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FAAH inhibition as a preventive treatment for migraine: A pre-clinical study

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ABSTRACT

Background: Fatty-acid amide hydrolase (FAAH) is an intracellular serine hydrolase that catalyzes the cleavage of endogenous fatty-acid amides, including the endocannabinoid anandamide (AEA). We previously reported that the peripherally restricted FAAH inhibitor URB937, which selectively increases AEA levels outside the central nervous system, reduces hyperalgesia and c-Fos expression in the trigeminal nucleus caudalis (TNC) and the locus coeruleus in an animal model of migraine based on nitroglycerin (NTG) administration.
Aim: To further investigate the relevance of FAAH inhibition in the NTG animal model of migraine by testing the effects of the globally active FAAH inhibitor URB597.
Methods: Our experimental approach involved mapping neuronal c-Fos protein expression, measurement of AEA levels in brain areas and in trigeminal ganglia, evaluation of pain-related behavior and quantification of molecular mediators in rats that received URB597 (2 mg/kg i.p.) either before or after NTG administration (10 mg/kg, i.p.).
Results: Pre-treatment with URB597 significantly reduced c-Fos immunoreactivity in the TNC and inhibited NTG-induced hyperalgesia in the orofacial formalin test. This behavioral response was associated with a decrease in neuronal nitric oxide synthase, calcitonin gene-related peptide and cytokine gene expression levels in central and peripheral structures. Administration of URB597 after NTG had no such effect.
Conclusions: The findings suggest that global FAAH inhibition may offer a therapeutic approach to the prevention, but not the abortive treatment, of migraine attacks. Further studies are needed to elucidate the exact cellular and molecular mechanisms underlying the protective effects of FAAH inhibition.

1. Introduction

Activation of the trigeminovascular system has long been implicated in the development of cephalic pain and alteration of pain sensitivity during migraine attacks (Holland et al., 2017). Many inflammatory mediators that are released during migraine attacks can activate and sensitize peripheral and central trigeminovascular neurons (Moskowitz, 1984; Bernstein and Burstein, 2012). Clinical and experimental evidences suggests that the endocannabinoid system (ECS), which is known to play an important role in pain modulation (Piomelli and Sasso, 2014), affects trigeminovascular activation by interacting with neuroinflammatory mediators such as calcitonin gene-related peptide (CGRP) and nitric oxide (NO) (Akerman et al., 2007). The endocannabinoid anandamide (AEA) may modulate trigeminovascular system activation through two distinct mechanisms: presynaptically, by modulating the release of CGRP from trigeminal sensory fibers, and postsynaptically, by reducing CGRP-induced NO release (Akerman et al., 2004). AEA counteracts hyperalgesia and c-Fos protein expression elicited in the trigeminal nucleus caudalis (TNC) by nitroglycerin (NTG) (Greco et al., 2011a), an established animal model of migraine (Demartini et al., 2019), possibly by decreasing neuronal NO synthase (nNOS) and nuclear factor kappa B (NF-kB) expression (Nagy-Grócz et al., 2016). NTG as a "NO donor" may exert its hyperalgesic effect via the activation of the trigeminovascular system (Demartini et al., 2019), thus inducing a condition of trigeminal sensitization. This latter can be evaluated by means of the orofacial formalin test, one of the main behavioral tests used to evaluate trigeminal hyperalgesia (Raboisson and Dallel, 2004), a condition observed also in migraineurs within and between episodes (Noseda and Burstein, 2013).

The effects of plant-derived or synthetic cannabinoids on pain

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modulation have been extensively documented, but a narrow therapeutic window limits the therapeutic use of these agents (Cousijn et al., 2018). Enhancing endocannabinoid tone via inhibition of their degradative enzymes may be an alternative means of activating the ECS with improved tolerability (Woodhams et al., 2017). AEA is released from macrophages during inflammation and is rapidly deactivated by a combination of transport into cells and intracellular hydrolysis (Piomelli, 2003). The latter reaction is catalyzed by the serine hydrolase fatty-acid amide hydrolase (FAAH), which thus offers a potential target for anti-migraine therapy (Greco et al., 2018a). In addition to eliminating AEA, FAAH also participates in the degradation of other bioactive fatty acid amides, including palmitoylethanolamide (PEA), an endogenous agonist of nuclear peroxisome proliferator-activated receptor-α (PPAR-α) that displays potent analgesic and antiinflammatory properties (Lo Verme et al., 2005, 2006; Piomelli and Sasso, 2014).

The potential of increasing ECS activity through inhibition of FAAH and other endocannabinoid-degrading enzymes has been explored in models of inflammatory and neuropathic pain (Woodhams et al., 2017). However, only few studies have investigated the potential application of this strategy to migraine pain (Nozaki et al., 2015; Greco et al., 2015a, 2018b). Previously, we reported that the peripherally restricted FAAH inhibitor URB937 alleviates hyperalgesia and neuronal activation induced by NTG (Greco et al., 2015a) in the TNC, probably by increasing the levels of AEA and other FAAH substrates in dura mater and trigeminal ganglia (TGs). To further explore the relevance of FAAH inhibition in migraine treatment, in the present study we evaluated the effects of the global FAAH inhibitor, URB597 (Kathuria et al., 2003), in the NTG model, focusing on several outcome measures that are relevant to the pathogenesis of migraine: pain-related behavior in the formalin test, neuronal c-Fos expression, expression of various neuroinflammatory mediators and accumulation of FAAH substrates AEA and PEA.

2. Material and methods

2.1. Animals

Adult male Sprague-Dawley rats (weight 175–200 g, Charles River, Calco, Como, Italy) were used for the present study. The IASP's guidelines for pain research in animals were followed (Zimmermann, 1983). Rats were housed in plastic boxes in groups of 2 with water and food available ad libitum and kept on a 12:12 h light-dark cycle at the Centralized Animal Facility of the University of Pavia. 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 (N°1032/2015-PR; 1019/2016-PR).

2.2. Drugs

NTG (Bioindustria L.I.M. Novi Ligure, Italy) was prepared from a stock solution of 5.0 mg/1.5 ml dissolved in 27% alcohol and 73% propylene glycol. The NTG solution was diluted in saline (0.9% NaCl) to reach the final concentration of alcohol about 6% and propylene glycol 16% and administered intraperitoneally (i.p.) at a dose of 10 mg/kg. The NTG vehicle contained saline, alcohol 6% and propylene glycol 16% (Greco et al., 2015a). URB597 (3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate, Cayman Chemical) was dissolved in dimethylsulfoxide (DMSO) (Rahn et al., 2008) and was injected i.p. at a dose of 2 mg/kg (Nozaki et al., 2015; Booker et al., 2012; Holt et al., 2005). DMSO was administered i.p. in a volume of 1 ml/kg (Greco et al., 2015a, 2017). The suitability of DMSO as a vehicle in our experimental paradigms (c-Fos expression and behavioral testing) was previously tested and validated (Greco et al., 2015a). Experimental groups with treatment combinations are illustrated in Fig. 1A.

2.3. Experimental design

Groups of 6 to 9 rats were randomly allocated to receive URB597 either 1 h before of 3 h after NTG or NTG vehicle. To minimize animal use, in a preliminary study we evaluated whether the timing of administration of DMSO (1 h before and 3 h after NTG or vehicle) could affect the nocifensive behavior at the orofacial formalin test. As the results showed no significant difference between pre- and post-treatment (data not shown), we used the post-treatment paradigm as a control group. Animals received URB597 1 h before (pre-treatment) or 3 h after (post-treatment) the administration of NTG or NTG vehicle. They were sacrificed 4 h after NTG or vehicle (Fig. 1B) to evaluate: (i) c-Fos protein expression, (ii) AEA and PEA levels, (iii) response in the orofacial formalin test (iv) and mRNA expression levels. The latency of 4 h for NTG injection was selected in agreement with previous observations showing that it corresponds to the timing of maximal neuronal activation in brain areas relevant for migraine and to the maximal inflammatory response at meningeal level induced by the NO donor (Demartini et al., 2019; Reuter et al., 2001). The timing of URB597 administration was chosen according to previous data, showing that the antinociceptive effect of URB597 occurs as early as 1 h after i.p. injection and persists up to 6 h in several animal models of pain (Holt et al., 2005; Jayamanne et al., 2006; Okine et al., 2012). Furthermore, it is worth noting that URB597 administered i.p., at a dose lower than the dose we used in our experiments, induced an increase in the brain levels of AEA and other fatty-acid ethanolamides already significant at 30 min and that lasted for several hours (Fegley et al., 2005).

2.4. c-Fos expression

Rats were anaesthetized with a lethal dose of anaesthetic (chloral hydrate) and perfused transcardially with saline followed by 270–300 ml of ice-cold 4% paraformaldehyde 4 h after NTG or NTG vehicle administration. Brains were removed, post-fixed for 12 h in the same fixative and subsequently transferred in solutions of sucrose at increasing concentrations (up to 30%) during the following 72 h. All brains were cut at 50 μm on a freezing sliding microtome. c-Fos expression was detected using the free floating immunohistochemical technique with a rabbit polyclonal antiserum directed against c-Fos protein (Greco et al., 2013). Coronal sections were incubated for 48 h at 4 °C with the c-Fos antibody (1:5000, Merck KGaA, Darmstadt, Germany). After thorough rinsing in potassium phosphate buffered saline containing Triton X-100, sections were processed with the avidin–biotin technique, using a commercial kit (Vector Labs, UK). c-Fos staining was visualized with nickel-intensified 3′,3′-diaminobenzidine tetrahydrochloride (DAB). For c-Fos expression evaluation, cell counts of individual nuclei were made from every sixth section throughout their rostrocaudal extent for each rat. The nuclei evaluated were identified with the rat brain atlas Paxinos and Watson 4th edition. TNC: from bregma −14.08 mm (interaural −5.08 mm) until −17 mm (interaural −8 mm); nucleus tractus solitarii (NTS): from bregma −13.24 mm (interaural −4.24 mm) until −13.68 mm (interaural −4.68 mm); locus ceruleus (LC): from bregma −9.80 mm (interaural −0.80 mm) until −10.04 mm (interaural −1.04 mm); parabrachial nucleus (PAB): from bregma −8.80 mm (interaural 0.20 mm) until −9.80 mm (interaural −0.50 mm); periaqueductal gray (PAG): from bregma −7.80 mm (interaural 1.20 mm) until −8.80 mm (interaural 0.20 mm).

In order to avoid differences related to the asymmetrical sectioning of the brain, c-Fos-immunoreactive cells were counted bilaterally (3 sections for each nucleus and the mean value obtained from the two sides was used for the statistical analysis). Image analysis was performed by an investigator unaware of the experimental design, using an AxioSkop 2 microscope connected to a computerized image analysis system (AxioCam MR5) equipped with dedicated software (AxioVision Rel 4.2) (Zeiss, Oberkochen, Germany).

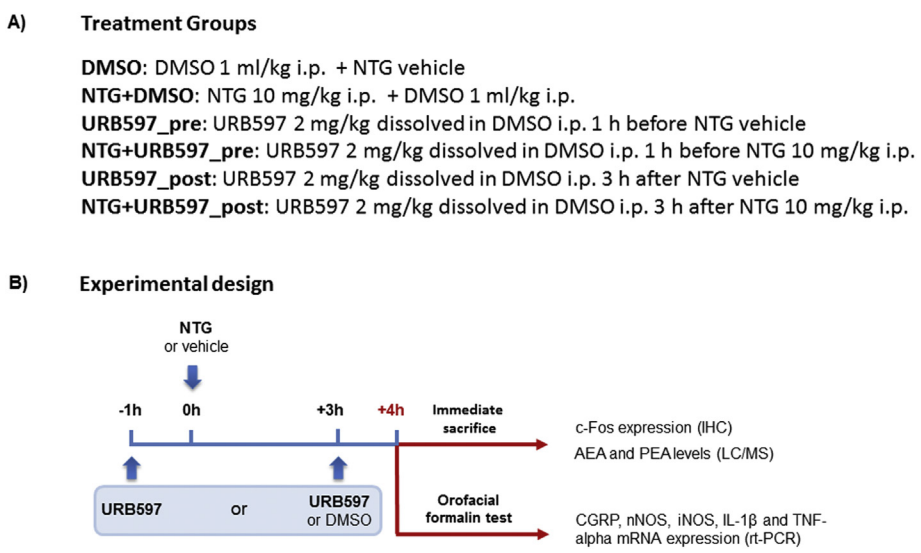

Fig. 1. Treatments groups (A) and experimental design (B). Four hours after NTG or NTG vehicle injection, an experimental group set of animals were immediately sacrificed for c-Fos expression and AEA and PEA essays, the other set of experimental groups underwent orofacial formalin test before been sacrificed for rt-PCR detection of CGRP, nNOS, iNOS, IL-1β and TNF alpha mRNA expression.

2.5. AEA and PEA levels

2.5.1. Tissue collection

Animals were sacrificed by decapitation after exposure to carbon dioxide (Clapper et al., 2010) 4 h after NTG or NTG vehicle injection. Since several factors may influence the concentrations of endogenous substances measured in samples from post mortem, medulla (bregma, −13.30 to −14.60 mm), cervical spinal cord (C1-C2, CSC) and TGs were quickly dissected out and snap-frozen in liquid nitrogen. Tissue parts were subsequently homogenized and AEA and PEA were extracted as described below.

2.5.2. Lipid extraction and fractionation

A Bligh-Dyer procedure, modified as described (Astarita and Piomelli, 2009), was used. Briefly, frozen tissues were weighed, transferred to glass vials and homogenized in cold methanol (2 ml) containing AEA-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_2. 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). AEA 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 N_2 and lipids were reconstituted in methanol/chloroform (50 µL; 9:1, v/v) and transferred to glass vials for LC/MS analyses.

2.5.3. LC/MS analyses

AEA and PEA levels were measured using a Xevo TQ UPLC-MS/MS 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. A representative chromatogram has been added as Supplementary figure.

2.6. Orofacial formalin test

Rats were acclimatized to the test chamber 20–25 min before testing. The observation box was a 30x30x30-cm glass chamber with mirrored sides. A camera, recording nocifensive behavior for off-line analysis, was located at a distance of 50 cm from the box 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 (Greco et al., 2015a, 2017; Raboisson and Dallel, 2004). Face rubbing was measured counting the seconds the animal spent grooming the injected area with the ipsilateral forepaw or hindpaw 0–6 min (Phase I) or 12–45 min (Phase II) after formalin injection. The observation time was divided into 15 blocks of 3 min each. Researchers who performed the evaluations were blind to treatments.

2.7. Gene expression

2.7.1. Tissue collection

Rats were sacrificed by decapitation after exposure to carbon dioxide (Clapper et al., 2010) at the end of the formalin test (i.e. 45′ after formalin injection). Rat decapitation guarantees more rapidity, which is extremely relevant when evaluating gene expression in order to minimize nuclease activity and preserve RNA. Dura mater, medulla (bregma, −13.30 to −14.60 mm), CSC and TG 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.

2.7.2. rt-PCR

All procedures were performed under RNase-free conditions; after RNA extraction, 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.

We evaluated the gene expression of CGRP, nNOS, interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-alpha) in the medulla, CSC and TG; the gene expression levels of IL-1β and inducible NOS (iNOS) in the dura mater. mRNA levels were measured by rt-PCR (Greco et al., 2015b, 2017). Primer sequences obtained from the AutoPrime software (http://www. autoprime.de/AutoPrimeWeb) are reported in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), whose expression remained constant in all experimental

Table 1
Primer sequences used.

Gene	Forward primer	Reverse primer
GAPDH	AACCTGCCAAGTATGATGAC	GGAGTTGCTGTTGAAGTCA
CGRP	CAGTCTCAGCTCCAAGTCATC	TTCCAAGGTTGACCTCAAAG
nNOS	CCGGCTACACTTCTCCTCAC	CACGAAGCAGGGGACTACAT
iNOS	TGGCCTCCCTCTGGAAAGA	GGTGGTCCATGATGGTCACAT
IL-1β	CTTCCTTGTGCAAGTGTCTG	CAGGTCATTCTCCTCACTGTC
TNF-alpha	CCTCACACTCAGATCATCTTCTC	CGCTTGGTGGTTTGCTAC

groups, was used for normalization. All samples were assayed in triplicate and the ΔΔCt method was used to investigate the differences in gene expression levels.

2.8. Statistical analysis

An a priori power analysis was conducted to determine the minimal 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 the second phase 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 ± 13.5; CT = 142 ± 18) and thus, we estimated a sample size of 6 rats in each experimental group with an effect size of 1.69. However, since the orofacial formalin test shows an intergroup variability, we used a maximum of 9 rats.

For nociceptive responses, gene expression and lipid levels, the statistical differences between groups were determined using the one-way ANOVA followed by post hoc Newman-Keuls Multiple Comparison. For c-Fos expression in each cerebral nucleus, the differences between groups were analysed using the Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. A probability level of $< 5\%$ was considered significant.

3. Results

3.1. c-Fos expression

As documented in previous studies (Greco et al., 2011b, 2015a, 2018b), NTG administration significantly increased c-Fos expression in brain areas involved in autonomic functions and in nociception – including the TNC (Fig. 2A, B), NTS, LC, PAG and PAB (Fig. 2). This effect was prevented by URB597 when administered before NTG in the majority of these structures, but not the PAG (Fig. 2E), reaching a statistical significance only in the TNC (NTG + DMSO 123.9 ± 10.1 vs NTG + URB597_pre 58.2 ± 7.1) (Fig. 2A).

In striking contrast with this finding, URB597 failed to affect NTG-induced c-Fos expression when injected 3 h after NTG (Fig. 2). Similarly, URB597 did not alter c-Fos expression when administered in the absence of NTG.

3.2. AEA and PEA levels

NTG did not affect AEA or PEA mobilization in any of the structures investigated in the present study (medulla, CSC and TGs).

URB597 administration significantly elevated AEA and PEA levels in these same structures, irrespective of whether it was administered before or after NTG (Fig. 3A–F).

URB597 administered before NTG vehicle increased AEA and PEA levels in all the areas investigated. URB597 administered 3 h after NTG vehicle significantly increased AEA levels in the medulla and CSC (Fig. 3A and B), and PEA levels in the medulla (Fig. 3D) as compared with DMSO and NTG + DMSO groups. No significant increase was instead observed in AEA and PEA levels in the TGs and in PEA levels in the CSC (Fig. 3C and F).

3.3. Orofacial formalin test

Consistent with previous studies (Greco et al., 2015a), formalin injection in NTG-treated rats elicited a nocifensive behavior (face rubbing) that achieved statistical significance compared to vehicle-treated controls in the second phase of the response (12–45 min following formalin administration). As illustrated in Fig. 4, URB597 reduced formalin-induced nocifensive behavior when administered 1 h before NTG (NTG + DMSO 186.0 ± 10.8 vs NTG + URB597_pre 82.6 ± 12.7), but not when injected 3 h after NTG. No significant effect was reported when URB597 was given before or after NTG vehicle compared with rats injected with DMSO.

3.4. Transcription of proinflammatory genes in the CNS

Formalin injection caused a marked increase in the transcription of CGRP, nNOS, IL-1β and TNF-alpha in the medulla and CSC ipsilateral to formalin injection in rats treated with NTG (Fig. 5). URB597 pretreatment prevented the increase of CGRP (medulla: 5.2 ± 0.6 vs 1.4 ± 0.3; CSC: 7.6 ± 1.6 vs 1.5 ± 0.2), nNOS (medulla: 15.0 ± 2.9 vs 2.1 ± 0.8; CSC: 7.3 ± 1.6 vs 1.8 ± 0.4), IL-1β (medulla: 15.1 ± 3.6 vs 2.3 ± 0.3; CSC: 5.1 ± 0.8 vs 2.4 ± 0.4) and TNF-alpha (medulla: 5.4 ± 0.7 vs 1.7 ± 0.2; CSC: 8.9 ± 1.7 vs 2.0 ± 0.5) compared to NTG + DMSO (Fig. 5). By contrast, treatment with URB597 3 h after NTG did not prevent NTG-induced increase in mRNA levels (Fig. 5). No effect on gene transcription was observed when URB597 was given with NTG vehicle.

3.5. Transcription of proinflammatory genes in peripheral structures

Similar to what we have observed in the CNS, NTG administration produced a substantial increase in the expression of CGRP, nNOS, IL-1β and TNF-alpha in TG ipsilateral to formalin injection and IL-1β and iNOS mRNA levels in dura mater, compared to the vehicle (DMSO) group (Fig. 6). The effect was partially, but significantly, prevented by URB597 only when the administration of the FAAH inhibitor preceded that of NTG (Fig. 6). In particular URB597 1 h before NTG reduced gene expression of CGRP (14.2 ± 4.2 vs 1.7 ± 0.2), nNOS (14.3 ± 2.9 vs 1.4 ± 0.1), IL-1β (11.0 ± 1.9 vs 1.0 ± 0.1) and TNF-alpha (8.32 ± 1.3 vs 1.3 ± 0.3) in TG; similarly it reduced IL-1β (7.2 ± 1.1 vs 1.5 ± 0.2) and iNOS (7.0 ± 0.9 vs 1.6 ± 0.2) mRNA levels in dura mater. No effect on gene transcription in the same regions was observed when URB597 was given with NTG vehicle.

4. Discussion

FAAH inhibitors may offer a promising option for the treatment of various painful pathologies (Woodhams et al., 2017), including migraine headache (Greco et al., 2018a; Tassorelli et al., 2019). In this condition, which is still unsatisfactorily managed by current standards of care (Martelletti and Giamberardino, 2019), FAAH blockade might have meaningful therapeutic benefits without eliciting the psychoactive effects related to direct cannabinoid 1 (CB_1) receptor activation (Piomelli and Sasso, 2014; Woodhams et al., 2017). As a further test of this hypothesis, in the present study we examined the actions of the global FAAH inhibitor URB597 on behavioral, cellular and molecular consequences of systemic NTG and local orofacial formalin administration in rats (Fig. 1B).

The results show that URB597 effectively prevents formalin-induced nocifensive responses in animals treated with NTG, along with neuronal activation and transcription of key neuroinflammatory genes. Of note, the FAAH inhibitor was only effective when administered before NTG, i.e. in a preventive protocol, but not when this order of administration was reversed. The potential mechanistic basis for the preventive effects of URB597 and its significance to migraine therapy are discussed below.

URB597 protects AEA from FAAH-mediated deactivation and, by

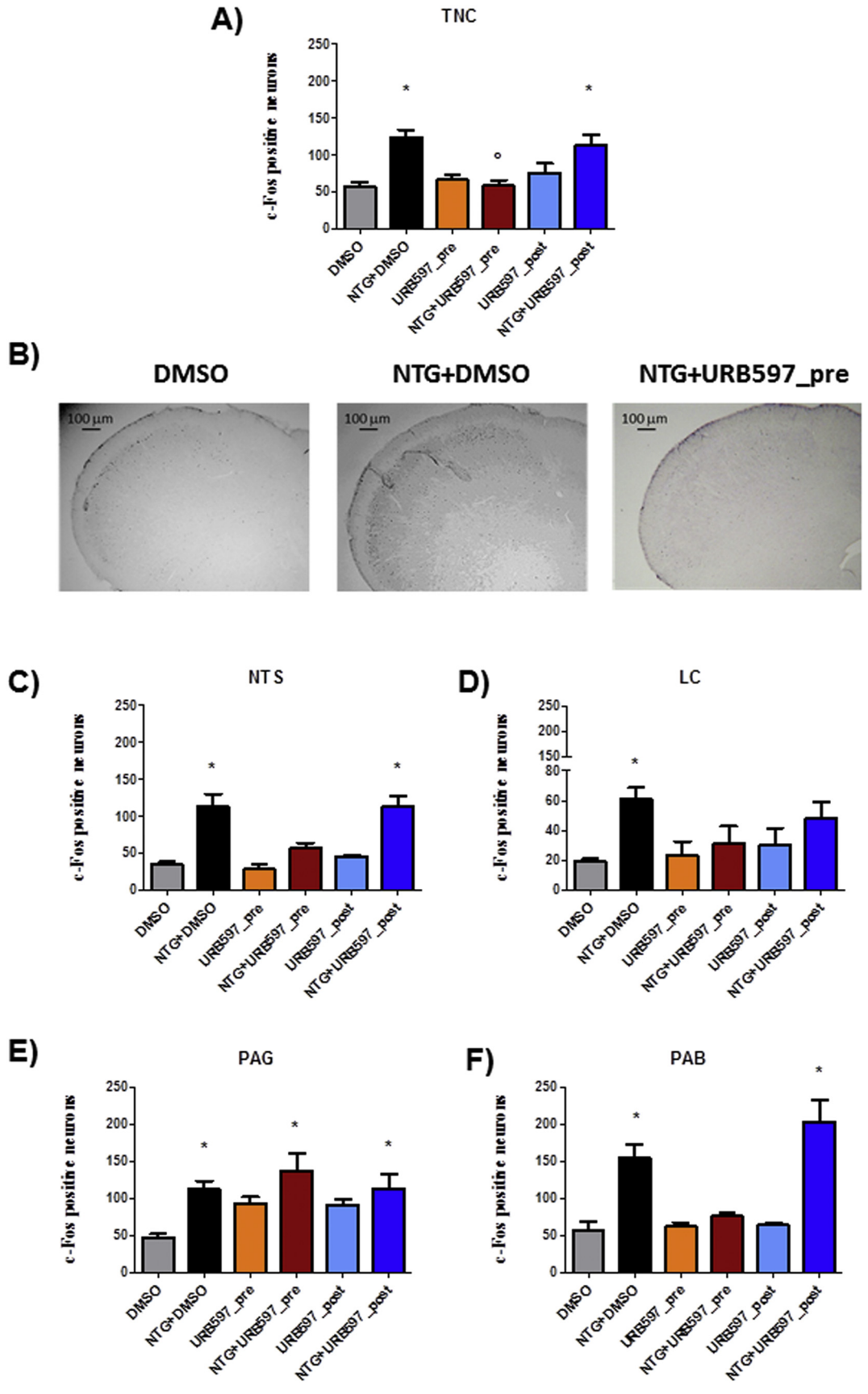

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Fig. 2. Expression of c-Fos protein in specific brain areas. Each group was formed by 6–9 rats.
NTG (NTG + DMSO) administration induced a significant increase in c-Fos expression in TNC (A), NTS (C), LC (D), PAG (E) and PAB (F) compared to DMSO group (NTG vehicle + DMSO). URB597, administered 1 h before NTG (pre-treatment), reduces c-Fos expression significantly only in the TNC compared to the NTG + DMSO group. URB597, administered 3 h after NTG (post-treatment), did not change c-Fos expression in the brain nuclei under evaluation, compared to the NTG + DMSO group.
B) Representative photomicrographs of TNC of rats treated with NTG vehicle + DMSO (DMSO group), or NTG + DMSO or URB597 1 h before NTG (NTG + URB597_pre group).
Data are expressed as mean ± SEM, Kruskal-Wallis test followed by Dunn's Multiple Comparison Test: $^{*}P < .05$ vs DMSO; $^{\circ}P < .05$ vs NTG + DMSO.
NTS = nucleus tractus solitarii; TNC = trigeminal nucleus caudalis; LC = locus ceruleus; PAG = periaqueductal gray; PAB = parabrachial nucleus.

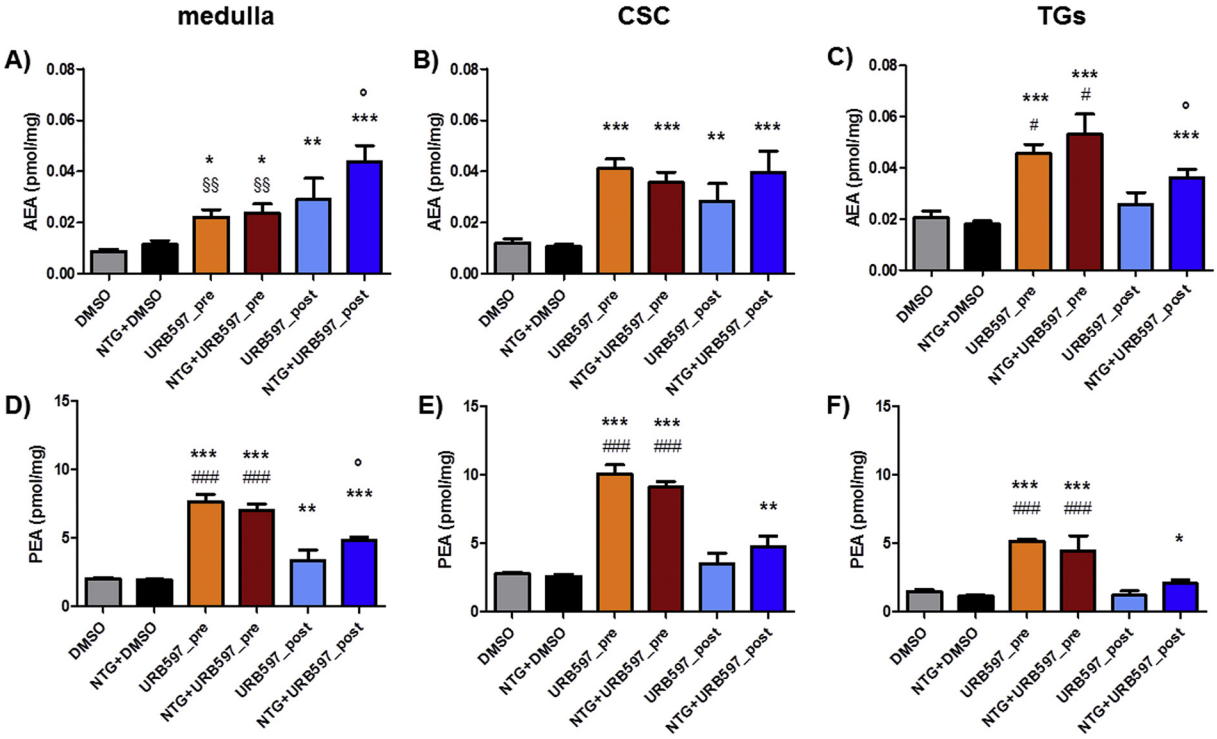

Fig. 3. AEA (A,B,C) and PEA (D,E,F) levels at medulla, cervical spinal cord (CSC) and trigeminal ganglia (TGs). Each group was formed by 6–9 rats. AEA and PEA levels did not differ significantly between NTG + DMSO and DMSO groups in all the areas evaluated. The groups that received URB597 before NTG or NTG vehicle showed significantly increased levels of AEA and PEA in central and peripheral areas as compared to NTG + DMSO and DMSO groups (A–F). When URB597 was administered after NTG or NTG vehicle we observed a different pattern of increase in the endocannabinoids levels: AEA and PEA levels were increased in all the areas under investigation in the NTG + URB597 group. When URB597 was associated to NTG vehicle, no significant increase was instead observed in AEA and PEA levels in the TGs (C and F) and in PEA levels in the CSC (E).
Data are expressed as mean ± SEM, one-way ANOVA followed by Newman-Keuls Multiple Comparison Test: $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$ vs DMSO and NTG + DMSO; $^{\circ}P < .05$ vs URB597_post; $^{\S\S}P < .01$ vs NTG + URB597_post; $^{\#}P < .05$ and $^{\#\#\#}P < .001$ vs URB597_post and NTG + URB597_post.

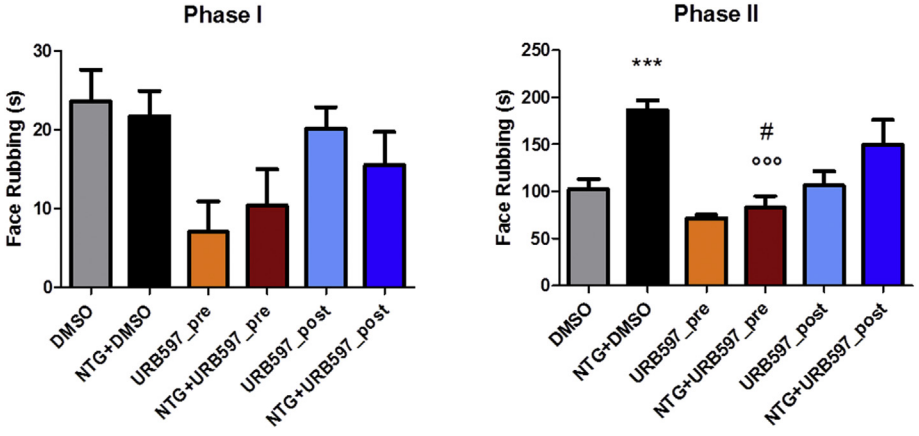

Fig. 4. Orofacial formalin test. Each group was formed by 6–9 rats. No significant differences between groups were seen during Phase I of the test. In Phase II, NTG administration (NTG + DMSO) significantly increased the face rubbing behavior compared to DMSO group. No changes were observed when URB597 was administered with NTG vehicle in both paradigms (URB937_pre and URB937_post). URB597 injected 1 h before NTG (NTG + URB597_pre) significantly decreased face rubbing behavior in Phase II compared to NTG + DMSO group. No changes were observed when URB597 was administered 3 h after NTG (NTG + URB597_post).
Data are expressed as mean ± SEM, one-way ANOVA followed by Newman-Keuls Multiple Comparison Test: $^{***}P < .001$ vs DMSO, URB597_pre and URB937_post; $^{\circ\circ\circ}P < .001$ vs NTG + DMSO; $^{\#}p < .05$ vs NTG + URB937_post.

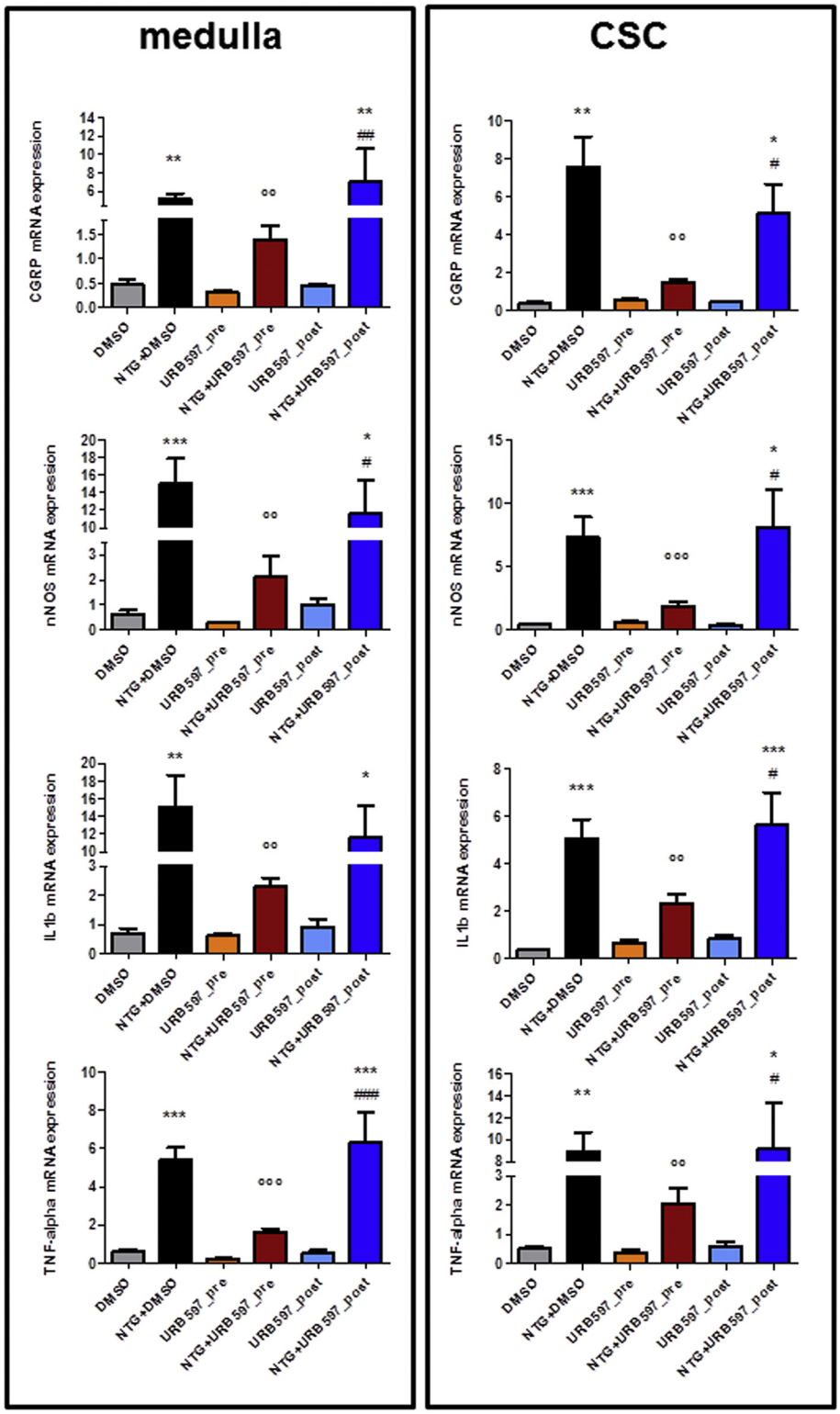

Fig. 5. mRNA expression levels of CGRP, nNOS, IL-1β and TNF-alpha in medulla and cervical spinal cord (CSC). Each group was formed by 6–9 rats. NTG treatment (NTG + DMSO group) significantly increased mRNA levels of all the genes evaluated compared to DMSO group in all areas. NTG-induced changes were prevented by administration of URB597 1 h before NTG (NTG + URB597_pre group); by contrast, administration of URB597 3 h after NTG (NTG + URB597_post group) did not affect NTG-induced increases in gene expression, as suggested by the absence of significant differences with NTG + DMSO group.

Data are expressed as mean ± SEM of the fold change. One-way ANOVA followed by Newman-Keuls Multiple Comparison Test: $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$ vs DMSO, URB597_pre and URB597_post; $^{\circ\circ}P < .01$ and $^{\circ\circ\circ}p < .001$ vs NTG + DMSO; $^{\#}P < .05$, $^{\#\#}P < .01$ and $^{\#\#\#}P < .001$ vs NTG + URB597_pre.

doing so, enhances the activity of this endocannabinoid lipid at CB_1 receptors in the brain and peripheral tissues (Kathuria et al., 2003; Fegley et al., 2005). The pharmacological properties of URB597 have been extensively characterized in animal models (Piomelli et al., 2006) and have been shown to include – among others – a combination of reduced anxiety-like and depressive-like symptoms (Kathuria et al., 2003; Gobbi et al., 2005; Bortolato et al., 2007), nicotine self-administration (Justinova et al., 2015) and pain-related behaviors (Russo et al., 2007). Studies with receptor antagonists and genetically modified mice have demonstrated that most, but not all, these effects are mediated by AEA activation of CB_1 receptors. Indeed, whereas the emotional and antinociceptive actions of URB597 are effectively suppressed by CB_1 receptor blockade or deletion (Kathuria et al., 2003; Gobbi et al., 2005; Bortolato et al., 2007), the FAAH inhibitor's ability to attenuate nicotine responses depends on activation of PPAR-α (Justinova et al., 2015; Pistis and Melis, 2010), which are engaged by the non-endocannabinoid FAAH substrates PEA and oleoylethanolamide (Fu et al., 2005; Lo Verme et al., 2005). The latter compounds, and particularly

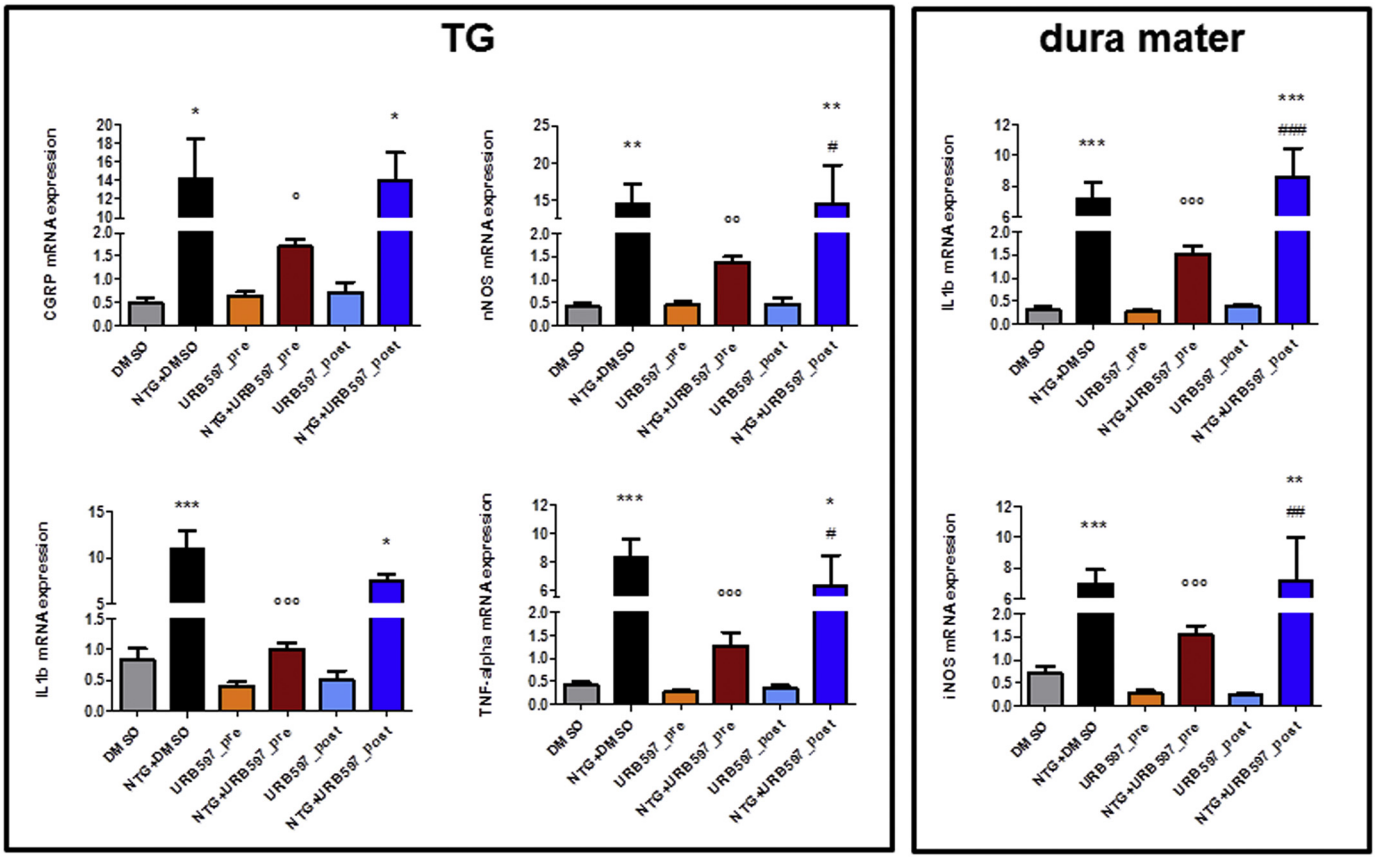

Fig. 6. mRNA expression levels of CGRP, nNOS, IL-1β and TNF-alpha in the trigeminal ganglion (TG); of IL-1β and iNOS in the dura mater. Each group was formed by 6–9 rats.

NTG treatment (NTG + DMSO group) significantly increased mRNA levels of all the genes evaluated compared to DMSO group in all areas. NTG-induced changes were prevented by administration of URB597 1 h before NTG (NTG + URB597_pre group); by contrast, administration of URB597 3 h after NTG (NTG + URB597_post group) did not affect NTG-induced increases in gene expression, as suggested by the absence of significant differences with NTG + DMSO group.

Data are expressed as mean ± SEM of the fold change. One-way ANOVA followed by Newman-Keuls Multiple Comparison Test: $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$ vs DMSO, URB597_pre and URB597_post; $^{\circ}P < .05$, $^{\circ\circ}P < .01$ and $^{\circ\circ\circ}p < .001$ vs NTG + DMSO; $^{\#}P < .05$, $^{\#\#}P < .01$ and $^{\#\#\#}P < .001$ vs NTG + URB597_pre.

PEA, display profound analgesic and anti-inflammatory properties, which have also been ascribed to PPAR-α activation (Lo Verme et al., 2005; Lo Verme et al., 2006). In the present study we show that systemic administration of URB597 before or after NTG results, as expected, in a marked elevation of AEA levels in central and peripheral neural structures (medulla, cervical spinal cord and trigeminal ganglia) that are involved in the pathophysiology of migraine. In addition, the FAAH inhibitor strongly increased PEA levels when its administration preceded that of NTG compared to post treatment. This puzzling finding requires additional consideration. Evidence indicates that PEA is cleaved not only by FAAH, but also by the cysteine hydrolase N-acylethanolamine acid amidase (NAAA) (Ueda et al., 2010). NAAA is expressed at high levels in cells of the monocyte lineage, including microglia (Ueda et al., 2010), where is thought to be the primary deactivating enzyme for PEA (Zhu et al., 2011). Since tissue NAAA levels increase during states of inflammation and neuroinflammation (Bonezzi et al., 2016), it is tempting to speculate that expression or activity of this enzyme may be enhanced by administration of NTG and might thus contribute to PEA hydrolysis following, but not prior to, exposure to this vasodilating and pro-inflammatory agent. URB597 does not inhibit NAAA activity (Solorzano et al., 2009), thus our present results could be explained by NTG-induced expression of NAAA that replaces FAAH as the primary effector of PEA degradation. Testing this hypothesis will require, of course, further experimentation. Previous studies have shown that acute noxious stimuli – including formalin injection or foot shock – stimulate AEA mobilization in brain structures implicated in nociceptive processing (Walker et al., 1999; Hohmann et al., 2005). Moreover, endocannabinoid levels and cannabinoid receptor expression are altered by nerve injury (Lim et al., 2003). In the present study, we did not observe any effect of NTG on AEA mobilization. A plausible explanation for this negative result is that a more robust noxious stimulus (e.g., formalin injection) may be needed for AEA to be mobilized. Alternatively, the timing of AEA mobilization in the brain, reported to be very rapid (7–15 min) in other experimental settings (Hohmann et al., 2005), might not coincide with the timing of animal sacrifice (4 h after NTG), selected to detect changes in c-Fos expression (Fig. 1B). Of note, previous findings indicate that FAAH activity may be increased in the medulla and other brain areas 4 h after NTG administration (Greco et al., 2010). Such an increase may be related to mechanism which contribute to offset any stimulation of AEA levels induced by NTG.

URB597 caused a marked reduction of nocifensive behavior (face rubbing) in the orofacial formalin test in animals treated with NTG, and of NTG-induced activation in structures involved in migraine pain such as the TNC. Accordingly, Nozaki et al. (2015) reported in mice that NTG-induced mechanical allodynia and c-Fos expression in TNC were completely abolished when URB597 was administered 2 h before NTG injection. Taken together, these data confirm the ability of URB597 to modulate the pro-nociceptive effects induced by NTG only when it is used preventively. Taken together, these data confirm the ability of URB597 to modulate the pro-nociceptive effects induced by NTG only

when it is used preventively. Moreover, the global FAAH inhibitor markedly attenuated NTG-induced increase in the transcription of key mediators of neuroinflammation: a significant reduction in mRNA levels of CGRP, nNOS, IL-1β and TNF-alpha in TG ipsilateral to formalin injection and IL-1β and iNOS gene expression in dura mater were found after FAAH inhibition. These effects were lost when URB597 was administered after the NTG challenge. The data gathered in this study do not warrant definite conclusions regarding the precise mechanisms that are engaged selectively by the pre-NTG dosing of URB597. One interesting hypothesis is that the simultaneous elevations of both AEA and PEA may be required to prevent the activation of the inflammatory pathway triggered by NTG (Greco et al., 2005). Further experimental data are needed to confirm this speculation. In this context, it is important to point out that the CNS-impermeant FAAH inhibitor URB937 attenuates nocifensive responses after NTG administration (Greco et al., 2015a), confirming previous data showing that peripheral FAAH inhibition may exert a tight regulatory control on pain processing and might offer a more effective strategy to pain management than does global FAAH inhibition (Sasso et al., 2012).

4.1. Limitations of the study

Here, we confirmed the use of FAAH inhibition as a potential therapy for migraine (Nozaki et al., 2015; Greco et al., 2015a). However, additional research is needed to elucidate the changes in AEA and PEA levels at the trigeminal level in more discrete cerebral areas (e.g. TNC, LC or PAG) and at earlier time points after acute and chronic NTG administration, also in relationship to the possible unwanted side-effects of chronic FAAH inhibitors. These findings contribute further evidence in favor of a possible role of the endocannabinoid system in migraine pathophysiology, but do not provide conclusive data regarding the relative importance of peripherally-restricted versus not peripherally-restricted inhibition of FAAH. Further direct comparison studies are necessary to this end. Additionally, to give more translational reliability, it is necessary to reproduce the same evaluations in female rats as well, since it is known that migraine prevalence is higher in female population (Global Burden of Diseases, 2016), in different ages, and, hopefully, in other animal models of migraine.

5. Conclusion

In conclusion, our study shows that pretreatment with the globally active FAAH inhibitor URB597 prevents NTG-induced hyperalgesia in the trigeminal region, while simultaneously elevating AEA levels and attenuating transcription of several neuroinflammatory genes in central and peripheral structures known to be involved in the pathophysiology of migraine. The results are consistent with a protective role for AEA-mediated endocannabinoid signaling in migraine and suggest that FAAH may offer a novel therapeutic option for the prevention of migraine attacks. Increasing the concentration of endocannabinoids via FAAH inhibition may be associated to a better tolerability than exogenous agonistic agents, as suggested by the careful characterization of the pharmacological profile of URB597 in rodents (Piomelli et al., 2006) and by the good tolerability reported in Phase I and II studies with FAAH inhibitors (Mallet et al., 2016). Careful ruling out of possible off-target effects (van Esbroeck et al., 2017) and cautious evaluation of tolerability is however mandatory, especially in the chronic administration modality.

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Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2019.104624.

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