



## Characterization of the peripheral FAAH inhibitor, URB937, in animal models of acute and chronic migraine

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### ARTICLE INFO

#### Keywords:

NTG  
Migraine  
Trigeminal hyperalgesia  
URB937

### ABSTRACT

Inhibiting the activity of fatty-acid amide hydrolase (FAAH), the enzyme that deactivates the endocannabinoid anandamide, enhances anandamide-mediated signaling and holds promise as a molecular target for the treatment of human pathologies such as anxiety and pain. We have previously shown that the peripherally restricted FAAH inhibitor, URB937, prevents nitroglycerin-induced hyperalgesia – an animal model of migraine – and attenuates the activation of brain areas that are relevant for migraine pain, e.g. trigeminal nucleus caudalis and locus coeruleus. The current study is aimed at profiling the behavioral and biochemical effects of URB937 in animal models of acute and chronic migraine. We evaluated the effects of URB937 in two rat models that capture aspects of acute and chronic migraine, and are based on single or repeated administration of the vasodilating drug, nitroglycerin (NTG). In addition to nocifensive behavior, in trigeminal ganglia and medulla, we measured mRNA levels of neuropeptides and pro-inflammatory cytokines along with tissue levels of anandamide and palmitoylethanolamide (PEA), an endogenous agonist of peroxisome proliferator-activated receptor type-a (PPAR-a), which is also a FAAH substrate. In the acute migraine model, we also investigated the effect of subtype-selective antagonist for cannabinoid receptors 1 and 2 (AM251 and AM630, respectively) on nocifensive behavior and on levels of neuropeptides and pro-inflammatory cytokines. In the acute migraine paradigm, URB937 significantly reduced hyperalgesia in the orofacial formalin test when administered either before or after NTG. This effect was accompanied by an increase in anandamide and PEA levels in target neural tissue, depended upon CB1 receptor activation, and was associated with a decrease in calcitonin gene-related peptide (CGRP), substance P and cytokines TNF-alpha and IL-6 mRNA. Similar effects were observed in the chronic migraine paradigm, where URB937 counteracted NTG-induced trigeminal hyperalgesia and prevented the increase in neuropeptide and cytokine transcription.

The results show that peripheral FAAH inhibition by URB937 effectively reduces both acute and chronic NTG-induced trigeminal hyperalgesia, likely via augmented anandamide-mediated CB1 receptor activation. These effects are associated with inhibition of neuropeptidergic and inflammatory pathways.

### 1. Introduction

Trigeminal hyperalgesia or facial allodynia have been used in animal models of migraine pain to simulate clinical symptomatology and to

investigate the mechanisms related to migraine pain (Harriott et al., 2019). Systemic administration of nitroglycerin (NTG), a vasodilator that acts as nitric oxide donor, in rodents is a well-known animal model of migraine that reproduces multiple symptoms of migraine and induces,

**Abbreviation:** CB, cannabinoid receptor; CGRP, calcitonin gene-related peptide; DMSO, dimethylsulfoxide; FAAH, fatty-acid amide hydrolase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; i.p., intraperitoneal; IL-6, Interleukin 6; LC/MS, liquid chromatography-mass spectrometry; NTG, nitroglycerin; PEA, palmitoylethanolamide; PPAR-a, peroxisome proliferator-activated receptor type-a; TNC, trigeminal nucleus caudalis; TNF-alpha, Tumour Necrosis Factor alpha; TRPV1, transient receptor potential vanilloid 1.

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<https://doi.org/10.1016/j.nbd.2020.105157>

Received 13 August 2020; Received in revised form 14 October 2020; Accepted 27 October 2020

Available online 28 October 2020

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**Table 1**

Experimental groups for each set. Sets A,B and C dealt with the acute migraine model; set D dealt with the chronic migraine model.

Groups	Experimental sets		
	A and B	C	D
DMSO (NTG vehicle+DMSO)	NTG + DMSO	CT chronic (NTG vehicle+URB937 vehicle)	
NTG + DMSO	NTG + URB937	NTG chronic (NTG + URB937 vehicle)	
URB937 pre (NTG vehicle+URB937)	NTG + URB937 + AM251	URB937 chronic (NTG vehicle+URB937)	
NTG + URB937 pre	NTG + URB937 + AM630	NTG + URB937 chronic	
URB937 post (NTG vehicle+URB937)			
NTG + URB937 post			

In set A, after the acute treatments indicated in the table, animals were sacrificed and samples collected for the quantification of AEA and PEA levels. In set B, after the acute treatments indicated in the table, the animals underwent the orofacial formalin test, then they were sacrificed, and samples collected for ex vivo analysis. In set C, URB937 was co-administered with CB1 and CB2 antagonists (AM251 or AM630) and animals underwent the orofacial formalin test. In set D, after chronic treatments indicated in the table, animals underwent the orofacial formalin test, then they were sacrificed, and samples collected for ex vivo analysis.

CT: control; DMSO: dimethylsulfoxide; NTG: nitroglycerin; NTG vehicle: 6% alcohol and 16% propylene glycol and saline; URB937 vehicle: 10% polyethylene glycol 200, 10% tween 80 and saline.

inter alia, hyperalgesia at the trigeminal and spinal levels (Demartini et al., 2019).

Modulation of endogenous cannabinoid signaling through inhibition of endocannabinoid deactivation has gained attention as a possible therapeutic target for several clinical disorders, including migraine pain (Ahn et al., 2009; Panlilio et al., 2013; Wortley et al., 2017; Greco et al., 2018a). Inhibitors of fatty-acid amide hydrolase (FAAH), the enzyme that catalyzes the cleavage of the endocannabinoid anandamide along with other endogenous fatty-acid amides, have demonstrated promising activity in animal models of migraine (Greco et al., 2015a, 2020). We have reported that the globally active FAAH inhibitor, URB597 (Kathuria et al., 2003), reduced c-Fos expression in the trigeminal nucleus caudalis (TNC), NTG-induced hyperalgesia at the orofacial formalin test and expression of neuronal nitric oxide synthase, calcitonin gene-related peptide (CGRP) and cytokine gene expression in various central and peripheral nervous structures (Greco et al., 2020).

Unlike URB597, its derivative URB937 is actively extruded from the central nervous system, and therefore increases anandamide levels solely in peripheral tissues (Clapper et al., 2010). We previously reported that this agent attenuated NTG-induced nocifensive behavior and neuronal activation in brain nuclei that are known to play a role in migraine (Greco et al., 2015a), suggesting that peripheral FAAH might offer a new molecular target for migraine pain (Greco et al., 2020). In this context, it is worth noting that URB937 exerts profound analgesic effects in animal models (Piomelli and Sasso, 2014) but does not display any of the intoxicating properties that are typical of direct-acting cannabinoid receptor agonists (e.g. sedation, motor dysfunction, appetite stimulation) (Vozella et al., 2019). To gain further insights into the mechanisms of anti-migraine action of URB937, in the present study we characterized the pharmacological effects of URB937 in two rat models of acute and chronic migraine, which are based on systemic administration of NTG (Greco et al., 2018b; Demartini et al., 2019).

## 2. Material and methods

### 2.1. Animals

A total of 144 adult male Sprague-Dawley rats, weighing 250–270 g, were used in this study and randomly allocated to experimental groups as reported in Table 1. The IASP guidelines for pain research in animals were followed (Zimmerman, 1983). All procedures were conducted in accordance with the European Convention for Care and Use of Laboratory Animals and the experimental protocols were approved by the Italian Ministry of Health (1032/2015-PR; 1019/2016-PR; 1239/2015-PR). All experiments were conducted in a randomized manner by an experimenter who was blinded to treatments.

### 2.2. Nitroglycerin (NTG)-based animal models

In this study, two NTG-based animal models were used to mimic episodic and chronic migraine pain (Buzzi and Tassorelli, 2010; Pradhan et al., 2014; Demartini et al., 2019). NTG (Bioindustria L.I.M. Novi Ligure (AL), Italy) was prepared from a stock solution of 5.0 mg/1.5 ml dissolved in 27% alcohol and 73% propylene glycol. For injections, NTG was further diluted in saline (0.9% NaCl) to reach the final concentration of 6% alcohol and 16% propylene glycol. These dilutions were used as vehicle in an equivalent volume. In the acute migraine model, NTG was injected intraperitoneally (i.p.) at the dose of 10 mg/kg 4 h before behavioral testing and/or ex vivo analysis (Greco et al., 2020). For the chronic model, NTG was administered i.p. at the dose of 5 mg/kg every 2 days over a 9-day period to a total of five injections (Greco et al., 2018b).

### 2.3. Experimental design

URB937 (N-cyclohexyl-carbamic acid, 3'-(aminocarbonyl)-6-hydroxy[1,1'-biphenyl]-3-yl ester) synthesized by Prof. Piomelli's team (Sasso et al., 2012), was administered at 1 mg/kg, i.p., (Greco et al., 2015a) in a single dose for the acute migraine model or according to a repeated dose schedule (for the chronic migraine model) (see below).

#### 2.3.1. Acute migraine model

For the acute model, URB937 was dissolved in 100% dimethylsulfoxide (DMSO, 1 ml/kg), and injected (10 mg/kg) either 1 h before or 3 h after NTG or its vehicle (16% propylene glycol, 6% alcohol and saline). Four hours after NTG/vehicle administration, a first set of rats (Fig. 1S-A, Supplementary material) were sacrificed to collect samples for quantification of anandamide and PEA levels in various regions of interest (see section "Anandamide and PEA levels"), while a second set of animals (Fig. 1S-B, Supplementary material) underwent the orofacial formalin test for behavioral assessment (see section "Orofacial formalin test"). At the end of the orofacial formalin test, rats were sacrificed to collect trigeminal ganglion and medulla (see section "Gene expression").

To keep animal numbers to a minimum, for the DMSO control group we only considered the 3 h post NTG timing, based on previous work which showed no difference when DMSO vehicle was administered 1 h before or 3 h after NTG (Greco et al., 2020). Similarly, since the data obtained for this study were conducted in part simultaneously with the URB597 study (Greco et al., 2020), wherever possible and reasonable, we ran 3 groups in parallel (URB937, URB597 and control).

The same approach was also used in the third set of experiments to evaluate the role of cannabinoid (CB) 1 and CB2 receptors in URB937 effect upon NTG-induced trigeminal hyperalgesia (Fig. 1S-C, Supplementary material). Specifically, NTG-injected rats received co-administration of URB937 and a CB1 or a CB2 receptor antagonist 3 h after NTG and, 1 h later they were exposed to the orofacial formalin test (see section "Orofacial formalin test").

To antagonize CB1 and CB2 receptors, we used AM251 ((N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-

pyrazole-3-carboxamide) and AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl](4-methoxyphenyl)) respectively. Both agents were dissolved in 100% DMSO and were administered at the dose of 1 mg/kg, i.p. (Borsani et al., 2007; Mohammadi Vosough et al., 2019). The final volume of DMSO for the co-administrations was kept at 1 ml/kg. AM251 exhibits 306-fold selectivity for CB1 over CB2 (Gatley et al., 1996), whereas the CB2 receptor inverse agonist AM630 exhibits 70–165-fold selectivity for CB2 over CB1 (Pertwee et al., 1995).

### 2.3.2. Chronic migraine model

In the chronic paradigm (Fig. 1S-D, Supplementary material), animals received URB937 (1 mg/kg, i.p.) or vehicle (1 ml/kg, i.p.) daily and NTG 5 mg/kg i.p. every other day for 9 days. Twenty-four hours after the last injection of NTG (or vehicle), rats underwent the orofacial formalin test. At the end of the behavioral test, the animals were sacrificed and trigeminal ganglion and medulla were collected for gene expression analysis (see section “Orofacial formalin test” and “Gene expression”).

Although the use of DMSO has been previously validated as a vehicle in our model of acute migraine (Greco et al., 2015a, 2020), in the chronic migraine model we opted to use 10% polyethylene glycol 200, 10% tween 80 and saline (1 ml/kg, i.p.) as a vehicle to dissolve URB937 due to the theoretical possibility that repeated administrations of DMSO might induce confounding effects (Kelava et al., 2011). We preliminarily evaluated the suitability of 10% polyethylene glycol 200, 10% tween 80 and saline as a vehicle in a subgroup of animals in the acute migraine model, reporting results comparable to DMSO. This was expected when considering that Clapper et al. (2010) showed in mice that URB937 (1 mg/kg, i.p. dissolved in PEG400/Tween-80/saline 1,1:18) attenuated behavioral responses induced by plantar formalin test, a finding that was confirmed by us in rats using the same dose of URB937 (1 mg/kg, i.p.) dissolved in 100%DMSO (Greco et al., 2015a, 2015b).

## 2.4. Anandamide and PEA levels

### 2.4.1. Tissue collection

For liquid chromatography-mass spectrometry (LC/MS) analyses the rats injected with URB937 or vehicle (DMSO) were sacrificed by decapitation after exposure to carbon dioxide (Clapper et al., 2010) 4 h after NTG or vehicle injection. Medulla (bregma, −13.30 to −14.60 mm) and trigeminal ganglia were quickly dissected out and snap-frozen in liquid nitrogen. Tissue parts were subsequently homogenized and anandamide and PEA were extracted as described below.

### 2.4.2. Lipid extraction and fractionation

Bligh-Dyer procedure, modified as previously described (Astarita and Piomelli, 2009), was used. Briefly, frozen tissues were weighed, transferred to glass vials and homogenized in cold methanol (2 ml) containing anandamide-d4 and PEA-d4 as internal standards. Lipids were extracted with chloroform (2 ml) and washed with LC/MS-grade water (1 ml). After centrifugation for 15 min at 2850 ×g and 4 °C, the organic phases were collected and transferred to a new set of glass vials. To increase extraction efficiency, the aqueous fractions were extracted again with chloroform (1 ml) and the centrifugation step was repeated. Both organic phases were pooled and dried under N<sub>2</sub>. Lipids were reconstituted in chloroform (2 ml) and the organic extracts were fractionated by Silica Gel G column chromatography (60-Å 230–400 mesh; Sigma-Aldrich, Milan, Italy). Anandamide and PEA levels were eluted from the silica column with 2 ml of chloroform/methanol (9:1, v/v) and then with 2 ml of chloroform/methanol (8:2, v/v). Both eluates were recovered in the same vial. The solvent was evaporated under nitrogen and lipids were reconstituted in methanol/chloroform (50 µL; 9:1, v/v) and transferred to glass vials for LC/MS analyses.

### 2.4.3. LC/MS analyses

Anandamide levels were measured using a Xevo TQ UPLC-MS/MS

**Table 2**  
Sequences of primers.

Gene	Forward primer	Reverse primer
GAPDH	AACCTGCCAAGTATGATGAC	GGAGTTGCTGTTGAAGTCA
CGRP	CAGTCTCAGTCCAAGTCATC	TTCCAAGGTTGACCTCAAAG
TNF-alpha	CCTCACACTCAGATCATCTTCTC	CGCTTGGTGGTTTGCTAC
IL-6	TTCTCTCCGCAAGACTTC	GGTCTGTGTGGGTGGTATC

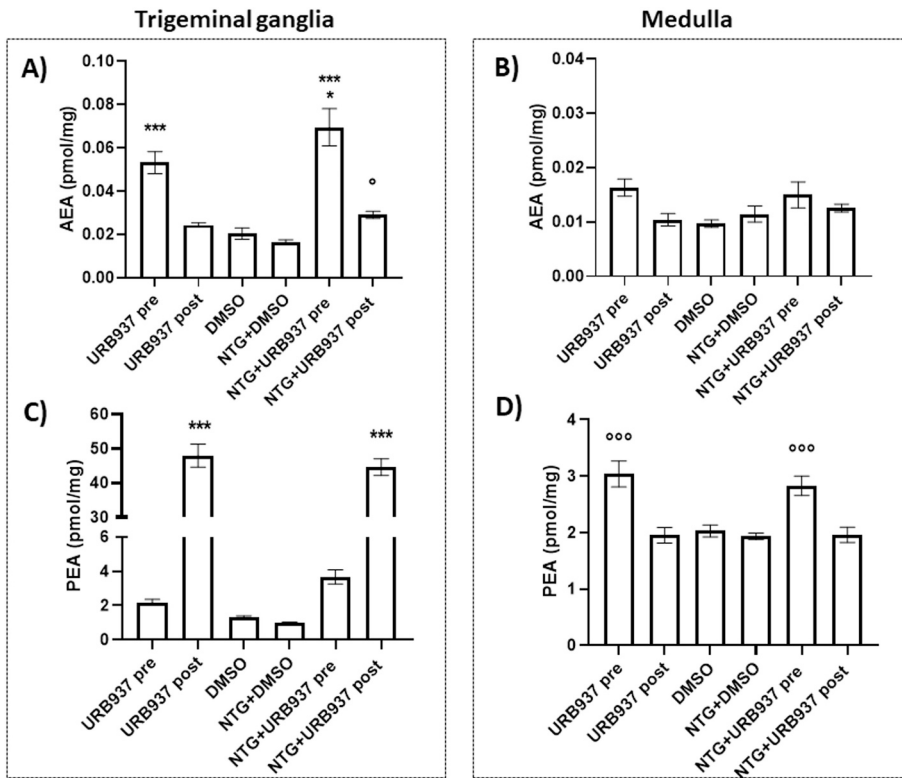
system equipped with a reversed-phase BEH C18 column (2.1 × 50 mm, 1.7 µm particle size) (Waters, Milford, USA). The mobile phase consisted of 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B. A linear gradient was used: 0.0–0.5 min 20% B; 0.5–2.5 min 20 to 100% B; and 2.5–3.0 min maintained at 100% B. The column was reconditioned to 20% B for 1 min. Analysis time was 4 min and the injection volume was 5 µL.

## 2.5. Orofacial formalin test

Rats were acclimatized to the test chamber 20–25 min before testing. A camera, recording nocifensive behavior for off-line analysis, was located at a distance of 50 cm from the observation box (30x30x30-cm glass chamber with mirrored sides) to provide a clear view of each rat. The subcutaneous injection of formalin (1.5%, 50 µL) was performed into the right upper lip. Immediately after formalin injection, each animal was placed into the observation box and its behavior recorded for a 45-min period (Raboisson and Dallel, 2004). Face rubbing was measured counting the seconds the animal spent grooming the injected area with the ipsilateral forepaw or hindpaw in the periods 0–6 min (Phase I) and 12–45 min (Phase II) after formalin injection. The observation time was divided into 15 blocks of 3 min each (45 min total). The researcher who performed the evaluations was blind to treatments.

## 2.6. Gene expression

At the end of the behavioral test (i.e. approximately 5 h after NTG/vehicle treatment) all rats were sacrificed with a lethal dose of anesthetic followed by decapitation and tissue samples were immediately collected. Rat decapitation guarantees the highest degree of rapidity, which is extremely relevant when evaluating gene expression in order to minimize nuclease activity and preserve RNA. Medulla in toto and trigeminal ganglion ipsilateral to the formalin injection were quickly dissected out, rinsed in cold sterile 0.9% sodium chloride solution, placed in cryogenic tubes and immediately frozen in liquid nitrogen. They were subsequently kept at −80 °C until rt-PCR processing. All procedures were performed under RNase-free conditions; samples were processed for the total RNA extraction with TRIzol® (Invitrogen, USA), in combination with tissue homogenization by means of ceramic beads (PRECELLYS, Berthin Pharma). RNA quality was assessed using a nanodrop spectrophotometer (Euroclone): the absorbance ratios (260/280 nm) ranged from 1.9 to 2.0 in all RNA samples, indicating no significant protein (including of blood origin) contamination. cDNA was generated using the iScript cDNA Synthesis kit (BIO-RAD) following the supplier's instructions. mRNA expression levels of the genes coding for CGRP, Tumour Necrosis Factor alpha (TNF-alpha) and Interleukin 6 (IL-6) were analyzed using the Fast Eva Green supermix (BIO-RAD) (Greco et al., 2015b; Greco et al., 2017). Sequences of the primers (Table 2) were selected from the AutoPrime software (<http://www.autoprime.de/AutoPrimeWeb>) and validated on BLAST in order to ensure the high efficiency. The amplification was performed through two-step cycling (95–60 °C) for 45 cycles with a light Cycler 480 Instrument Real Time PCR Detection System (Roche) following the supplier's instructions. Different cDNA samples – NTG/controls and replicates of both - in every assay we amplified each gene of interest and housekeeping gene (Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) from the same cDNA sample to ensure accurate normalization for each data set in the



**Fig. 1.** Anandamide (AEA) (A-B) and PEA (C-D) levels (expressed in pmol/mg) in trigeminal ganglia and medulla following acute systemic administration of NTG/vehicle and URB937/DMSO. AEA levels were significantly higher only in the trigeminal ganglia especially in URB937 pre groups (URB937 pre and NTG + URB937 pre). PEA levels were higher in the trigeminal ganglia in URB937 post groups (URB937 post and NTG + URB937 post), while in the medulla they were higher in the pre-treatment groups (URB937 pre and NTG + URB937 pre). Data are expressed as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparisons test: \*\*\**p* < 0.001 vs NTG + DMSO, DMSO, URB937 post and NTG + URB937 post; \**p* < 0.005 vs URB937 pre; °*p* < 0.05 vs NTG + DMSO; °°*p* < 0.001 vs NTG + DMSO, DMSO, URB937 post and NTG + URB937 post.

assay. All samples were assayed in triplicate and gene expression levels were calculated according to  $2^{-\Delta Ct} = 2^{-(Ct \text{ gene} - Ct \text{ housekeeping gene})}$  formula by using Ct (cycle threshold) values. Ct value for the housekeeping gene remained within 0.5 cycles in the same cDNA samples and between real time assays. All real time assay amplification data were normalized with housekeeping data derived from the same cDNA sample.

**2.7. Statistical analysis**

An a priori power analysis was conducted to determine the required sample size needed to obtain a statistical power of 0.80 at an alpha level of 0.05 (GPower 3.1). We hypothesized a difference in total nociceptive response in Phase II of the orofacial formalin test (face rubbing time) between rats injected with NTG and rats injected with NTG vehicle of at least 27 s ( $169 \pm 13.5$ ;  $CT = 142 \pm 18$ ) and thus, we estimated a sample size of six rats in each experimental group with an effect size of 1.69. However, since the orofacial formalin test shows an intergroup

variability, we increased the number to nine rats *per* group. An excessive response (animals vocalization and/or jumps) to the formalin injection would determine the immediate sacrifice of the animal and the data would not be considered for analysis.

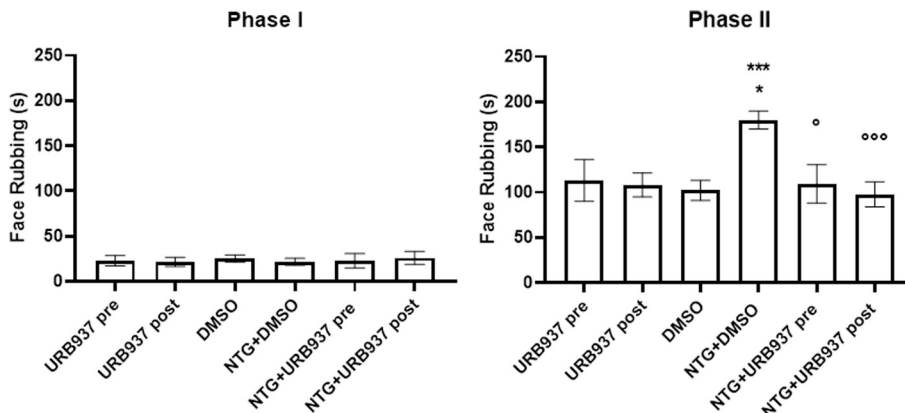
All data were tested for normality using the Kolmogorov-Smirnov (K-S) normality test and considered normal. For nociceptive responses, gene expression and lipid levels, the statistical differences between groups were determined using one-way ANOVA followed by post hoc Tukey's multiple comparisons test. Data are expressed as mean ± SEM, and a probability level of less than 5% was regarded as significant. The researcher performing the data analysis was blinded to treatments.

**3. Results**

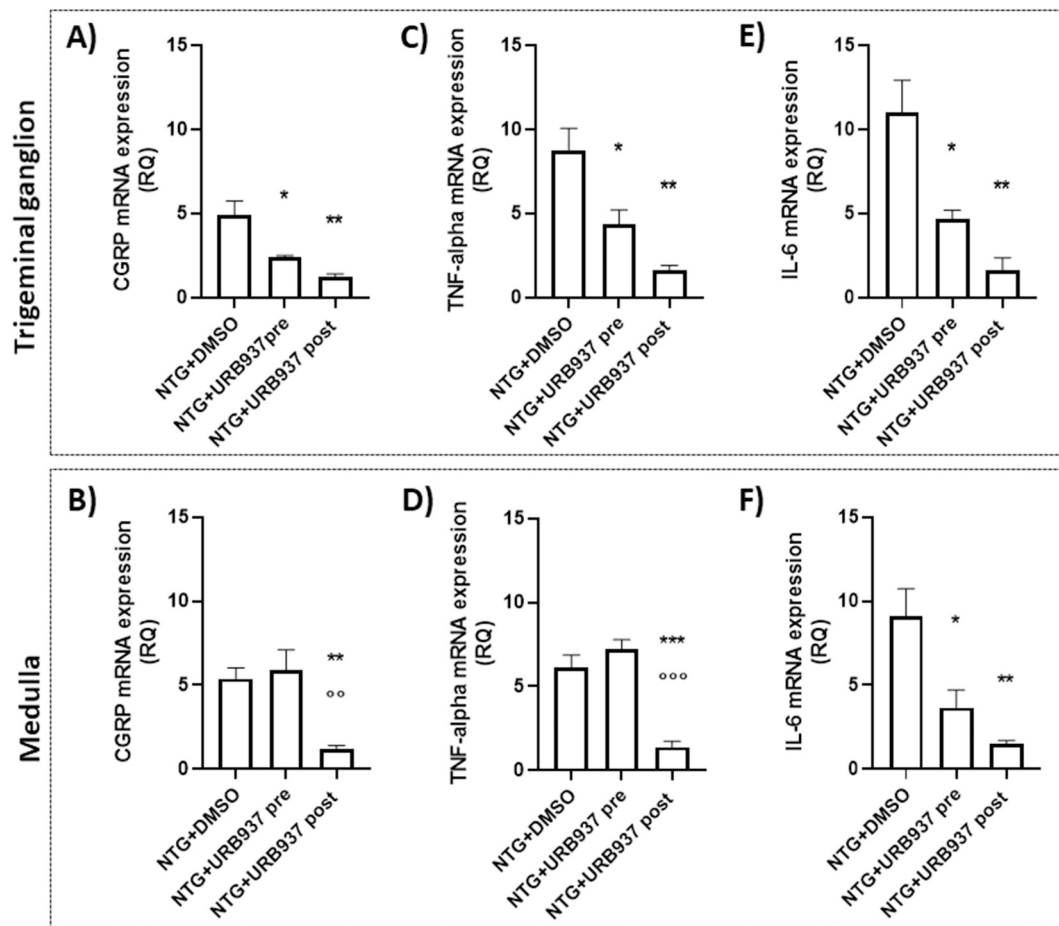
**3.1. Acute migraine model**

**3.1.1. Effect of URB937 on anandamide and PEA levels**

NTG did not affect anandamide or PEA mobilization in trigeminal



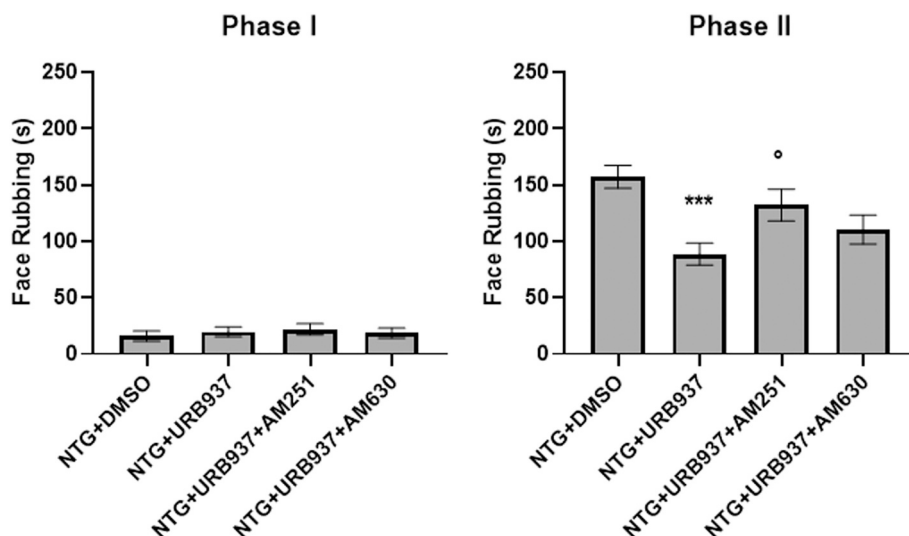
**Fig. 2.** Time of face rubbing (expressed in seconds) during Phase I and II of the orofacial formalin test following acute systemic administration of NTG/vehicle and URB937/DMSO. URB937, administered either 1 h before or 3 h after NTG reduced NTG-induced hyperalgesia in Phase II. Data are expressed as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparisons test: \**p* < 0.05 vs URB937 pre and URB937 post; \*\*\**p* < 0.01 vs DMSO; °*p* < 0.05 and °°°*p* < 0.001 vs NTG + DMSO.



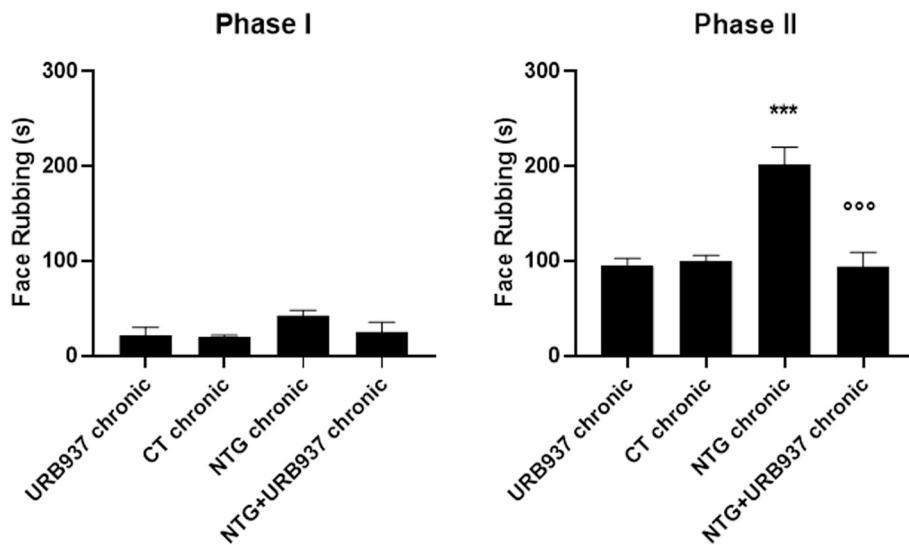
**Fig. 3.** Gene expression in trigeminal ganglion ipsilateral to formalin injection and medulla in toto, after acute treatment of NTG and URB937/DMSO. CGRP (A-B), TNF-alpha (C-D) and IL-6 (E-F) mRNA levels, expressed as relative quantification (RQ). URB937 administration 3 h after NTG reduced all the evaluated genes in both areas. Administration of URB937 1 h prior to NTG reduced all genes in the trigeminal ganglion, and only IL-6 in the medulla. Data are expressed as mean ± SEM; one-way ANOVA followed by Tukey's multiple comparisons test. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs NTG + DMSO; °° $p < 0.01$  and °°° $p < 0.001$  vs NTG + URB937 pre.

ganglia and medulla-pons (Fig. 1), confirming a previous report (Greco et al., 2020). URB937 administration significantly increased anandamide levels in trigeminal ganglia, especially when the FAAH inhibitor was administered before NTG or its vehicle (Fig. 1A). No change in

anandamide levels was seen in the medulla (Fig. 1B). URB937 also increased PEA levels in the trigeminal ganglia only when administered after NTG or NTG vehicle (Fig. 1C); by contrast, in the medulla, the FAAH inhibitor increased PEA levels only when it was administered 1 h



**Fig. 4.** URB937 attenuates nocifensive behavior via peripheral CB1 receptors. Time of face rubbing (expressed in seconds) during Phase I and II of the orofacial formalin test after co-administration of URB937 with CB1 or CB2 inverse agonists (AM251 or AM630 respectively) in the animal model of migraine. During Phase II, only the co-administration of AM251 counteracted the anti-hyperalgesic effect of URB937 in NTG-sensitized rats. Data are expressed as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparisons test: \*\*\* $p < 0.001$  vs NTG + DMSO; ° $p < 0.05$  vs NTG + URB937.



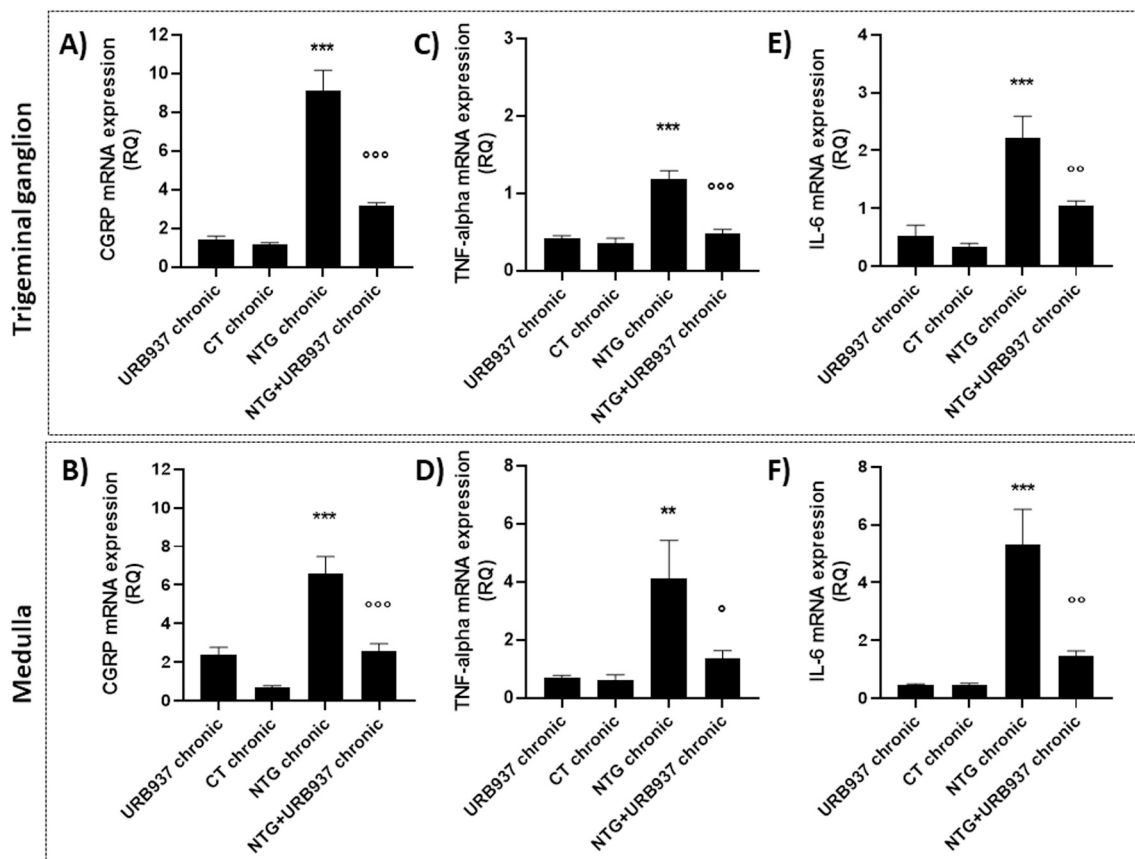
**Fig. 5.** Time of face rubbing (expressed in seconds) during Phase I and II of the orofacial formalin test after chronic treatment of NTG/vehicle and URB937/vehicle. Chronic treatment with URB937 prevented NTG-induced hyperalgesia during Phase II. Data are expressed as mean ± SEM. One-way ANOVA followed by Tukey’s multiple comparisons test: \*\*\**p* < 0.001 vs CT chronic and URB937 chronic; °°°*p* < 0.005 vs NTG chronic.

before NTG or vehicle (Fig. 1D).

**3.1.2. Effect of URB937 on NTG-induced trigeminal hyperalgesia (orofacial formalin test)**

As expected, NTG injection was followed by a hypersensitive state, which was detectable as an increase in nocifensive behavior during

Phase II of the orofacial formalin test. URB937 reduced formalin-induced Phase II nocifensive behavior when it was administered either 1 h before or 3 h after NTG (Fig. 2). No significant effect was observed when URB937 was injected with vehicle compared with DMSO group (Fig. 2). No significant differences among groups were seen during Phase I of the test.



**Fig. 6.** Gene expression in the trigeminal ganglion ipsilateral to formalin injection and in the medulla in toto, after chronic treatment of NTG/vehicle and URB937/vehicle. The panels illustrate mRNA levels of CGRP (A-B), TNF-alpha (C-D) and IL-6 (E-F). Chronic treatment with URB937 counteracted NTG-induced increase of all genes evaluated in both areas.

Data are expressed as relative quantification (RQ) and indicated as mean ± SEM. One-way ANOVA followed by Tukey’s multiple comparisons test: \*\**p* < 0.01 and \*\*\**p* < 0.001 vs CT chronic and URB937 chronic; °*p* < 0.05, °°*p* < 0.01 and °°°*p* < 0.001 vs NTG chronic.

### 3.1.3. Effect of URB937 on gene expression

URB937 decreased mRNA expression levels of the genes coding for CGRP, TNF- $\alpha$  and IL-6 in the trigeminal ganglion when administered either 1 h before or 3 h after NTG (Fig. 3A,C,E). In the medulla, when administered 1 h before NTG, URB937 only reduced IL-6 mRNA levels in rats injected with NTG compared to NTG + DMSO (Fig. 3F). When URB937 was administered after NTG we observed a significant reduction in mRNA levels of all evaluated genes (Fig. 3B,D,F).

No changes were instead observed in the gene expression in all the evaluated areas when URB937 was administered (either in the pre- or post-paradigm) with NTG vehicle (Fig. 2S, Supplementary material).

### 3.1.4. Effects of URB937 co-administered with AM251 or AM630 on the orofacial formalin test

As illustrated in Fig. 4, co-administration of the CB1 inverse agonist, AM251, counteracted the anti-hyperalgesic effect of URB937 in NTG-sensitized rats during Phase II of the test. By contrast, no significant change in the effect of URB937 was seen when the inhibitor was co-administered with the CB2 antagonist, AM630. Similarly, no significant differences between groups were noted during Phase I of the test.

## 3.2. Chronic migraine model

### 3.2.1. Effect of URB937 on NTG-induced trigeminal hyperalgesia (orofacial formalin test)

As expected from prior studies (Greco et al., 2018b), the rats treated with repeated intermittent NTG developed orofacial hyperalgesia in Phase II of the orofacial formalin test, which was prevented by chronic administration of URB937 (NTG chronic vs NTG + URB937 chronic groups). No statistically detectable effect was observed in the URB937 chronic group compared with rats injected with vehicle (CT chronic group) (Fig. 5). No significant differences between groups were seen during Phase I of the test.

### 3.2.2. Effect of URB937 on gene expression

Repeated intermittent NTG treatment increased gene expression of CGRP in trigeminal ganglion and medulla (Greco et al., 2018b). We also noted an increase in TNF- $\alpha$  and IL-6 mRNA levels in the same areas when compared to the control group (Fig. 6). Chronic administration of URB937 prevented NTG-induced expression of all genes in both trigeminal ganglion and medulla (Fig. 6). Chronic URB937 administration with NTG vehicle did not cause any statistically detectable change.

## 4. Discussion

It is generally accepted that repeated activation of trigeminovascular afferents and the associated release of neurotransmitters at the meningeal neurovascular endings induces peripheral and central sensitization, which in turn predisposes patients to develop migraine attacks and, in some migraineurs, leads to chronic migraine (Akerman et al., 2007; Zubrzycki et al., 2015; Burstein et al., 2017). In recent years, research has been focused mostly on the CGRP pathways, which has yielded the first class of migraine-specific preventive treatment: monoclonal antibodies targeting CGRP (Raffaelli and Reuter, 2018). These agents have proved effective in approximately 50% of subjects, however, which leaves ample room for searching additional therapeutic targets by investigating the role of other pathways that might be potentially involved. In this frame, our group has obtained promising results on the role of the endocannabinoid system in migraine pain (Greco et al., 2011, 2015a, 2015b, 2018a, 2020). The endocannabinoid system may control the release of several mediators involved in migraine pain, including neuropeptides and pro-inflammatory mediators (Donvito et al., 2018; Tassorelli et al., 2019). FAAH inhibitors – whose proximal action is to increase endogenous levels of anandamide and other fatty acyl ethanolamides – thus prolonging the duration of their biological effect – may thus represent a potential therapeutic strategy for the treatment of

several clinical disorders, including migraine (Greco et al., 2018a, 2020).

In the present study, we report that increased endocannabinoid tone in trigeminal afferents, achieved by systemic administration of the peripherally restricted FAAH inhibitor, URB937, alleviates trigeminal hyperalgesia elicited in rats by NTG administration. We further show that the anti-hyperalgesic effects of URB937 require CB1, thus confirming the role of this cannabinoid receptor subtype in the response (Clapper et al., 2010; Kathuria et al., 2003). CB1 receptors are located on fibers in the spinal trigeminal tract, as well as on large diameter myelinated fibers. They are also present in the TNC (Tsou et al., 1998; Price et al., 2003). Based on our findings, it is likely that CB1 receptors are activated by increased levels of anandamide in trigeminal ganglia, produced by URB937 administration pre- or post-NTG. Interestingly, in the trigeminal ganglia we also found a significant increase in PEA levels, though only in the post-NTG paradigm. The different temporal pattern of increase observed for anandamide ('pre' paradigm) and PEA ('post' paradigm) in the trigeminal ganglion, and to a lesser extent in the medulla, suggests differential biochemical pathways of interaction with the biological effects of NTG. PEA produces its anti-nociceptive and anti-inflammatory effects by engaging the nuclear receptor PPAR- $\alpha$  (Lo Verme et al., 2005; Pontis et al., 2016). These receptors are expressed in many inflammatory-related cells, including macrophages (Chawla, 2010) and satellite glial cells (Chiocchetti et al., 2019) located in the ganglia, which suggests a role of PEA in the modulation of inflammatory processes (Lo Verme et al., 2005; Pontis et al., 2016). In addition to activating CB1, elevated anandamide may result in the desensitization of transient receptor potential vanilloid 1 (TRPV1) (Lizanecz et al., 2006), a receptor-channel that mediates pain transmission and CGRP release and is involved in sensitization (Capuano et al., 2014).

In contrast with the findings obtained in trigeminal ganglia, URB937 did not affect anandamide levels in the medulla. This was expected, because URB937 has no direct access to the central nervous system (Clapper et al., 2010; Moreno-Sanz et al., 2011). Quite unexpectedly, however, in this same area we observed a significant increase in PEA when URB937 was administered before NTG. This may be related to a moderate inhibition of FAAH activity in the area postrema, a structure located in the medulla that is not protected by the blood-brain barrier. In agreement, in an animal model of acute nausea, URB937 reduced partially, but significantly, FAAH activity in this area, where it also increased the levels of anandamide, PEA and oleoylethanolamide (Rock et al., 2017). Together, these findings add more evidence on the molecular mechanism responsible for the peripheral effect of URB937 and provide additional data on its biological effects.

Our findings show that the anti-hyperalgesic actions of URB937 in the NTG model were associated with a reduction in the expression of neuropeptides and pro-inflammatory cytokines. Together with the data obtained with the lipids assays, this finding suggests that URB937 elevates the levels of anandamide and PEA in trigeminal ganglia, which leads, in turn, to a reduced activation of the neuropeptidergic (CGRP) and inflammatory (IL-6) pathways triggered by NTG, in agreement with our previous studies (Greco et al., 2005, 2020). Moving centrally, we speculate that changes in lipid availability in the trigeminal ganglion, together with the putative reduction in the release of neuropeptides and inflammatory mediators may reverberate on the second order neurons in the TNC (located in the medulla). Much of what we detected in the trigeminal ganglia may also reflect the activity of URB937-induced changes in the trigemino-vascular endings on the meninges. Indeed, an increase in anandamide and PEA levels at the meningeal level may contribute to a reduction of trigeminal activation. Testing this hypothesis will require, however, further and specifically targeted studies.

It is worth mentioning that, since anandamide may be peripherally synthesized at the orofacial level, the action of URB937, via CB1 receptors expressed on cutaneous nociceptors, cannot be excluded. Thus, it is possible that synergistic interactions among several mechanisms may be necessary for anandamide and PEA to produce their effects.

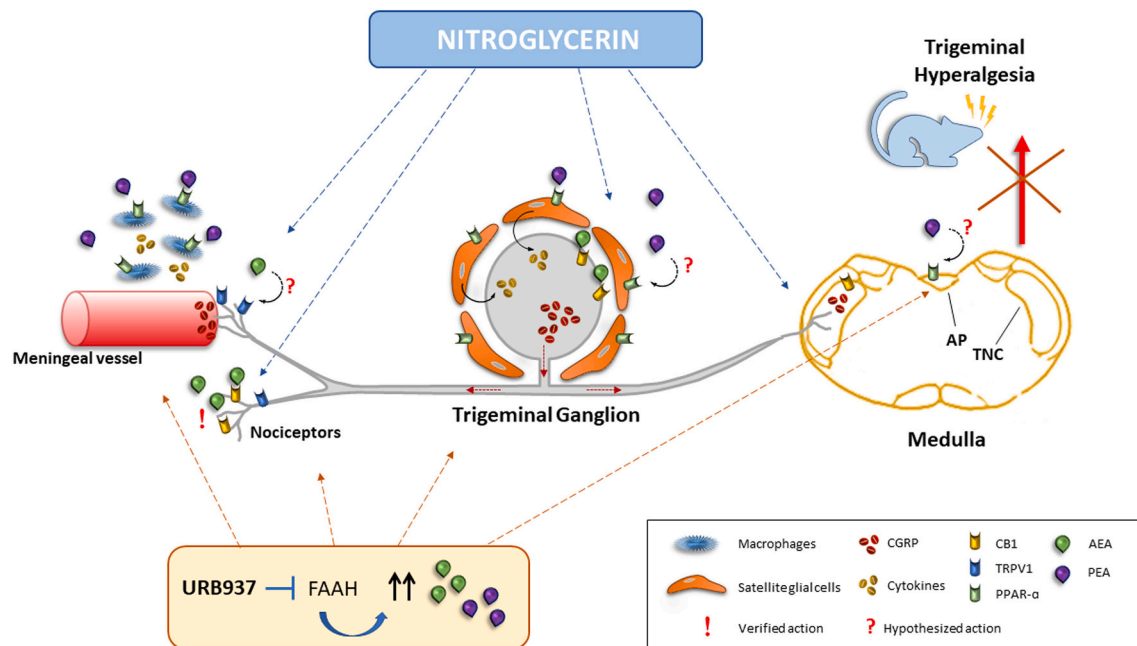


Fig. 7. Trigeminal pain processing in the animal model of migraine with putative sites and mediators of URB937 effect.

The ability of URB937 to interfere with migraine-related mechanisms was confirmed in the animal model of chronic migraine, where chronic treatment with URB937 significantly reduced NTG-induced trigeminal hyperalgesia; this effect was associated with a down-regulation of CGRP and cytokine genes expression in the trigeminal ganglion and the medulla.

The hypothetical biological role of URB937 in migraine pain and the areas of putative crosstalk with CGRP and inflammatory mediators are schematically illustrated in Fig. 7.

Trigeminal afferents arising in the trigeminal ganglion convey sensory information from the intracranial and extracranial sites to the trigeminal nucleus caudalis (TNC) causing trigeminal hyperalgesia. This pathway is activated by nitroglycerin administration (Demartini et al., 2019) and inhibited by CB1 receptors activation in the trigeminal ganglion, reducing the expression of pain biomarkers (e.g. cytokines, CGRP). URB937 - which peripherally inhibits FAAH activity - may counteract nitroglycerin-induced trigeminal hyperalgesia possibly through TRPV1 or PPAR- $\alpha$  receptors located on peripheral trigeminal afferents (e.g. on meningeal vessels), on macrophages, on satellite glial cells in the trigeminal ganglion and/or in the area postrema (AP).

## 5. Conclusions

Peripheral inhibition of FAAH by URB937 showed an anti-hyperalgesic effect in two complementary rat models of episodic and chronic migraine. The increased availability of anandamide and PEA in the trigeminal ganglion blocks NTG-induced inflammatory mediators and neuropeptides gene expression in the trigeminal ganglion itself and in medulla, thus reducing trigeminal nociception. The present findings provide new insights into the complexity of mechanisms that may be involved in trigeminal, migraine-related nociception and suggest that FAAH inhibition might qualify as a therapeutic target for migraine pain, confirming the close relationship between migraine and the endocannabinoid system in nociceptive processing at the trigeminal level.

## Funding

This study was supported by grants from the Italian Ministry of Health to the IRCCS Mondino Foundation, Pavia, Italy (RF2013-

02355704, Ricerca Corrente 2018–2019).

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## Declaration of Competing Interest

The authors report no competing interests.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nbd.2020.105157>.

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