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Transient receptor potential ankyrin 1 mediates headache-related cephalic allodynia in a mouse model of relapsing–remitting multiple sclerosis

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Abstract

Primary headache conditions are frequently associated with multiple sclerosis (MS), but the mechanism that triggers or worsens headaches in patients with MS is poorly understood. We previously showed that the proalgesic transient receptor potential ankyrin 1 (TRPA1) mediates hind paw mechanical and cold allodynia in a relapsing–remitting experimental autoimmune encephalomyelitis (RR-EAE) model in mice. Here, we investigated the development of periorbital mechanical allodynia (PMA) in RR-EAE, a hallmark of headache, and if TRPA1 contributed to this response. RR-EAE induction by injection of the myelin oligodendrocyte peptide fragment$_{35-55}$ (MOG$_{35-55}$) and Quillaja A adjuvant (Quil A) in C57BL/6J female mice elicited a delayed and sustained PMA. The PMA at day 35 after induction was reduced by the calcitonin gene–related peptide receptor antagonist (olcegepant) and the serotonin 5-HT1$_{B/D}$ receptor agonist (sumatriptan), 2 known antimigraine agents. Genetic deletion or pharmacological blockade of TRPA1 attenuated PMA associated with RR-EAE. The levels of oxidative stress biomarkers (4-hydroxynonenal and hydrogen peroxide, known TRPA1 endogenous agonists) and superoxide dismutase and NADPH oxidase activities were increased in the trigeminal ganglion of RR-EAE mice. Besides, the treatment with antioxidants (apocynin or α-lipoic acid) attenuated PMA. Thus, the results of this study indicate that TRPA1, presumably activated by endogenous agonists, evokes PMA in a mouse model of relapsing–remitting MS.

Keywords: Headache, Sumatriptan, 4-Hydroxynonenal, Hydrogen peroxide, Calcitonin gene–related peptide, NADPH oxidase

1. Introduction

Multiple sclerosis (MS) is characterized by a chronic demyelinating and inflammatory process[38] that results in several debilitating symptoms, including different types of pain.[47,71] Several studies have reported that primary headaches, such as migraine tension-type headaches, are more frequent in patients with MS than in the general population.[33,46,56] Besides, various studies demonstrated a range from 4 to 61.8% of headaches in patients with MS, although the MS mechanisms that result in headaches are poorly known.[46]

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.painjournalonline.com).

PAIN 00 (2021) 1–10

http://dx.doi.org/10.1097/j.pain.0000000000002520

The transient receptor potential ankyrin 1 (TRPA1) is a cationic channel expressed in peripheral pain-detecting sensory neurons.[7,49,54,68] Transient receptor potential ankyrin 1 has emerged as a specific target for several exogenous headache triggers, and some pieces of evidence demonstrated that various antimigraine medicines have an inhibitory action on TRPA1 channel activity.[7,37,50,53,54] Besides, TRPA1 is also a recognized sensor of the redox state in the cellular environment[57] because it is activated by oxidative stress by-products, such as hydrogen peroxide (H_2O_2),[64] 4-hydroxynonenal (4-HNE),[69] and nitric oxide.[51]

TRPA1 activation in primary sensory neurons also evokes the peripheral release of the calcitonin gene–related peptide (CGRP), and this neuropeptide is the primary mediator of migraine headache.[44] Calcitonin gene–related peptide subcutaneous injection in the mouse periorbital area or rat trigeminal ganglion (intraganglionar) elicited prolonged periorbital mechanical allodynia (PMA).[4,44] It was also reported that CGRP released from periorbital trigeminal terminals caused PMA because of the gating of TRPA1 by the promigraine agent, glyceryl trinitrate, in cell bodies of trigeminal neurons.[49]

Facial mechanical allodynia (whisker pad and periorbital region) was detected in a mouse model of progressive MS induced by immunization with the MOG$_{35-55}$ antigen and complete Freund adjuvant (CFA).[16,18,19] Besides, we recently showed that TRPA1 mediates plantar mechanical and cold allodynia in a mouse model of RR-EAE induced by the immunization with MOG$_{35–55}$ and Quil A.[15] However, until now, no study has evaluated the development of PMA in a RR-EAE model or evaluated the mechanisms involved in this nociceptive behavior.

The aim of this study was 2-fold. First, we explored whether the mouse model of RR-EAE induced by the immunization with $MOG_{35–55}$ and Quil A caused PMA and whether classical antimigraine agents could inhibit this nociceptive response. Second, we investigated whether oxidative stress and TRPA1 were implicated in RR-EAE–induced PMA. Hence, our results indicate that mice developed PMA after RR-EAE induction and antimigraine drugs reduced the periorbital nociception. Furthermore, pharmacological and genetic approaches to reduce TRPA1 activation are able to reduce PMA in this model. Thus, TRPA1 channel seems to be activated by endogenous agonists to cause periorbital nociception after RR-EAE induction.

2. Materials and methods

2.1. Animals

The following mouse strains were used: C57BL/6J, littermate wild-type (*Trpa1*$^{+/+}$), and TRPA1-deficient (*Trpa1*$^{-/-}$) (KWAN et al., 2006) (female, 20-30 g, 4-6 weeks). Women present a high prevalence of developing RRMS[72]; thus, the RR-EAE model was optimized in female C57BL/6J.[31] All the animals (5 per cage) were maintained in controlled temperatures (22 ± 2°C) and bred in-house with a 12-h light–dark cycle (lights on from 7:00 AM to 7:00 PM) and were accommodated with wood shaving bedding and nesting material. Tap water and laboratory standard animal food (Puro Lab 22 PB pellet form, Puro Trato, Rio Grande do Sul, Brazil, and Charles River, Milan, Italy) were provided ad libitum. The animals were moved and acclimatized to the experiment room for at least 1 hour before each procedure. Experiments were performed according to the ethical guidelines to investigate pain in conscious animals (ZIMMERMANN, 1983), and the Institutional Committee for Animal Care and Use of the Federal University of Santa Maria (protocols #8640200617/2017 and #6412121218/2018) and the Italian Ministry of Health (protocol #1194/2015-PR) approved the experimental procedures. Behavioral studies followed the Animal Research Reporting In Vivo Experiments (ARRIVE) guidelines (MCGRATH; LILLEY, 2015). All experiments were performed by an operator blinded to drug administration and genotype. Besides, more information about the experimental protocols is provided in **Figure 1** Supplementary. The protocols described in Supplementary Fig 1 (A and B, available at http://links.lww.com/PAIN/B525) were performed only for this study. Besides the protocols described in Supplementary Fig 1(C) (available at http://links.lww.com/PAIN/B525), the results for mechanical and cold allodynia in the paw were previously published.[16] The total sample size for each experiment set was calculated by GPower 3.1 software. The GPower 3.1 software defined a sample size of n = 8 animals per group. This calculation agrees with other articles published in the pain research and studies using the EAE model.[1,15,30,60,61,63]

2.2. Reagents

If not otherwise indicated, all reagents were from Merck Life Science SRL (St. Louis, MO). Mouse myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) was synthesized by EZBiolab (Carmel, CA).

2.3. Relapsing–remitting experimental autoimmune encephalomyelitis mouse model

A mouse model of RR-EAE was performed by the subcutaneous injection of a mixed solution of $MOG_{35–55}$ antigen (200 μg) and

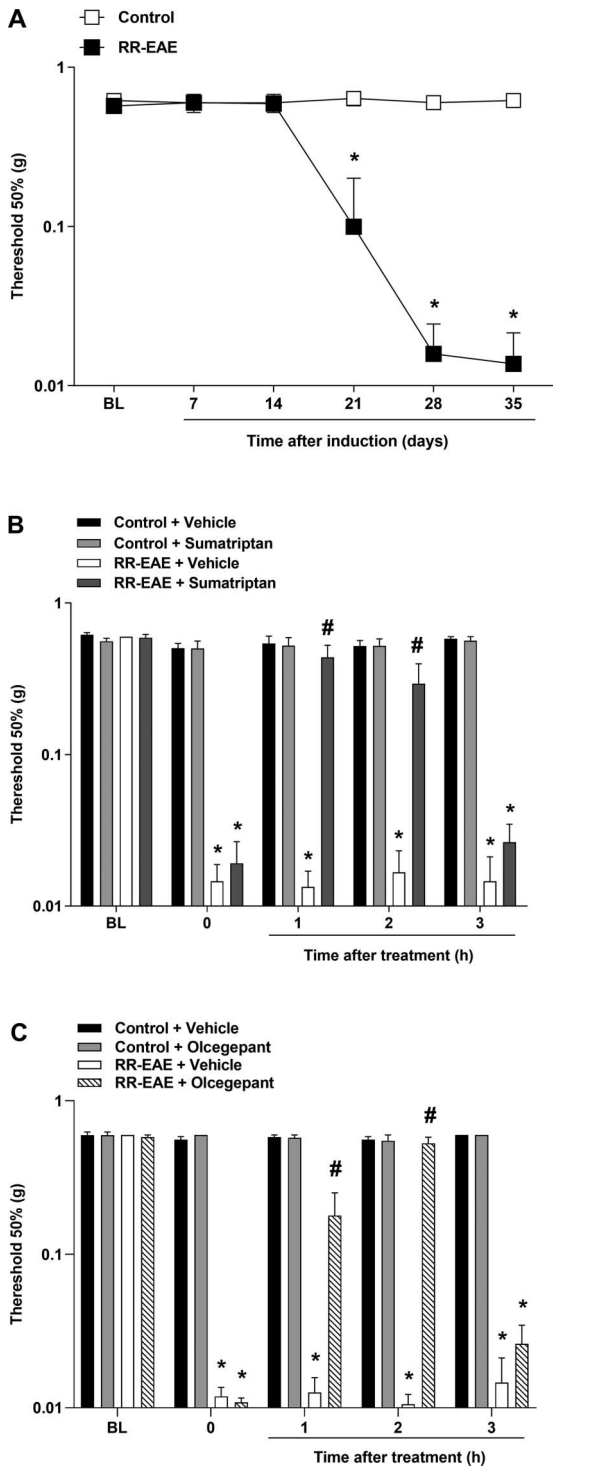

Figure 1. Mice developed periorbital mechanical allodynia (PMA) after relapsing–remitting experimental autoimmune encephalomyelitis (RR-EAE) induction, and sumatriptan or olcegepant administration showed an anti-allodynic effect. (A) PMA was detected on days 21 to 35 after RR-EAE induction. The vehicle group received isotonic saline 0.9% i.g. (sumatriptan) or dimethyl sulfoxide (DMSO) 1% in isotonic saline 0.9% i.p. (olcegepant). The treatment with (B) sumatriptan (0.6 mg/kg, intragastric, i.g.) and (C) olcegepant (1 mg/kg, intraperitoneal, i.p.) or vehicle (4% DMSO plus 4% Tween 80 in isotonic saline 0.9%.) was given on day 35 postinduction (p.i., time 0) of the RR-EAE model. Baseline measurements (described as BL in the graph) were observed before induction. Data are expressed as mean + SEM. (n = 8) for graphs B or C. *$P < 0.05$ when compared with the control group or baseline (BL) values; and #$P < 0.05$ when compared with RR-EAE vehicle-treated group [two-way ANOVA, followed by the Bonferroni post hoc test]. ANOVA, analysis of variance.

Quil A (45 μg) in phosphate-buffered saline solution (100 μL).[15,29,31] The mixture, containing MOG_{35-55} and Quil A, was injected in 2 equal amounts (50 μL) into both flanks on day 0. On day 0 and 48 hours (day 2) postinduction (p.i), mice received pertussis toxin (250 ng) diluted in phosphate-buffered saline (1 ng/μL) by intraperitoneal (i.p.) injection. Control mice only received equal doses of Quil A and pertussis toxin.[15,29,31]

2.4. Assessment of relapsing–remitting experimental autoimmune encephalomyelitis clinical signs

The assessment of the RR-EAE clinical signs in immunized mice and the control group was performed once a week over an experimental period of 35 days in a randomized, blinded manner using the clinical disease scoring paradigm. The score was assessed according to the following scale: 0, normal behavior; 0.5, limpness of the distal tail region and hunched appearance; 1, completely limp tail or developing weakness in the hind limbs; 1.5, limp tail and distinct hind limbs weakness recognized by unsteady gait and poor grip of hind limbs while hanging on cage underside; 2, limp tail with unilateral partial hind limb paralysis; 2.5, limp tail and partial paralysis of bilateral hind limbs; 3, complete paralysis of bilateral hind limbs; 3.5, complete bilateral hind limbs paralysis and unilateral forelimb paralysis; and 4, quadriplegia. Clinical scores $\leq$ 0.5 were indicative of no disease or disease remission,[31] and if clinical scores were $>$ 1.5, animals were excluded from the study. Besides, if an animal showed a weight loss of 20 to 30% of the initial weight, the animal was excluded from the experiments. Mice were monitored weekly after RR-EAE post-induction for the assessment of the RR-EAE clinical signs and weight.

2.5. Behavioral studies

2.5.1. Rotarod test

Mice were trained on the rotarod apparatus 1 day before induction. Mice were individually placed on the rotarod apparatus (fixed speed 16 rmp and 180 seconds), and the latency to the first fall was recorded.[58–60] This session was repeated 2 times. The rotarod test was performed on days 7, 14, 21, 28, and 35 p.i. of RR-EAE. Animals that failed to stay 180 seconds in the rotarod were removed from the study.[31,59] All mice developed the RR-EAE clinical scores without significant weight loss or reduction of locomotor function or coordination, and no animals were excluded from the study, as reported in previous studies.[15,31]

2.5.2. Periorbital mechanical allodynia

The periorbital mechanical threshold was evaluated using an up-and-down paradigm.[12,68] Mice were individually placed in a restrained apparatus designed for the evaluation of the periorbital mechanical threshold. One day before the first behavioral observation, mice were habituated to the apparatus. On the day of the experiment, after 60 minutes of adaptation inside the chamber, a series of von Frey filaments in logarithmic increments of force (0.008, 0.02, 0.04, 0.07, 0.16, 0.4, and 0.6 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 stroking its face with the forepaw, head withdrawal from the stimulus, or head shaking. The test was initiated with the 0.07 g filament. The absence of response after 5 seconds led to the use of a filament with increased force, whereas a positive response led to the use of a weaker filament. Six measurements were collected for each mouse or until 4 consecutive positive or negative responses occurred. The periorbital mechanical withdrawal threshold (expressed in g) was then calculated from the resulting scores.[17]

2.6. Treatment protocols

At day 35, induced and control mice received TRPA1 antagonists, HC-030031, A-967079,[3,15,63,68] metamizole ,or propyphenazone[52,68]; the antioxidants, α-lipoic acid or apocynin[15,63,68] (all, 100 mg/kg); sumatriptan[11,26] (0.6 mg/kg); or their vehicles (dimethyl sulfoxide, DMSO 1%; in isotonic saline 0.9%) by oral gavage (intragastric, i.g., 10 mL/kg). Olcegepant (1 mg/kg)[49] or its vehicle (4% DMSO plus 4% Tween 80 in isotonic saline 0.9%) was administered by i.p. injection (10 mL/kg). PMA was evaluated from 1 to 3 hours after vehicle or compound administration because no compound showed an antinociceptive effect 3 hours after injection.

2.7. Determination of oxidative biomarkers

On day 35, after RR-EAE induction or control, mice were killed, and the trigeminal ganglion and brainstem were dissected. The samples were homogenized in Tris-HCl buffer (50 mM, pH 7.4) and centrifuged at 3000 rpm for 10 minutes at 4°C to determine oxidative stress biomarkers.

2.7.1. Four-hydroxynonenal and H_2O_2 levels determination

According to the manufacturer's protocol, the content of 4-HNE was analyzed using an OxiSelect HNE Adduct Competitive Elisa Kit (Cell Biolabs, Inc, San Diego, CA).[15] The levels of 4-HNE were expressed in the percentage of 4-HNE when compared with the control group.

The levels of H_2O_2 were determined using the phenol red-horseradish peroxidase (HRPO) method.[9] In brief, 25 mM of sodium azide was added to supernatants to inhibit the cytochrome c oxidase enzyme present in samples.[39] The homogenate-containing sodium azide was centrifuged at 12.000 xg for 20 minutes at 4°C. A mixture containing supernatant, 25 μL of phenol red (100 mg/mL), and 5 μL of HRPO (50 mg/mL) was incubated in the dark for 10 minutes at 25°C. The reaction was stopped by adding NaOH (1 M, 20 uL). The absorbance of the enzymatic reaction was read at 610 nm using a SpectraMax i3 Platform (Molecular Devices, LLC, San Diego, CA) microplate reader. H_2O_2 levels were expressed as nanomoles (nmol H_2O_2) per mg protein compared with a standard H_2O_2 sample.

2.7.2. Superoxide dismutase and nicotinamide adenine dinucleotide phosphate oxidase activity evaluation

To analyze the SOD activity, samples were incubated for 2 minutes with adrenaline and glycine buffer at 30°C, and the absorbance was measured at 480 nm.[5] The reaction was read in a microkinetic reader (Fisher Biotech, Waltham, MA; BT, 2000). The values of SOD activity were reported as U/mL of the sample.[2,68] The activity of NADPH oxidase was observed in samples using an appropriate assay kit (CY0100, cytochrome c reductase, NADPH Sigma-Aldrich, Milan, Italy). The NADPH oxidase activity was expressed as U/mL/mg of tissue.

2.8. Quantitative real-time polymerase chain reaction

RNA was purified from trigeminal ganglion and brainstem. According to the manufacturer's protocol, the standard TRIzol extraction method was used together with an RNeasy Mini Kit

(QIAGEN, 74106). RNA concentration and purity were assessed spectrophotometrically by measuring the absorbance at 280 nm. The RNA was then reverse transcribed using a SuperScript IV One-Step RT-PCR System (Thermo Fisher Scientific, Waltham, MA; 12595025) according to the manufacturer's protocol. For relative quantification of mRNA compared with the housekeeping gene, real-time PCR was conducted using Rotor-Gene Q (Qiagen, Germantown, MD). The sets of mouse primers were as presented in **Table 1**.

2.9. Statistical analysis

Data were expressed as mean + SEM and analyzed statistically by the parametric and nonparametric Student *t* test, 1-way or 2-way analysis of variance according to the experimental protocol, followed by the posttest Bonferroni when needed. The maximal inhibition (I_{max}) was calculated using the following formula: 100 × (h posttreatment − basal postinduction mean)/(basal preinduction mean − basal postinduction mean). The individual values were inserted as column statistics in Prism GraphPad and calculated the mean of these values. To meet parametric assumptions, data of mechanical threshold scores were log-transformed before analyses. Differences between groups were considered significant when *P* values were less than 0.05 ($P < 0.05$), using the GraphPad Prism 5.0 program.

3. Results

3.1. Periorbital mechanical allodynia evoked by relapsing–remitting experimental autoimmune encephalomyelitis was reduced by sumatriptan and olcegepant administration

Administration of $MOG_{35–55}$ and Quil A elicited an increase in clinical scores that started at day 14 and peaked at day 35 (supplementary Fig. 2A, available at http://links.lww.com/PAIN/B525), indicating the onset of RR-EAE. However, no changes in locomotor activity (supplementary Fig. 2B, available at http://links.lww.com/PAIN/B525) or body weight (supplementary Fig. 2C, available at http://links.lww.com/PAIN/B525) between RR-EAE and the control group were detected during the 35 days of observation.

Relapsing–remitting experimental autoimmune encephalomyelitis mice developed a time-dependent increase in PMA from day 21 to 35 p.i. (**Fig. 1A**). At day 35 after immunization, treatment with the CGRP receptor antagonist, olcegepant (1 mg/kg, i.p.),[49] or the serotonin $5\text{-HT}1_{B/D}$ receptor agonist, sumatriptan (0.6 mg/kg, i.g.),[11] which have shown efficacy in the acute treatment of headache migraine attacks,[6,48,55] produced a reduction (maximum inhibition was 91% and 73% for olcegepant and sumatriptan, respectively) of PMA, without affecting the mechanical threshold of control mice (**Fig. 1B and C**).

3.2. Transient receptor potential ankyrin 1 genetic deletion and pharmacological inhibition decreased periorbital mechanical allodynia in relapsing–remitting experimental autoimmune encephalomyelitis mice

At day 35 after immunization, 2 chemically unrelated selective TRPA1 antagonists, HC-030031 (100 mg/kg, i.g.) and A-967079 (100 mg/kg, i.g.), diminished PMA (**Fig. 2A and B**). HC-030031 and A-967079 reduced PMA from 1 to 2 hours after i.g. administration, and the maximum inhibition of PMA produced by HC-030031 and A-967079 was 73% and 80%, respectively (**Fig. 2A and 2B**). Similar inhibition was produced by 2 analgesic drugs that have been recently identified as TRPA1 antagonists,[52] metamizole (100 mg/kg, i.g.) and propyphenazone (100 mg/kg, i.g.) (**Fig. 2B and C**), which induced a maximum inhibition of 89% and 100% of PMA induction, respectively.

3.3. Transient receptor potential ankyrin 1 genetic deletion impairs periorbital mechanical allodynia development in relapsing–remitting experimental autoimmune encephalomyelitis mice

Further and conclusive proof of the role of TRPA1 in the mouse model of RR-EAE was obtained with mice with genetic deletion of the channel. Immunization with $MOG_{35–55}$ and Quil A adjuvant of female *Trpa1*$^{+/+}$ mice produced a PMA similar to that obtained in C57BL/6, which started at day 21 and was maintained until day 35 (**Fig. 3A**). By contrast, *Trpa1*$^{-/-}$ mice did not develop PMA (**Fig. 3A**). The TRPA1-dependent PMA did not parallel an increase in TRPA1 mRNA expression in the peripheral (trigeminal ganglion) or central nervous system tissue (brainstem) (**Fig. 3B**).

3.4. Oxidative stress mediates periorbital mechanical allodynia in relapsing–remitting experimental autoimmune encephalomyelitis–induced mice

The TRPA1 channel is an oxidative stress sensor activated by an extensive series of reactive oxygen, nitrogen, and carbonyl species, including H_2O_2 and 4-HNE.[53] The RR-EAE model induction enhanced the 4-HNE and H_2O_2 levels (**Figs. 4A and B**) and the activities of 2 ROS-catalyzing enzymes, NADPH oxidase and SOD (**Fig. 4C and 4D**), in the trigeminal ganglion. Changes of 4-HNE and H_2O_2 levels or NADPH oxidase and SOD activities were confined to the peripheral neurons as the 4 parameters were not different in the brainstem of immunized mice compared with control mice (**Fig. 4E–G**).

3.5. Treatment with α-lipoic acid and apocynin reduced periorbital mechanical allodynia in the relapsing–remitting experimental autoimmune encephalomyelitis–induced mice

Systemic (i.g) administration of 2 different antioxidants, α-lipoic acid and apocynin (all, 100 mg/kg), induced a (from 1 to 2 hours after administration) reduction of PMA in mice with RR-EAE (**Fig. 5A and B**). The 2 antioxidants did not produce any change in the mechanical threshold compared with control mice (**Fig. 5B**). Maximum inhibition of PMA was 82% and 67% for α-lipoic and apocynin, respectively (**Fig. 5B**). Thus, we hypothesized that

Table 1

List of forward and reverse primers used in reverse transcription-qualitative polymerase chain reaction assays and their respective sequences (5′–3′).

Gene	Sequence forward (5′-3′)	Sequence reverse (5′-3′)	Accession number
Trpa1	GCAGGTGGAACTTCATACCAACT	CACTTTGCGTAAGTACCAGAGTGG	NM_177,781
Actb	CATTGCTGAC AGGATGCAGAAGG	TGCTGGAAGGTGGACAGT GAGG	NM_007,393

TRPA1, transient receptor potential ankyrin 1.

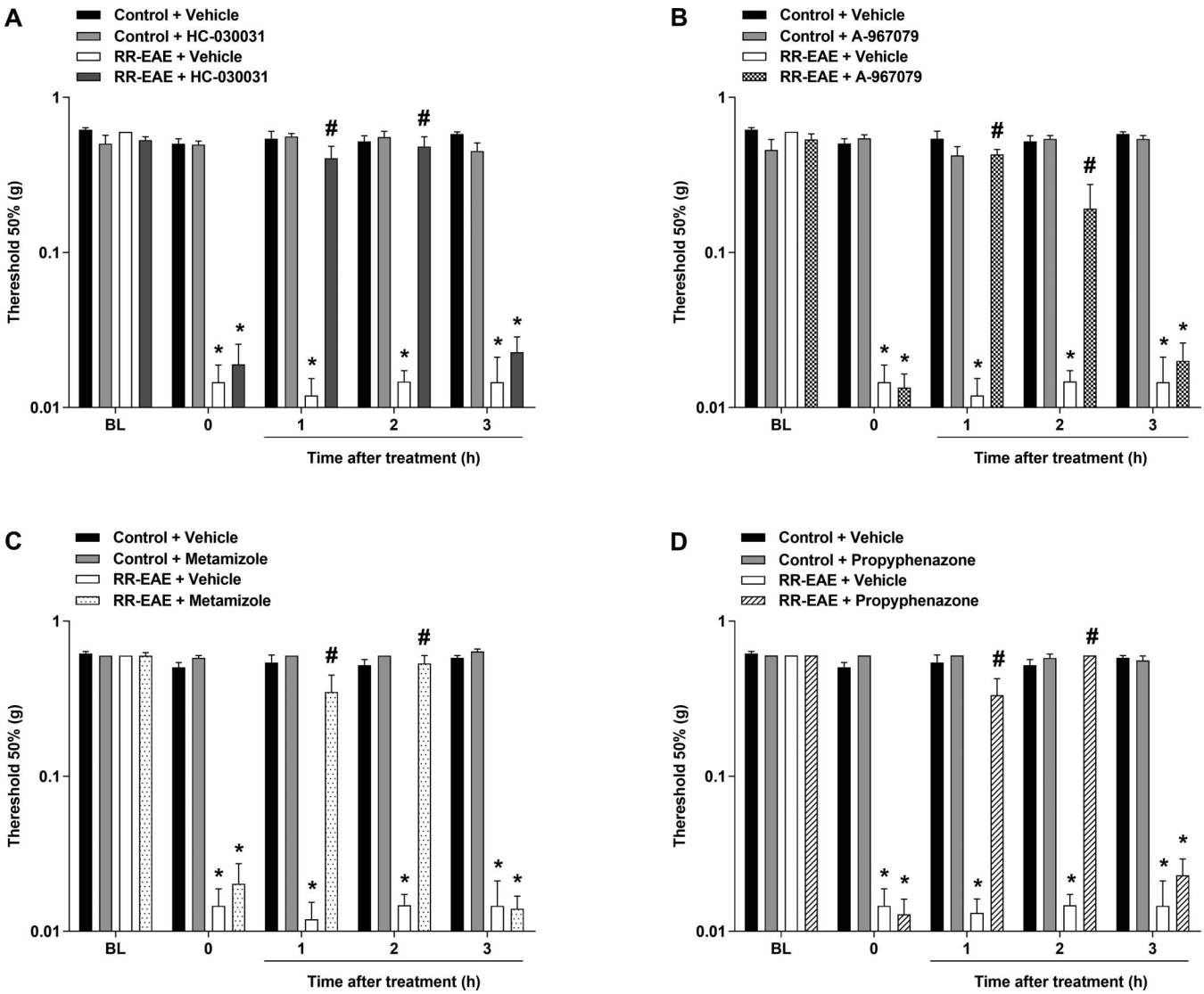

Figure 2. Selective and nonselective TRPA1 antagonists reduced periorbital mechanical allodynia (PMA) caused by a model of relapsing–remitting experimental autoimmune encephalomyelitis (RR-EAE) induction in mice. Selective TRPA1 antagonists (A) HC-030031 or (B) A-967079 and nonselective TRPA1 antagonists, (C) metamizole or (D) propyphenazone (all 100 mg/kg, intragastric, i.g.), were administered on day 35 postinduction (p.i., time 0) of the RR-EAE model. Baseline measurements (described as BL in the graph) were observed before induction. The vehicle group received dimethyl sulfoxide (DMSO) 1% in isotonic saline 0.9% by i.g injection. Data are expressed as mean + SEM. (n = 8). $^*P < 0.05$ when compared with the group or baseline values; $\#P < 0.05$ when compared with the RR-EAE vehicle-treated group [two-way ANOVA, followed by the Bonferroni post hoc test]. ANOVA, analysis of variance; TRPA1, transient receptor potential ankyrin 1.

oxidative stress targeting of TRPA1 in trigeminal ganglion neurons is implicated in PMA evoked by RR-EAE in mice.

4. Discussion

Headache, a common symptom in the initial phases of MS,[25] has been associated with demyelinating lesions in the central nervous system.[47] Despite the high prevalence and deterioration of primary headaches in patients with MS,[46,47,71] the mechanisms underlying MS-associated headaches are poorly understood. We previously reported that in a model of RR-EAE, mice developed hind paw mechanical and cold allodynia.[15] Here, in the same model, we showed for the first time the development of a delayed and sustained PMA. Allodynia in the periorbital and other cutaneous areas is considered a hallmark of headache in migraine attacks.[20,27] Recently, using a different model of EAE (progressive EAE), which reproduces a progressive multiple sclerosis model, we detected the role of TRPA1 in periorbital nociception.[16] Thus, the present finding might be considered the first optimizing model of headache-related cephalic allodynia associated with a RR-EAE model.

Sumatriptan, a mainstay in the acute treatment of migraine attacks,[6,48] has been found to reduce periorbital nociceptive behaviors in different mouse headache-like models in mice.[22,26] Currently, to treat headache in humans, only sumatriptan,[67] metamizole,[62] and propyphenazone[23] are used by oral route.[10,48,62] Usually, the antimigraine pharmacotherapy presents a short effect, approximately 4 hours after administration.[65] In humans, sumatriptan half-time is around 2 hours and has a concentration peak of 45 minutes after oral intake.[43,65] In addition, in the umbellulone migraine-like model in mice, the sumatriptan showed an antinociceptive effect that lasted 4 hours after i.g. treatment for PMA reduction.[35] Olcegepant was discontinued in clinical trials because of problems in oral formulation development[55] and is used as a CGRP antagonist in headache and migraine-like rodent models by i.p. injection for

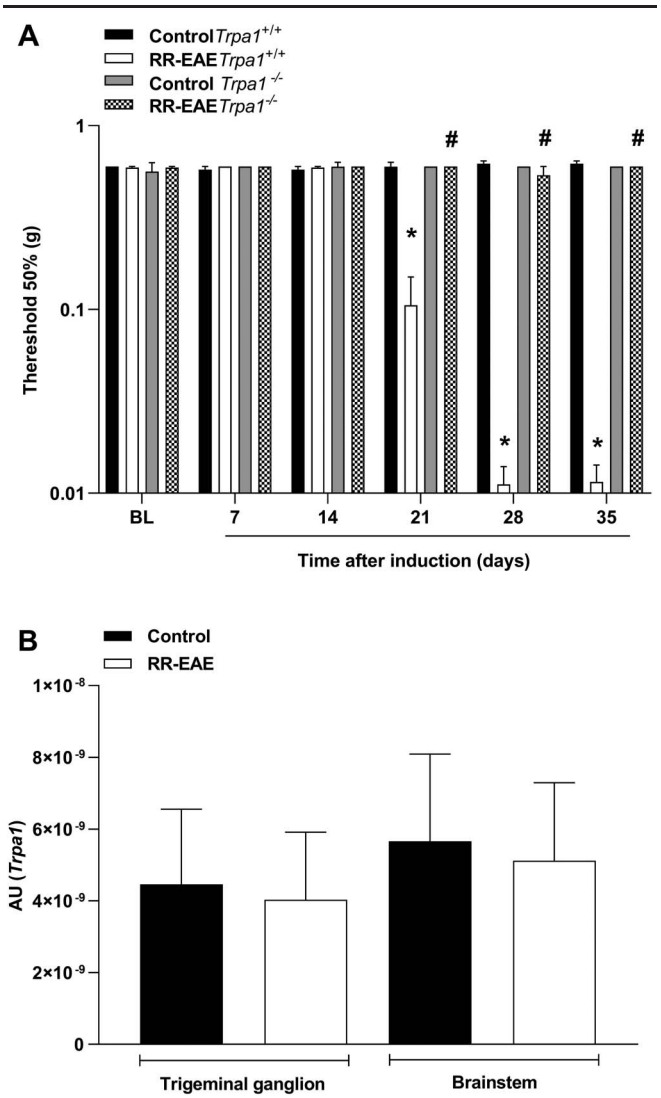

Figure 3. TRPA1 genetic deletion reduced periorbital mechanical allodynia (PMA) in relapsing–remitting experimental autoimmune encephalomyelitis (RR-EAE) in mice. (A) PMA in TRPA1 knockout mice (*Trpa1*$^{-/-}$) was abolished in RR-EAE–induced mice on day 35 postinduction (p.i), and *Trpa1*$^{+/+}$ showed nociception after 21 to 35 days of induction. Baseline measurements (described as BL in the graph) were observed before induction. (B) No alteration in *Trpa1* mRNA levels in control and RR-EAE mice was detected in trigeminal ganglion and brainstem samples. Data are expressed as mean + SEM. (von Frey test n = 6; qRT-PCR n=4-7). For von Frey test, *$P < 0.05$ when compared with the group or baseline values; #$P < 0.05$ when compared with the RR-EAE wild-type group [two-way ANOVA, followed by the Bonferroni post hoc test (A)]. For qRT-PCR, $P > 0.05$ when compared with the control group [nonparametric Student *t* test (B and C)]. ANOVA, analysis of variance; TRPA1, transient receptor potential ankyrin 1.

physiopathology investigations. In humans, the half-time of olcegepant is 2.5 hours after intravenous administration.[28] In other studies, using experimental models in rodents, the antinociceptive effect of olcegepant was around 3 to 4 h after i.p. injection.[35,44]

The validity of the present RR-EAE as a headache-related cephalic allodynia model was further strengthened by the efficacy in reducing PMA of the serotonin 5-$HT_{1B/D}$ receptor agonist, sumatriptan. Here, it was observed that an antinociceptive effect of sumatriptan lasted 2 hours after i.g. administration. Furthermore, olcegepant, a CGRP receptor antagonist reduced PMA in the present model of RR-EAE during 2 hours after i.p. administration. This CGRP antagonist has shown beneficial effects in relieving the pain of the migraine attack[12,49] and attenuating allodynia in different migraine models in rodent.[34,44,49] A large body of evidence reports the implication of TRPA1 in rodent models of trigeminal pain and migraine. The facial allodynia that follows constriction of the infraorbital nerve was attenuated by TRPA1 antagonism or genetic deletion.[68] PMA evoked by dural application or inhalation of the prohead-ache volatile compound, umbellulone,[7,21,54] and systemic exposure to the promigraine drug, glyceryl trinitrate,[49] were also reduced by genetic and pharmacological inhibition of the TRPA1 channel. However, glyceryl trinitrate may also cause facial nociception by perivascular target activation (endothelium, mast cells, and leukocytes),[24] Nrf-2 modulation,[13] and protease-activated receptor 2 activation[36] mechanisms. In addition, umbellulone-induced PMA was prevented by other compounds, including propranolol (a beta-blocker) and nor-binaltorphimine (a kappa opioid receptor antagonist).[35]

Metamizole and propyphenazone are atypical nonsteroidal anti-inflammatory drugs because their analgesic effect dissoci-ates from their anti-inflammatory action.[14,45] The notion that metamizole is one of the commonest analgesics used for acute migraine treatment and the recent identification of metamizole and propyphenazone as TRPA1 antagonists[52] support the channel role in allodynia associated with RR-EAE. Our present data showing attenuated PMA in mice treated with a variety of TRPA1 antagonists (for 2 hours after i.g. injection) or in mice with genetic TRPA1 channel deletion indicate the crucial role of this receptor in PMA evoked by the RR-EAE model.

In clinical research, 4-methylamino-antipyrine, the main meta-mizole metabolite, showed a half-time of 2.6 to 3.5 hours,[42] and for propyphenazone, a half-time of approximately 2.8 hours was described,[72] both after oral administration. Metamizole and propyphenazone were recently discovered as nonselective TRPA1 antagonists that showed an antinociceptive effect until 1 hour after i.g. administration in mice in this study.[52] The selective TRPA1 antagonists are used only in experimental investigation, and a half-life around 30 minutes for HC-030031 and a distribution half-life of 1.8 hours for A-967079 were observed in rats.[69] The antinociceptive effect of HC-030031 and A-967079 (same dose used in our study) was 1 to 2 hours when administered in mice in other pain models.[1,68] Furthermore, in mechanical and cold allodynia tests performed after a neuropathic pain model induced by RR-EAE or PMS-EAE, the TRPA1 antagonists showed an antinociceptive effect during 1 and 2 hours after their administration.[15,63]

Transient receptor potential ankyrin 1 is gated by an un-precedented series of endogenous agents generated under inflammatory circumstances and oxidative stress.[53] NADPH oxidase activity is found in neurons, astrocytes, and microglia,[8] and SOD, whose activity is associated with TRPA1 stimulation,[1,3] are critical enzymes in the oxidative stress pathway and ROS generation. The α-lipoic acid pharmacokinetic profile showed a half-life of 2 hours,[70] and for apocynin, the half-time was approximately 6 hours,[73] both by intragastric route in rats. However, the antinociceptive effect of the α-lipoic acid and apocynin was previously detected for 1 and 2 hours in these RR-EAE and PMS-EAE models.[15,63] Moreover, the antinociceptive action in mice for these antioxidant compounds was detected for 1 and 2 hours after i.g. administration in mice.[1,3,68] The observation that activities of 2 key enzymes for oxidative stress modulation, SOD and NADPH oxidase, were increased in the trigeminal ganglion and 2 antioxidants, apocynin and α-lipoic

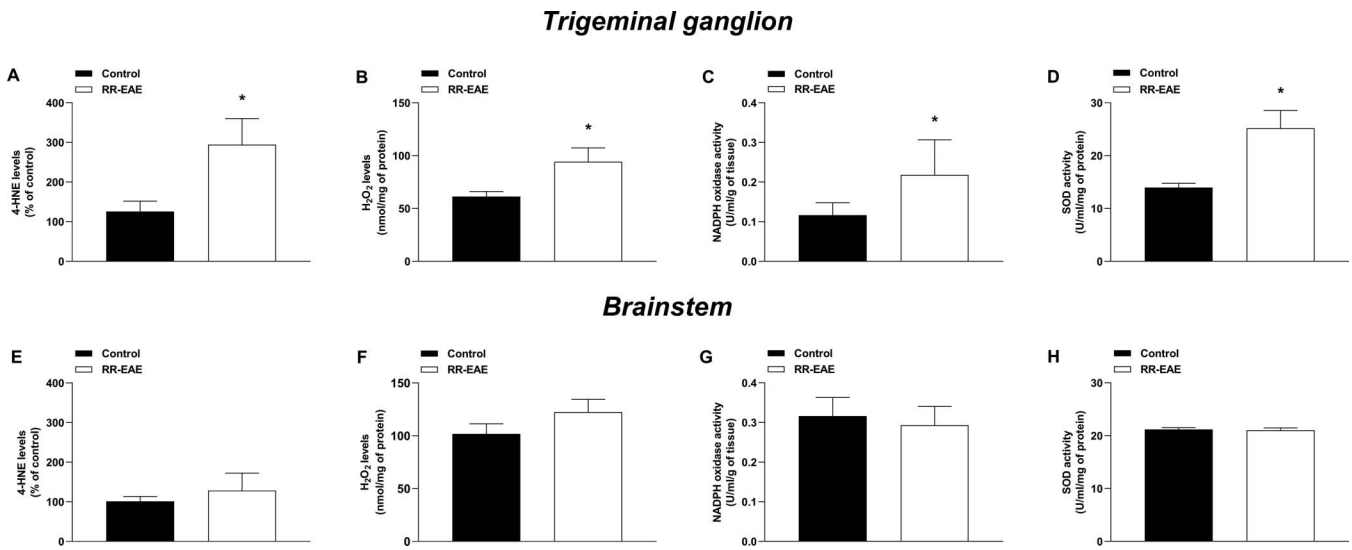

Figure 4. TRPA1 endogenous agonist production, NADPH oxidase, and superoxide dismutase (SOD) activities were increased in the trigeminal ganglion of relapsing–remitting experimental autoimmune encephalomyelitis (RR-EAE)-induced mice. Measurement of (A) 4-hydroxynonenal (4-HNE) and (B) hydrogen peroxide (H_2O_2) levels, (C) NADPH oxidase, and (D) SOD activity in trigeminal ganglion samples 35 days after RR-EAE mice induction. Measurement of (E) 4-HNE, (F) H_2O_2 levels, (G) NADPH oxidase, and (H) SOD activity in brainstem samples 35 days after RR-EAE mice induction. Data are expressed as mean + SEM. (n = 5-6). *$P < 0.05$ when compared with the control group [nonparametric Student *t* test (A, B, E, and F) and parametric Student *t* test (C, D, G, and H)]. TRPA1, transient receptor potential ankyrin 1.

acid, attenuated PMA associated with RR-EAE suggests that activation of TRPA1 channel by oxidative stress sustains PMA.

TRPA1 is implicated in nociception induction in different models of periorbital pain in mice.[7,49,54,68] Here, we found an increase of SOD and NADPH activity and 4-HNE and H_2O_2 levels in the trigeminal ganglion of RR-EAE–induced mice compared with the control group, and no alteration in brainstem samples was found for these oxidative markers. Thus, our results showed the hypothesis that TRPA1 activation in trigeminal ganglion by oxidative agonists maintains PMA in this model of RR-EAE, differently from the result obtained previously for the spinal cord and induction of mechanical and cold allodynia in the hind paw.[15] Similarly, in a previous study, glyceryl trinitrate i.p. injection failed to increase 4-HNE in the brainstem but increased the levels of this TRPA1 endogenous compounds in the trigeminal ganglion.[49] Moreover, in a model of EAE in mice, no alteration in microglial activation in the spinal trigeminal nucleus has been found, but an increase in this neuroinflammatory parameter in the dorsal horn spinal cord has been shown, although it has been described as facial and hind paw mechanical allodynia. However, the study described immune cell infiltration in trigeminal ganglion after EAE induction in mice.[19] Thus, the neuroinflammation caused by EAE could be different in the brainstem and spinal cord areas. The TRPA1 is expressed in the brainstem, specifically in the astrocytes and neurons of the superficial laminae of the trigeminal caudal nucleus (Vc) in rats[32,40] and also in the trigeminal ganglion in mice and rats.[32,49] Besides, trigeminal ganglion neuron cell bodies that mediate PMA in this RR-EAE model could be included in meningeal nociceptors. This class of sensory neurons contributes to the periorbital sensitization to different inflammatory mediators and TRPA1 agonists.[21,41,49,66,74] However, more investigation is necessary to elucidate other specific brainstem areas which could be involved in the TRPA1 mediates headache-related cephalic allodynia in this RR-EAE model.

It was also reported that CGRP released from periorbital trigeminal terminals caused PMA because of the gating of TRPA1 by the promigraine agent, glyceryl trinitrate, in trigeminal neuron cell bodies.[49] Nevertheless, the TRPA1 channel contribution in endogenous pathways implicated in rodent models of headache-related cephalic allodynia is unknown. Here, for the first time, we show that TRPA1, presumably activated by oxidative stress associated with RR-EAE, is crucial for sustaining PMA. These results are in accordance with our previous study showing that TRPA1 is also involved in periorbital allodynia caused by a PMS-EAE model in mice.[16] Whether this mechanism is implicated in the de novo onset or worsening of a preexisting primary headache in patients with MS will be the object of future studies.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Acknowledgements

Fellowships from the Conselho Nacional de Desenvolvimento Científico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) are also acknowledged. Diéssica Padilha Dalenogare fellowship from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) [process # 88882.428089/2019-01] and Programa Institucional de Internacionalização CAPES [process #88887.374020/2019-00]. G. Trevisan is recipient of a fellowship from CNPq [process #303531/2020-7], L'ORÉAL - ABC - UNESCO Para Mulheres na Ciência, 2016 and Prêmio Capes de Teses - Ciências Biológicas II, CAPES, 2014 [process #23038.006930/2014/59]. This work was also supported by European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement No. 835286) (Pierangelo Geppetti). The funding source was not involved in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. Author contributions: All the authors discussed the results, commented on the manuscript, and approved this final version. (1) Substantial contributions to conception and design, data

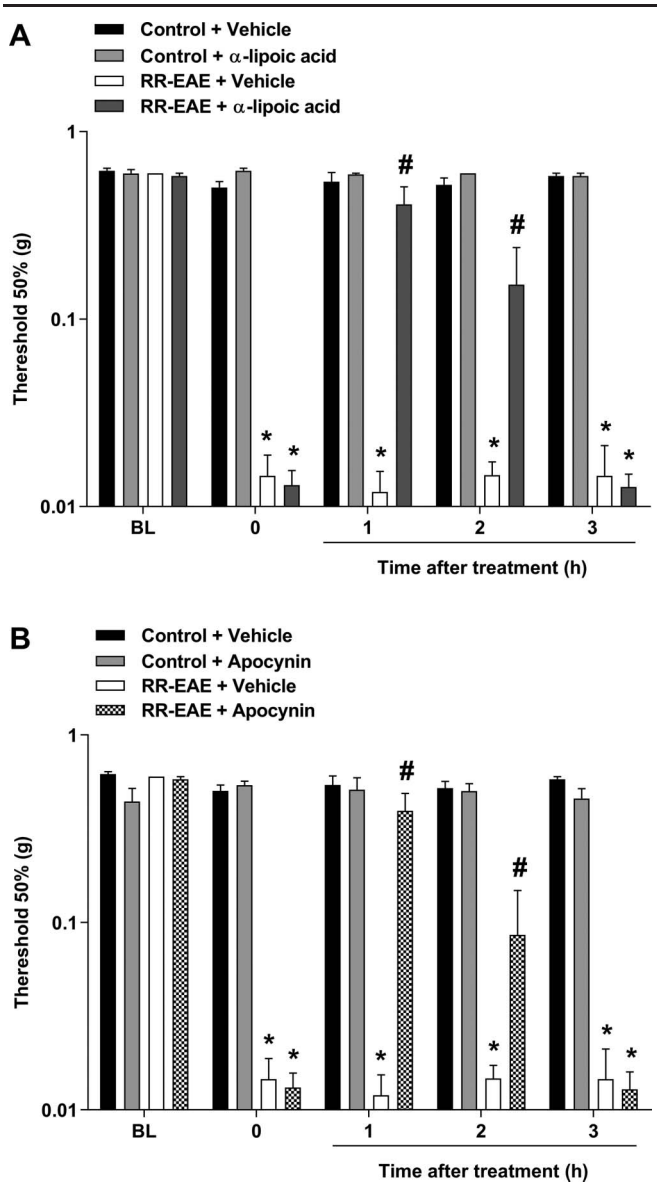

Figure 5. Treatment with α-lipoic acid and apocynin reduced periorbital mechanical allodynia (PMA) in the relapsing–remitting experimental autoimmune encephalomyelitis (RR-EAE)-induced mice. The treatment with (A) α-lipoic acid (100 mg/kg, intragastric, i.g.) and (B) apocynin (100 mg/kg, i.g.) was given on day 35 postinduction (p.i., time 0) of the RR-EAE model, and baseline measurements (described as BL in the graph) were observed before induction. The vehicle group received dimethyl sulfoxide (DMSO) 1% in isotonic saline 0.9%. Data are expressed as mean + S.E.M. (n = 8). * $P < 0.05$ when compared with the group or baseline values; # $P < 0.05$ when compared with the group treated with a vehicle [two-way ANOVA, followed by the Bonferroni post hoc test]. ANOVA, analysis of variance.

acquisition, analysis, and interpretation: D.P. Dalenogare, M.C. Theisen, D.S. Peres, M.F.P. Fialho, N. Andriguetto, L. Barros, L. Landini, M. Titiz, F. de Logu, S.M. Oliveira, R. Nassini, P. Geppetti, and G. Trevisan. (2) Drafting and critically revising the article for important intellectual content: D.P. Dalenogare, M.C. Theisen, D.S. Peres, M.F.P. Fialho, N. Andriguetto, L. Barros, L. Landini, M. Titiz, F. de Logu, S.M. Oliveira, R. Nassini, P. Geppetti, and G. Trevisan. (3) Final article approval: D.P. Dalenogare, M.C. Theisen, D.S. Peres, M.F.P. Fialho, N. Andriguetto, L. Barros, L. Landini, M. Titiz, F. de Logu, S.M. Oliveira, R. Nassini, P. Geppetti, and G. Trevisan. (4) Acquisition of funding and general supervision of the research group: S.M. Oliveira, R. Nassini, P. Geppetti, and G. Trevisan.

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B525.

Article history:
Received 20 May 2021
Received in revised form 1 October 2021
Accepted 7 October 2021
Available online 26 October 2021

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