, *Plp-CreERT⁺;Trpa1^{fl/fl}* and Control mice. (n=6 mice per group). Data are mean \pm s.e.m. 2-way ANOVA, Bonferroni correction. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ vs. Veh, Control/Veh. # $p < 0.05$ ## $p < 0.01$ ### $p < 0.001$ #### $p < 0.0001$ vs. Control/Exemestane, Control/Anastrozole.

Discussion

Previous findings have shown that systemic exposure to AIs (10) or their substrate, androstenedione (11), cause pain-like behaviors in mice localized to both fore paw and hind paw, indicating a widespread and diffuse localization of a proalgesic phenotype associated with the use of these anti-breast cancer medicines. The present study shows that systemic exposure to AIs can target the periorbital area of mice as they dose-dependently cause PMA. The observation that the three AIs, letrozole, anastrozole, and exemestane, exert similar TRPA1-dependent mechanical allodynia supports their role as channel agonists that, acting primarily on the neuronal TRPA1, elicit the release of the pro-migraine neuropeptide, CGRP (16, 17). Thus, the common pathway and the widespread localization of their proalgesic activity suggest that, everywhere they target peptidergic nerve terminals, they produce sustained mechanical allodynia.

The use of pharmacological tools and genetic strategies to selectively silence regulatory proteins in specific cell types, such as scs of primary sensory neurons, has been instrumental to propose their critical role in mouse models of neuropathic (15) and cancer (18, 19) pain. The implication of peptidergic nerve terminals, most likely C-fiber nociceptors and the ensuing CGRP release, the surrounding scs, and either the C-fibers or, most probably, other adjacent nerve terminals with Ad-fiber or C-fiber, has been proposed to play a critical role in a mouse model of migraine pain (14). Here, the two antagonists, A967079 (the TRPA1 antagonist) and olcegepant (the CLR/RAMP1 antagonist), reduced PMA evoked by letrozole, suggesting the most parsimonious hypothesis that letrozole, targeting TRPA1, releases CGRP that promotes PMA. Selective *Ramp1* or *Trpa1* silencing in scs, which caused CLR/RAMP1 and TRPA1 unresponsiveness, reduced PMA elicited by both single or repeated (mimicking prolonged treatment schedule in patients) letrozole administration, supports this hypothesis. However, we recently showed that CGRP sustains PMA via the activation of neuronal TRPA1 by the ROS generated by a feedforward mechanism promoted by SC TRPA1, which sustains PMA (14). Thus, the observation that neuronal *Trpa1* silencing attenuates PMA might indicate that letrozole directly activates neuronal TRPA1.

A series of pharmacological agents, such as the adenylyl cyclase inhibitor, SQ-22536, the NOS inhibitor, L-NAME, and the free-radical spin trapping agent, PBN, reduced PMA by letrozole. All these interventions have been shown to inhibit PMA in mice *in vivo*, and in cultured human scs *in vitro* the increases in cyclic AMP, NO, and ROS evoked by CGRP (14). Thus, the most parsimonious hypothesis suggests that, due to its nitrile moiety (13, 20), letrozole directly targets TRPA1 in C-fiber trigeminal neurons to release CGRP in the periorbital area. Then, CGRP, as previously shown for CGRP released, via TRPV1, by capsaicin (14), initiates a proalgesic pathway in scs that encompasses increased cyclic AMP levels and the release of NO that, engaging TRPA1, generates ROS that, targeting TRPA1 in adjacent nociceptors, converts these nerves in an allodynic phenotype. The observation that PMA induced by both exemestane and anastrozole was reduced in mice with selective silencing of *Ramp1* or *Trpa1* in scs indicates that all AIs encode the same SC-dependent proalgesic pathway reported for letrozole. While the paper is supported by robust *in vivo* findings, we acknowledge that it has been performed in mice only. Thus, the translational value of the present findings requires further proof in patients treated with AIs.

Conclusions

Migraine attacks are characterized by pulsating and throbbing head pain that is aggravated by common daily activities, such

as walking or climbing stairs (21). Facial cutaneous allodynia is one of the symptoms of migraine attack, and allodynia is a feature of chronic migraine (22, 23). The present study shows that PMA, a surrogate of headache-like pain produced by AIs, is mediated by the CGRP receptor and the TRPA1 channel in scs. Should the mechanisms, identified here in mice, be present in humans, the novel anti-CGRP medicines may alleviate headaches that manifest in women with breast cancer undergoing AI treatment.

Materials and Methods

Animals. Male mice were used throughout (25-30 g, 6-8 weeks). In several mouse pain models where mechanical allodynia was mediated by CGRP release and/or TRPA1 activation, a similar response was found between male and female mice; therefore, mechanical allodynia was determined to be unrelated to sex (14, 24-26). Thus, in accordance with the 3R guidelines to minimize the number of animals and avoid possible confounding effects of hormone fluctuation in pain perception, only male mice were used. The following mouse strain was used: C57BL/6J mice (Charles River, RRID:IMSR_JAX:000664). To generate mice in which the *Trpa1* and *Ramp1* genes were conditionally silenced in Schwann cells/oligodendrocytes, homozygous 129S-*Trpa1*^{tm2Kyk/J} (*floxed TRPA1*, *Trpa1*^{fl/fl}, RRID:IMSR_JAX:008649 Jackson Laboratory) and C57BL/6N-*Ramp1*^{<tm1c(EUCOMM)Wtsi>/H} (*floxed Ramp1*, *Ramp1*^{fl/fl} Stock No: EM:07401, MRC HARWELL Mary Lion Center) (14) were crossed with hemizygous B6.Cg-Tg(*Plp1-creERT*)3Pop/J mice (*Plp1-creERT*, RRID:IMSR_JAX:005975 Jackson Laboratory), expressing a tamoxifen-inducible Cre in myelinating cells (*Plp1*, proteolipid protein myelin 1) (15). The progeny (*Plp1-creERT*;*Trpa1*^{fl/fl} and *Ramp1-creERT*;*Trpa1*^{fl/fl}) were genotyped by PCR for *Trpa1*, *Ramp1*, and *Plp1-creERT*. Mice negative for *Plp1-creERT* (*Plp1-creERT*⁻;*Trpa1*^{fl/fl} and *Plp1-creERT*⁻;*Ramp1*^{fl/fl}) were used as control. Both positive and negative mice to *creERT* and homozygous for *floxed Trpa1* (*Plp1-creERT*⁺;*Trpa1*^{fl/fl} and *Plp1-creERT*⁻;*Trpa1*^{fl/fl}, respectively) and *floxed Ramp1* (*Plp1-creERT*⁺;*Ramp1*^{fl/fl} and *Plp1-creERT*⁻;*Ramp1*^{fl/fl}) mice were treated with intraperitoneal (i.p.) 4-hydroxytamoxifen (4-OHT) (1 mg/100 µl in corn oil, once a day for 3 consecutive days). Treatments resulted in Cre-mediated ablation of *Trpa1* and *Ramp1* in PLP-expressing Schwann cells/oligodendrocytes. To selectively delete the *Trpa1* gene or *Ramp1* in primary sensory neurons, *Trpa1*^{fl/fl} or *Ramp1*^{fl/fl} mice were crossed with hemizygous *Advillin-Cre* mice (*Adv-Cre*) (27, 28). Both positive and negative mice to *Cre* and homozygous for *floxed Trpa1* (*Adv-Cre*⁺;*Trpa1*^{fl/fl} and *Adv-Cre*⁻;*Trpa1*^{fl/fl}, respectively) or *Ramp1* (*Adv-Cre*⁺;*Ramp1*^{fl/fl} and *Adv-Cre*⁻;*Ramp1*^{fl/fl}) were used.

The group size of n=6 animals for behavioral experiments was determined by sample size estimation using G*Power (v3.1) (29) to detect size effect in a post-hoc test with type 1 and 2 error rates of 5 and 20%, respectively. Mice were allocated to vehicle or treatment groups using a randomization procedure (<http://www.randomizer.org/>). Investigators were blinded to the identities (genetic background) and treatments, which were revealed only after data collection. No animals were excluded from experiments. The behavioral studies followed the animal research reporting *in vivo* experiment (ARRIVE) guidelines (30). Mice were housed in a temperature- and humidity-controlled vivarium (12 hr dark/light cycle, free access to food and water, 5 animals per cage). At least 1 hr before behavioral experiments, mice were acclimatized to the experiment room and behavior was evaluated between 9:00 am and 5:00 pm. All the procedures were conducted following the current guidelines for laboratory animal care and the ethical guidelines for investigations of experimental pain in conscious ani-

imals set by the International Association for the Study of Pain (31). Animals were euthanized with inhaled CO₂ plus 10-50% O₂; confirmation of death was achieved by a physical method of killing (decapitation) (AVMA Guidelines for the Euthanasia of Animals, 2020).

Reagents and treatment protocols. If not otherwise indicated, reagents were obtained from Merck Life Science (Milan, Italy). Letrozole (0.05, 0.1, 0.5 mg/kg), exemestane (1, 5, 10 mg/kg), and anastrozole (0.02, 0.1, 0.2 mg/kg), or their vehicle (0.5% carboxymethyl cellulose, CMC), were given by intragastric (i.g.) route of administration. Antagonists and inhibitors were administered locally, by periorbital (10 µl/site, p.orb.) or intraplantar (10 µl/site, i.pl.) route of administration. Olcegepant (1 nmol, p.orb. or i.pl.), A967079 (300 nmol, p.orb. or i.pl.), SQ-22536 (25 nmol, p. b.), [(1E,3E)-1-(4-Fluorophenyl)-2-methyl-1-penten-3-one oxime], phenyl-N- tert-butyl nitron (PBN) (670 nmol, p.orb.), L-NAME (1 µmol, p.orb.), indomethacin (280 nmol, p.orb.), or vehicle (4% dimethyl sulfoxide, 4% Tween 80 in nacl 0.9%) were administered 30 minutes before letrozole (0.5 mg/kg, i.g.), exemestane (10 mg/kg, i.g.) and anastrozole (0.2 mg/kg, i.g.). In a second series of experiments, mice were treated with letrozole (0.5 mg/kg, i.g.) or veh once a day for 15 consecutive days. PMA was assessed at 2 h after the daily letrozole administration.

Behavioral assays. Periorbital mechanical allodynia. The measurement of mechanical thresholds in the periorbital area is considered a reliable method to investigate the mechanisms underlying headache and migraine pain (32). PMA was assessed using the up-down paradigm (33). Briefly, mice were placed in a restraint apparatus designed for the evaluation of periorbital mechanical thresholds (25). 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) before (basal threshold) and after treatments (25). On the day of the experiment, after 20 min 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, 0.6 and 1.0 g) were applied to the periorbital area perpendicular to the skin, with sufficient force to cause slight buckling, and held for approximately 5 s 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. Mechanical stimulation started with the 0.16 g filament. Absence of response after 5 s led to the use of a filament with increased force, 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.

Hindpaw mechanical allodynia. The mechanical paw-withdrawal threshold was measured using von Frey filaments of increasing stiffness (0.02-2 g) applied to the plantar surface of the mouse hind paw, according to the up-and-down paradigm (33). The 50% mechanical paw-withdrawal threshold (g) response was then calculated from the resulting scores. Mechanical paw-withdrawal threshold was measured at baseline and at different times following treatments.

Statistical analysis. The results are expressed as the mean ± standard error mean. For behavioral experiments with repeated measures, a two-way mixed-model ANOVA followed by a *post-hoc* Bonferroni's test was used. Statistical analyses were performed on raw data using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). P-values less than 0.05 were considered significant. The statistical tests used and sample size for each analysis are shown in the figure legends.

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Online supplementary material:

Supplementary Figure 1. a) Dose- and time-dependent hind paw mechanical allodynia (HMA) evoked by letrozole (0.05, 0.1, 0.5 mg/kg, intragastric, i.g.) or vehicle (veh) in C57BL/6J mice. b) HMA evoked by letrozole (0.5 mg/kg, i.g.) or veh in C57BL/6J mice pretreated (30 min before) with olcegepant (Olceg, 1 nmol, i.pl.) or veh. (n=6 mice per group).

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