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# Dual action of the cannabinoid receptor 1 ligand arachidonyl-2'-chloroethylamide on calcitonin gene-related peptide release

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## Abstract

**Background:** Based on the current understanding of the role of neuropeptide signalling in migraine, we explored the therapeutic potential of a specific cannabinoid agonist. The aim of the present study was to examine the effect of the synthetic endocannabinoid (eCB) analogue, arachidonyl-2'-chloroethylamide (ACEA), on calcitonin gene-related peptide (CGRP) release in the dura and trigeminal ganglion (TG), as cannabinoids are known to activate G<sub>v</sub>o-coupled cannabinoid receptors type 1 (CB1), resulting in neuronal inhibition.

**Methods:** The experiments were performed using the hemi-skull model and dissected TGs from male Sprague-Dawley rats. CGRP release was induced by either 60 mM K<sup>+</sup> (for depolarization-induced stimulation) or 100 nM capsaicin (for transient receptor potential vanilloid 1 (TRPV1)-induced stimulation) and measured using an enzyme-linked immunosorbent assay. The analysis of CGRP release data was combined with immunohistochemistry in order to study the cellular localization of CB1, cannabinoid receptor type 2 (CB2), CGRP and receptor activity modifying protein 1 (RAMP1), a subunit of the functional CGRP receptor, in the TG.

**Results:** CB1 was predominantly expressed in neuronal somas in which colocalization with CGRP was observed. Furthermore, CB1 exhibited colocalization with RAMP1 in neuronal A $\delta$ -fibres but was not clearly expressed in the CGRP-immunoreactive C-fibres. CB2 was mainly expressed in satellite glial cells and did not show substantial colocalization with either CGRP or RAMP1. Without stimulation, 140 nM ACEA per se caused a significant increase in CGRP release in the dura but not TG, compared to vehicle. Furthermore, 140 nM ACEA did not significantly modify neither K<sup>+</sup>- nor capsaicin-induced CGRP release. However, when the TRPV1 blocker AMG9810 (1 mM) was coapplied with ACEA, K<sup>+</sup>-induced CGRP release was significantly attenuated in the TG and dura.

**Conclusions:** Results from the present study indicate that ACEA per se does not exhibit antimigraine potential due to its dual agonistic properties, resulting in activation of both CB1 and TRPV1, and thereby inhibition and stimulation of CGRP release, respectively.

**Keywords:** Cannabinoid receptors, CGRP, Immunohistochemistry, Trigeminal ganglion, ACEA

## Introduction

Migraine is a highly debilitating neurological condition linked to the sensory innervation of cranial blood vessels by the trigeminal nerves, together known as the trigeminovascular system (TVS). A pioneering study revealed that plasma levels of calcitonin gene-related

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peptide (CGRP), a vasodilator found in trigeminal neurons, increase significantly in craniovascular regions during the headache phase of migraine attacks, suggesting a crucial role for CGRP in migraine pathology [1].

Previous research has demonstrated CGRP release from the dura, the trigeminal ganglion (TG) and the trigeminal nucleus caudalis (TNC) in rats [2–6]. The TNC constitutes the major relay station for nociceptive afferent input from peripheral cranial structures, and is located in the brainstem in the trigeminocervical complex (TCC) [7–9]. According to the current view on migraine pathophysiology, hypothalamic activation during the premonitory phase of a migraine attack results in activation of the TNC, which in turn leads to activation of the TG. Hereafter, CGRP is released from the TG, resulting in vasodilation [10, 11].

CGRP is localized to small and medium sized (30–60  $\mu\text{m}$ ) neuronal somas and varicose, unmyelinated C-fibres [12], from which it is directly released [13]. C-fibres run parallel to A $\delta$ -fibres in the TG and dura, and since A $\delta$ -fibres are the only peripheral nerve fibres known to express the CGRP receptor (CLR/RAMP1), it has been suggested that the pain experienced by migraineurs originates from A $\delta$ -fibres [11].

Activation of the CGRP receptor in the TG has been shown to implicate several signalling pathways known to mediate downstream physiological and pathophysiological effects. Among these is the cyclic adenosine monophosphate (cAMP) pathway, which is currently considered an antimigraine target [10, 11]. Triptans were originally developed for their vasoconstrictive properties, and later found to act by reducing intracellular cAMP levels through activation of 5-HT<sub>1B/D</sub> [10]. In spite of their success, 25% of migraine patients display unresponsiveness to triptans and 40% of migraine attacks are not alleviated by triptan intervention [14], thus highlighting the need for alternative drugs for the acute treatment of migraine. Further, the contraindication of triptans for migraineurs with cardiovascular disorders prompted the development of Lasmiditan, a specific 5-HT<sub>1F</sub> receptor agonist, which does not have vasoconstrictive effects [10, 15]. Although Lasmiditan does not activate 5HT<sub>1B</sub>, recent data suggest that part of the Lasmiditan effect might partially be through 5HT<sub>1D</sub> activation [16]. The 5-HT<sub>1B/1D/1F</sub> receptor subtypes are all G<sub>i/o</sub>-coupled G-protein coupled receptors (GPCRs) and elicit inhibitory effects on the adenylate cyclase (AC), causing a decrease in intracellular cAMP and thereby CGRP release [17]. Interestingly, the purinergic P2Y<sub>13</sub> receptor, with adenosine diphosphate (ADP) as its ligand, also exhibits G<sub>i/o</sub>-coupling and has been shown to suppress CGRP release with results similar to that of Sumatriptan in preclinical models of migraine [5].

The endocannabinoid system (ECS) has been found in even the most primitive animals with a neuronal network, such as Hydra of the phylum Cnidaria, considered to be the first animal species possessing a neuronal network, thus illustrating the phylogenetic ancientness of the ECS [18, 19]. The two major GPCRs in the ECS are cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2). CB1 is expressed in most brain structures as well as in the peripheral nervous system (PNS), while CB2 expression is mainly restricted to immune cells of central and peripheral tissues [20, 21]. The endocannabinoids (eCBs) 2-arachidonoylglycerol (2-AG) and N-arachidonylethanolamine (Anandamide, AEA) are well characterized agonists of CB1 and CB2 [22].

Immunohistochemistry (IHC) on different regions of the rat brain has previously demonstrated presynaptic localization of CB1, indicating a role in regulation of neurotransmission [22, 23]. Indeed, eCBs are viewed as key regulators of synaptic function in the central nervous system (CNS). Their role in retrograde suppression of presynaptic neurotransmitter release has been well established, and studies provide evidence that presynaptic CB1 activation can lead to inhibition of neurotransmitter release (e.g., glutamate, dopamine and acetylcholine) in both human and rodent assays [24, 25]. More specifically, CB1 activation has been shown to inhibit AC activity and thereby cAMP production [26], similar to the action of triptans. In addition to activation of G<sub>i/o</sub>-coupled CB1 receptors, cannabinoids have also been shown to stimulate G<sub>q/11</sub>-coupled CB1 receptors, highlighting the complexity of cannabinoid signalling [27].

Previous studies have shown that anandamide, the endogenous non-selective cannabinoid receptor agonist, is able to inhibit trigeminovascular dilation, C- and A $\delta$ -fibre activity as well as transient receptor potential ankyrin 1 (TRPA1) and transient receptor potential vanilloid 1 (TRPV1)-mediated CGRP release [28–31]. At higher concentrations, anandamide has also been shown to stimulate CGRP release through activation of TRPV1 [31]. The TRPV1 receptor, also known as the capsaicin receptor, has been demonstrated to colocalize with CGRP, substance P (SP) and nitric oxide (NO) in the human TG [32]. In a previous study by Fischer et al., it was examined whether methanandamide (mAEA), a synthetic anandamide analogue, modulates heat-induced CGRP release in the dura. mAEA was found to either depress (at non-noxious temperatures) or facilitate (at noxious temperatures) heat-induced CGRP release, indicating opposing dual actions of eCBs on CGRP release in the dura [33]. Since heat specifically activates TRPV1, one may ask whether eCBs exert the same dual effects on CGRP release when more general mechanisms, such as cellular depolarization, are employed to stimulate release.

Using the rat hemi-skull model, this study aims to determine the effect of arachidonyl-2'-chloroethylamide (ACEA), a synthetic anandamide analogue with high potency and selectivity for CB1 [34], on K<sup>+</sup>- and capsaicin-induced CGRP release from the dura and TG of male Sprague-Dawley rats. In addition, we examine the expression of CB1, CB2, receptor activity modifying protein 1 (RAMP1) and CGRP in the TG utilizing IHC.

## Methods

### Animals

Sprague-Dawley rats (300 – 400g) purchased from Taconic (Ejby, Denmark) were maintained on a 12/12h light/dark cycle (with dark beginning at 7am) and housed at constant temperature (22 ± 2°C) and humidity (55 ± 10%), with free access to food and water. Rats were generally housed in Eurostandard cages (Type VI with 123-Lid) 2–6 together.

### In situ/in vitro CGRP release

Rats were anaesthetized by CO<sub>2</sub> inhalation and decapitated. All procedures are approved by the Danish Animal Experimentation Inspectorate. The protocol is described in detail elsewhere [35]. Following decapitation and removal of skin, skulls were immersed in synthetic interstitial fluid kept on ice to maintain metabolism at low levels. Skulls were cut mid-sagittally and brains were removed whilst the cranial dura and TGs were left attached to the hemi-skulls. The TGs were subsequently dissected from the hemi-skulls and transferred to polypropylene tubes containing 10mL synthetic interstitial fluid (SIF) and kept in a water bath at +37°C for 30 min. Hemi-skulls were transferred to beakers containing SIF and placed in a water bath at +37°C for 15 min and then washed twice with intervals of 15 min by renewing the SIF solution.

TGs were randomized, placed in Eppendorf tubes on a heating block from Eppendorf (Hamburg, Germany) at +37°C, and washed five times with 300 µL SIF with intervals of 10 min. Hemi-skulls were randomized, placed on polystyrene cell culture plates from Sarstedt (Europe) in a water bath from Memmert (Bayern, Germany) at +37°C and washed five times with 300 µL SIF with intervals of 10 min. After the last 10 min incubation with 300 µL SIF, 200 µL samples (measuring baseline CGRP release) were extracted from the dura and TG and placed in Eppendorf tubes. The described procedures (i.e., washing and baseline extraction) were performed in all experiments prior to incubation with test compounds. It was previously demonstrated by Bhatt et al. that there is no significant difference between left and right CGRP release from the dura and TG [35], thus in the present experiments, one side functioned as a control for the other. In

each experiment, one side was used for vehicle application, whereas the other was used to examine the effect of ACEA/AMG9810.

### ELISA

To measure the amount of CGRP release from both tissues (dura and TG), samples were processed using commercial CGRP (human) Enzyme Immunoassay (EIA) kits from SPIbio (Paris, France). The antibody in the EIA kit is meant for human CGRP but has 100% cross reactivity with rat CGRP [5] and was therefore utilized in this experiment. 50 µL EIA buffer was added to each of the 200 µL samples as well as to the CGRP standards. EIA buffer contains protease inhibitors and prevents CGRP degradation [5]. The CGRP tissue concentrations were determined based on the standard curve. The experiments were performed following the manufacturer's protocol. Optical density was determined at 410 nm using a micro-plate photometer (Tecan, Infinite M200) and software from Magellan, version v.6.3 (Männedorf, Switzerland). Data was exported to Excel for analysis.

### Synthetic interstitial fluid

SIF was made using distilled water and was of the following composition (in mM): NaCl 108, KCl 3.48, MgSO<sub>4</sub> 3.5, NaHCO<sub>3</sub> 26, NaH<sub>2</sub>PO<sub>4</sub> 11.7, CaCl<sub>2</sub> 1.5, Na<sup>+</sup> Glucuronate 9.6, Glucose 5.55 and Sucrose 7.6. For K<sup>+</sup>-induced nerve fibre stimulation, a second SIF solution was prepared in which the KCl concentration was increased to 60 mM. To maintain similar osmolarities in the two solutions, the NaCl concentration was reduced appropriately. NaCl, NaH<sub>2</sub>PO<sub>4</sub> and CaCl<sub>2</sub> were supplied from EMSURE (Darmstadt, Germany) and the rest from Sigma-Aldrich (Darmstadt, Germany). All solutions were bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

### Immunohistochemistry

Adult, male Sprague-Dawley rats were anaesthetized by CO<sub>2</sub> inhalation and decapitated. TGs were carefully dissected and fixated with 4% paraformaldehyde (Sigma, St Louis, USA) diluted in phosphate buffered saline (PBS) for 2–4 h. To ensure cryoprotection the fixated TGs were submerged in a 10% sucrose in Sorensen's phosphate buffer (pH 7.2) at +4°C for 3–4 h. Subsequently, the TGs were submerged in a 25% sucrose in Sorensen's phosphate buffer (pH 7.2) at +4°C overnight. The following day, the TGs were embedded in a gelatine medium (30% egg albumin, 3% gelatine) and stored at –20°C. The TGs were then sectioned at 10 µm, collected on microscope slides (Superfrost, ThermoFisher) and stored at –20°C until used.

TG sections were thawed at room temperature (RT) and subsequently washed in PBS containing 0.25%

Triton-X (PBS-T) for  $2 \times 15$  minutes. A primary antibody was applied to each sample and the slides were incubated overnight at  $+4^\circ\text{C}$  in moisturized incubation chambers. The following day, the sections were washed with PBS-T  $2 \times 15$  minutes prior to incubation with secondary antibodies for 1 h at RT. Finally, the sections were washed  $2 \times 15$  minutes and a coverglass was mounted with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame CA, USA). The antibodies used and concentrations are given in Table 1.

For double IHC, the procedure was repeated twice before mounting. Negative controls (Supplementary Fig. 1) were performed by omitting the primary antibody. Any resulting immunofluorescence would suggest unspecific binding of the secondary antibodies or insufficient washing. The sections were examined in a light and epifluorescence microscope (Nikon 80i, Tokyo, Japan) equipped with a Nikon DS-2MV camera. Lastly, figure montages were processed in Adobe Photoshop CC20 (Adobe Systems, Mountain View, CA, USA).

### Compounds

ACEA, supplied pre-dissolved in anhydrous ethanol (5 mg/mL, 13.66 mM), was purchased from Tocris (Bristol, UK). Ethanol was used as vehicle for ACEA throughout all experiments and did not exceed 0.001%. Capsaicin was supplied from Tocris (Bristol, UK), dissolved in a 10 mM stock solution in 100% ethanol and was further diluted to a 0.001% ethanol containing solution of 100 nM capsaicin. AMG9810 was supplied from Tocris (Bristol, UK) and was dissolved at 1 mM in a 10% ethanol and 90% DMSO containing solution, which was also used as vehicle. When diluted the concentration of ethanol and DMSO was 0.01% and 0.09%, respectively. All remaining compounds were purchased from Sigma Aldrich/Merck (Darmstadt, Germany).

### Statistics

All data was subject to Grubb's test ( $\alpha = 0.05$ ) which was performed using the GraphPad outlier calculator. All the CGRP and Substance P release data passed the Shapiro-Wilk normality test ( $\alpha = 0.05$ ). Further, all identified outliers were removed from the dataset prior to performing Student's T-test and calculating mean and SEM values. Data for CGRP release is shown with mean  $\pm$  SEM, where 'n' represents the sample size (i.e., number of animals).

### Results

#### CGRP release

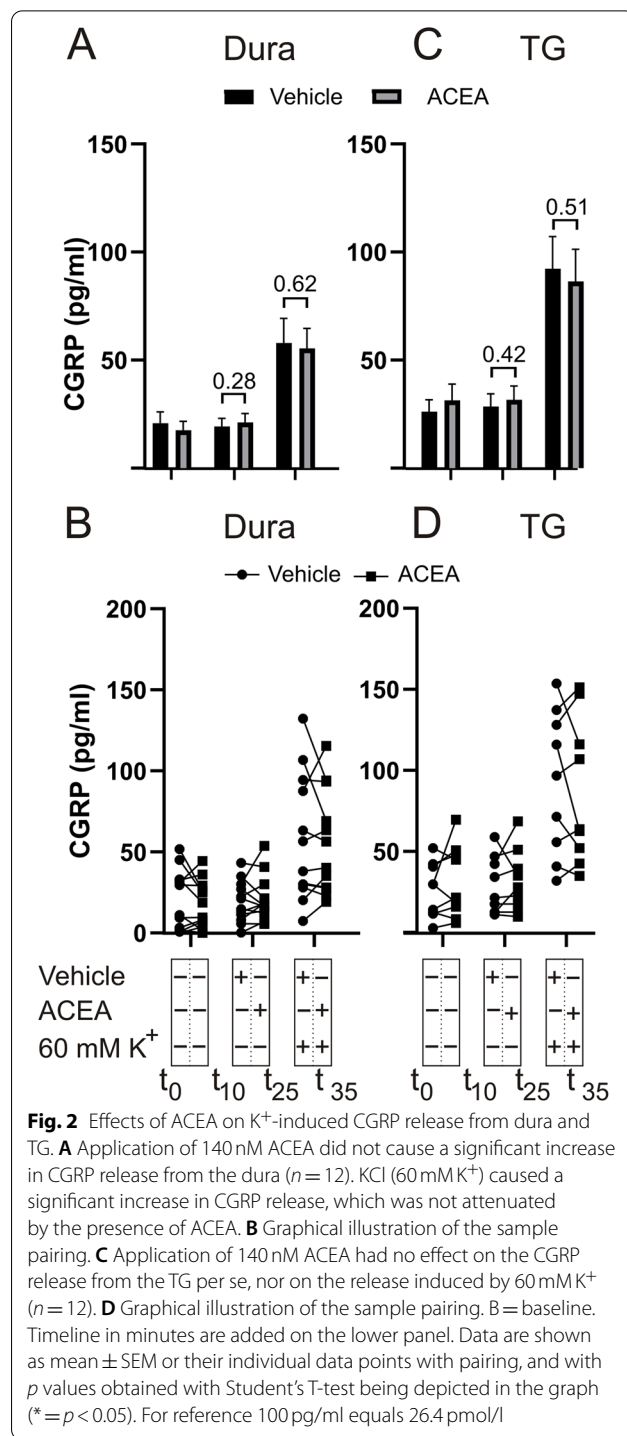
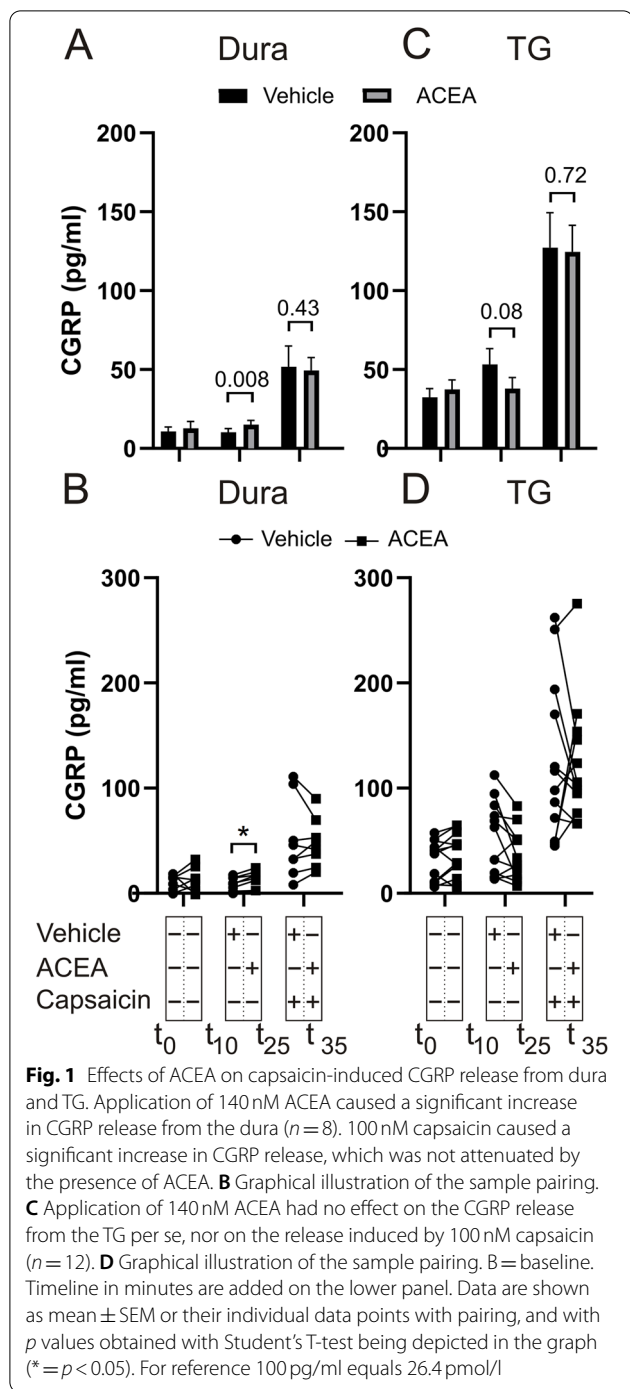
It has been well established in both human and rodent assays that  $G_{i/o}$ -coupled CB1 receptors are involved in retrograde suppression of neurotransmitter release [24, 25]. Since ACEA is a potent and highly selective CB1 agonist [34], we sought to examine the therapeutic potential of ACEA in migraine pathology by investigating whether ACEA per se, affects CGRP levels in the dura and TG of male Sprague-Dawley rats.

140 nM ACEA per se caused a significant increase in CGRP release from the dura (w/o ACEA:  $10.3 \pm 2.2$  pg/mL; w/ACEA:  $15.0 \pm 2.8$  pg/mL;  $p = 0.008$ ,  $n = 8$ ) compared to vehicle (Fig. 1A-B). In the TG however, the difference was non-significant (w/o ACEA:  $53.3 \pm 10.1$  pg/mL; w/ACEA:  $37.9 \pm 7.1$  pg/mL,  $p = 0.08$ ,  $n = 12$ ) (Fig. 1C-D). In continuation, we performed an experiment in which 100 nM capsaicin was used as a TRPV1-specific stimulus (Fig. 1). 140 nM ACEA did not cause significant inhibition of capsaicin-induced CGRP release in the TG (w/o ACEA:  $127.2 \pm 22.1$  pg/mL; w/ACEA:  $124.6 \pm 16.9$  pg/mL,  $p = 0.72$ ,  $n = 12$ ) or dura (w/o ACEA:  $51.7 \pm 13.3$  pg/mL; w/ACEA:  $49.4 \pm 8.2$  pg/mL,  $p = 0.43$ ,  $n = 8$ ) (Fig. 1). Considering that ACEA is the synthetic analogue of anandamide, which has been shown to stimulate CGRP release through activation of TRPV1 [31], it is possible that ACEA stimulated CGRP release through a higher degree of TRPV1 than CB1 activation.

Due to the potential implication of TRPV1 in CGRP release, we performed another experiment in which

**Table 1** Primary and secondary antibodies used

Product	Code	Source	Dilution	Immunogen	Company
CB1	ACR-001	Rabbit	1:100	N-terminal of rat CB1 receptor	Alomone Labs, Jerusalem, Israel
CB2	ACR-002	Rabbit	1:100	3rd intracellular loop of rat CB2 receptor	Alomone Labs, Jerusalem, Israel
CGRP	Ab81887	Mouse	1:100	Rat $\alpha$ -CGRP	Abcam, Cambridge, UK
RAMP1	844	Goat	1:100	C-terminal of human RAMP1	Merck & Co., Inc., West Point, PA, USA
Product		Source	Dilution	Against	Company
FITC		Goat	1:100	Anti-rabbit	Cayman Chemical, Ann Arbor, MI, USA
Alexa 594		Goat	1:100	Anti-mouse	Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA
Cy3		Donkey	1:200	Anti-goat	Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA



60mMK<sup>+</sup> was used as depolarizing stimulus. Addition of 140nM ACEA per se showed a slight but similar tendency to stimulate CGRP release in the dura (w/o ACEA: 19.4 ± 3.7 pg/mL; w/ACEA: 21.2 ± 4.0 pg/mL,  $p = 0.28$ ,  $n = 12$ ) as well as in the TG (w/o ACEA: 28.6 ± 5.9 pg/mL; w/ACEA: 31.7 ± 6.4 pg/mL,  $p = 0.42$ ,  $n = 9$ ) compared to vehicle (Fig. 2). Further, due to the difference observed

in these experiments, we combined all data after adding ACEA and compared to vehicle. The direct effect of adding ACEA per se was a change from baseline of 4.1 ± 1.9 pg/mL compared to vehicle at -0.7 ± 1.7 pg/mL being significant ( $p = 0.023$ ,  $n = 20$ ). When we further tested whether the addition of 140nM ACEA would



affect  $K^+$ -induced CGRP release, no significant inhibition was observed in the dura (Fig. 2A-B) or TG (Fig. 2C-D).

Since no significant inhibition was observed using  $K^+$  as depolarizing stimulus, it was considered whether TRPV1 competes with CB1 for binding to ACEA. Therefore, we performed an experiment using AMG9810, a potent and selective, competitive antagonist of TRPV1. Upon  $K^+$ -stimulation in the presence of 1 mM AMG9810, 140 nM ACEA demonstrated significant inhibition of CGRP release in the dura (w/o AMG9810:  $60.5 \pm 5.1$  pg/mL; w/AMG9810:  $47.2 \pm 4.1$  pg/mL,  $p=0.011$ ,  $n=12$ ) (Fig. 3A-B) and TG (w/o AMG9810:  $117.7 \pm 9.6$  pg/mL; w/AMG9810:  $86.6 \pm 10.6$  pg/mL,  $p=0.04$ ,  $n=9$ ) (Fig. 3C-D) compared to ACEA w/o AMG9810.

### Immunohistochemistry

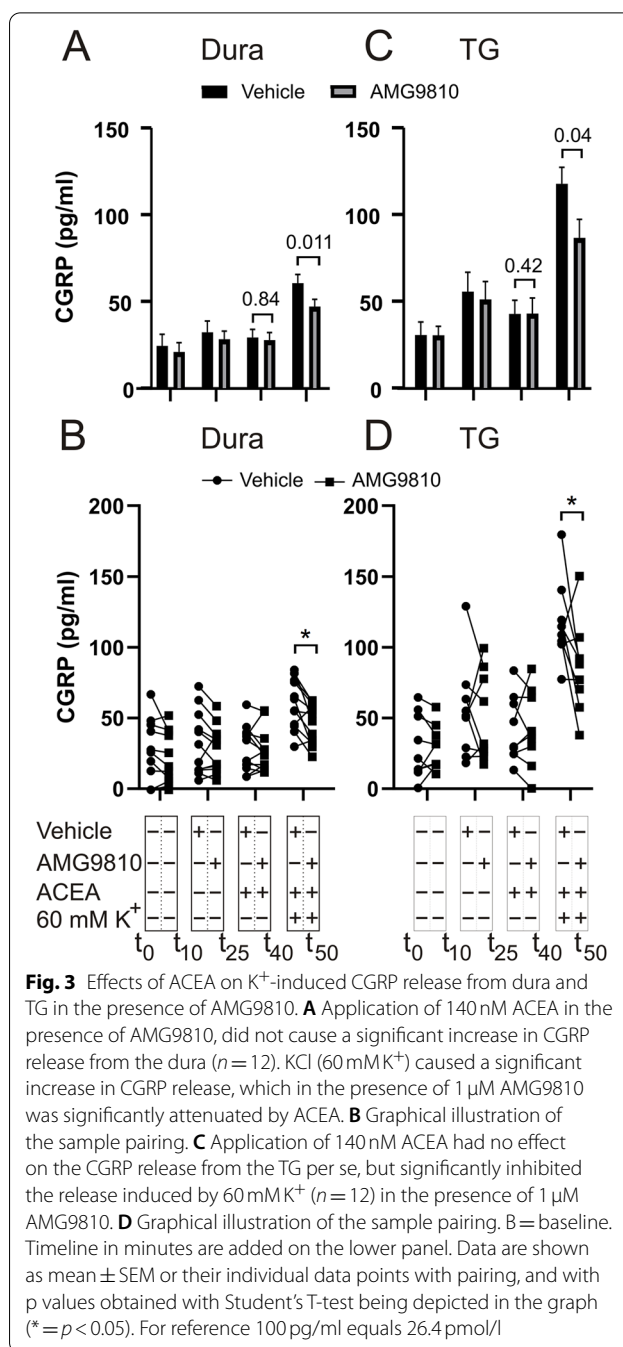
IHC images were used to determine the cellular location of CB1, CB2, CGRP and RAMP1 in the TG. RAMP1 is an essential part of the functional CGRP receptor [36] and therefore represents potential localization of the CGRP receptor, although it could also label neurons with the Amylin 1 receptor [37]. Nevertheless, Fig. 4 shows a high expression level of CB1 in neuronal somas of the TG relative to that observed in satellite glial cells (SGCs) and neuronal fibres. In contrast, CGRP was highly expressed in C-fibres with limited localization to neuronal somas of the TG (Fig. 4A), whereas RAMP1 exhibited high expression levels in both A $\delta$ -fibres and neuronal somas (Fig. 4B). Furthermore, CB1 was found to colocalize with CGRP in neuronal somas, whereas colocalization in the CGRP associated C-fibres was not clearly detected (Fig. 4A). Colocalization of CB1 and RAMP1 was observed in neuronal somas and A $\delta$ -fibres of the TG (Fig. 4B).

CB2 was found to be highly expressed in many SGCs relative to that observed in neuronal somas and fibres of the TG (Fig. 5). In addition, CB2 did not demonstrate colocalization with neither CGRP (Fig. 5A) nor RAMP1 (Fig. 5B) in the TG, supporting the notion that ACEA exerts its effect through CB1, and not CB2.

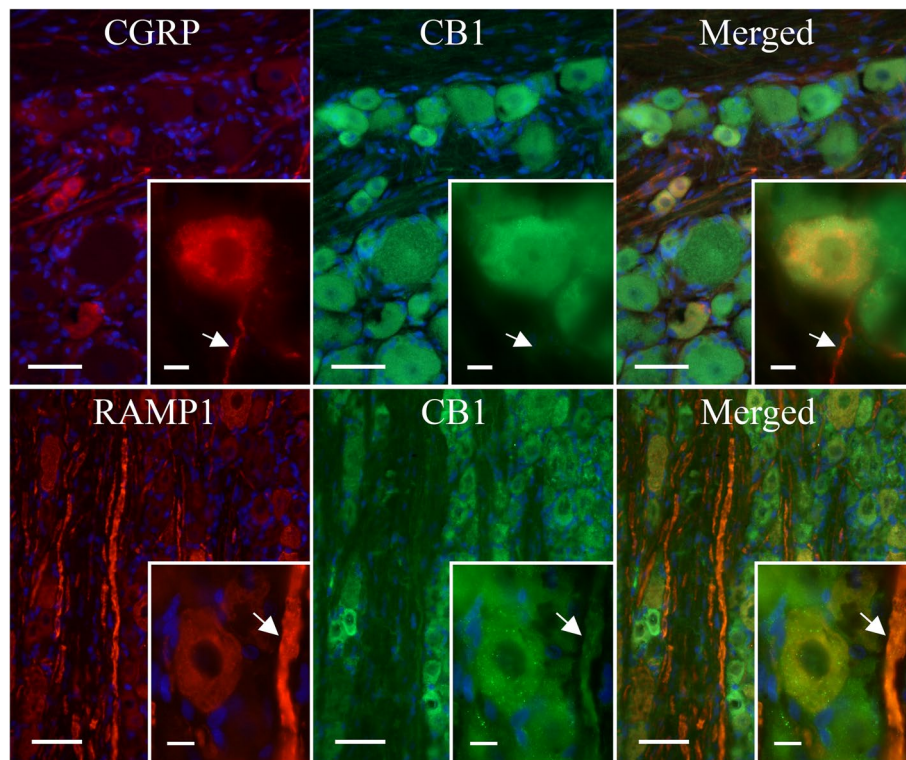
### Discussion

The data presented in the present study shows that the cannabinoid system in the TVS must be seen in light of TRPV1 crosstalk. CB1 activation is able to reduce CGRP release, but only when TRPV1 is blocked. This adds to the notion that receptors which couple negatively through cAMP have antimigraine potential, but off target effects could hide such responses.

IHC results from the TG revealed abundant expression of CB1 in neuronal somas, and to a lesser extent in C- and A $\delta$ -fibres (Fig. 4). In addition, IHC results showed



colocalization of CB1 with CGRP and RAMP1 (an indicator of CGRP receptor expression) in neuronal somas and to some extent in fibres of the TG, supporting the notion that CB1 may act through retrograde feedback inhibition of CGRP. We cannot rule out a possible interaction with neurons containing the Amylin 1 receptors, which has been detected in TG [38, 39]. Our findings are in agreement with those of Price et al. which demonstrate predominant colocalization of CB1 with markers



**Fig. 4** The expression of CB1, CGRP and RAMP1 in the TG. IHC localization of CB1, CGRP and the functional CGRP receptor (by staining for RAMP1) in the TG. Blue represents cell nuclei; orange represents colocalization of CGRP (upper panel) or RAMP1 (lower panel) with CB1; globular regions represent neuronal somas and fibrous regions represent individual neuronal fibres. Large image scale corresponds to 50  $\mu$ m, and the insert scale corresponds to 10  $\mu$ m. The arrows mark unmyelinated C-fibres (upper panel) and myelinated A $\delta$ -fibres (lower panel)

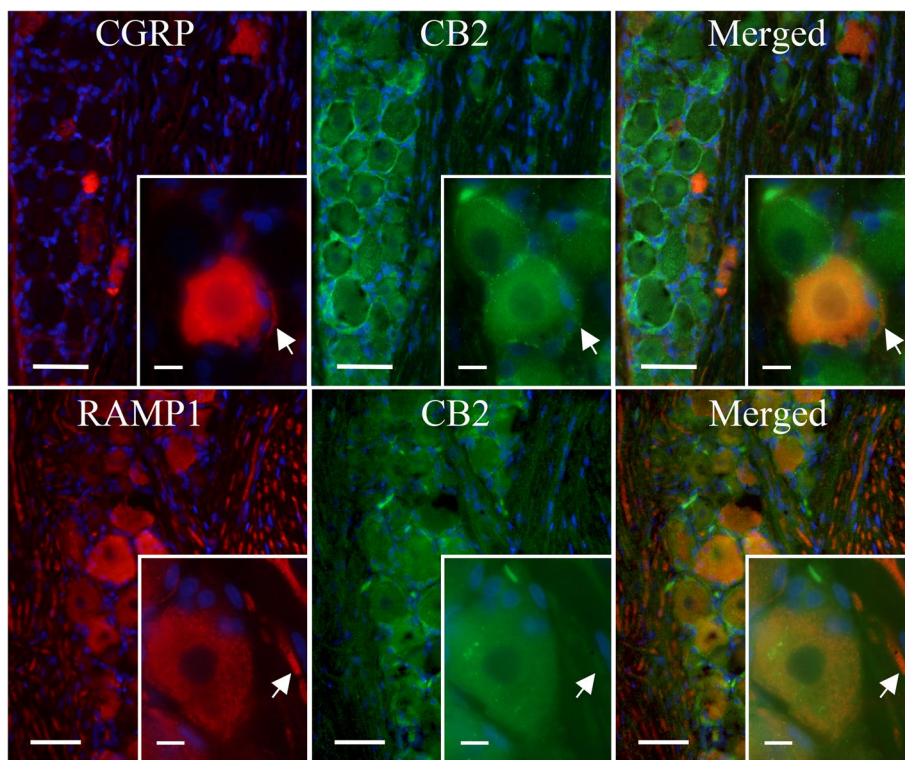
for myelinated large diameter fibres in the TG, and to a lesser extent with CGRP containing small diameter fibres [40]. In addition, Price et al. show that CB1 rarely colocalizes with TRPV1 in the TG [40], suggesting that CB1 and TRPV1 do not act in a concerted manner, but rather as part of two distinct systems both governing modulation of nociception and CGRP signalling.

Looking at expression data of the receptors, we re-analysed previously published data that was constructed using publicly available sequencing data generated by Hougaard Pedersen and collaborators [41]. RNA counts are depicted relative to 5-HT<sub>1B</sub>, since 5-HT<sub>1B</sub> agonists are the gold standard in migraine treatment and have shown to effectively inhibit cAMP production and CGRP release [17]. As can be seen in Supplementary Fig. 2 the average CB1 expression level is considerably higher than that of 5-HT<sub>1B/1D</sub>. CB2 expression only constitutes approximately 10% of 5-HT<sub>1B/1D</sub> expression. Furthermore, it was observed that TRPV1 is expressed almost double that of CB1 in the TG, roughly corresponding to an expression level 10 times that of 5-HT<sub>1B/1D</sub>.

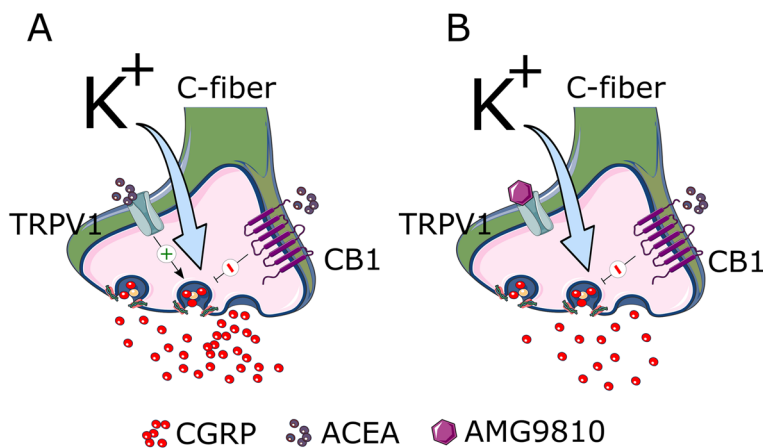
The initial application of ACEA demonstrated a significant increase in CGRP release from the dura, whereas

the response from the TG was non-significant. Although ACEA is a relatively specific agonist of CB1, it has been shown to activate TRPV1 [42]. When AMG9810, a blocker of TRPV1, was added (Fig. 3) there was no CGRP release caused by ACEA, suggesting that the effect was mediated by TRPV1 activation. This indicates that the slight tendency of ACEA to augment the capsaicin-induced response was caused by additional activation of TRPV1, and that the postulated ACEA-mediated CB1 activation did not constitute the main effect. The non-significant response observed in the TG upon ACEA application may be explained by the dual action of ACEA acting on G<sub>i/o</sub>-coupled CB1 receptors as well as on TRPV1 to inhibit and stimulate CGRP release, respectively, leading to a balanced response (Fig. 6).

When using K<sup>+</sup>-induced depolarization as stimulation, we hypothesized that CB1 activation and subsequent inhibition of CGRP release would be the predominant pathway. This was based on the earlier notion that anandamide is able to inhibit K<sup>+</sup> (and capsaicin)-induced CGRP release [43]. However, no significant inhibition was observed in this setting, although a tendency of inhibition was seen in the TG (Fig. 2). There is contrasting



**Fig. 5** The expression of CB2, CGRP and RAMP1 in the TG. IHC localization of CB2, CGRP and the functional CGRP receptor (by staining for RAMP1) in the TG. Blue represents cell nuclei; orange represents colocalization of CGRP (upper panel) or RAMP1 (lower panel) with CB2; globular regions represent neuronal somas and fibrous regions represent individual neuronal fibres. Large image scale corresponds to 50 mm, and the insert scale corresponds to 10 μm. The arrows mark unmyelinated C-fibres (upper panel) and myelinated Aδ-fibres (lower panel)



**Fig. 6** Graphical illustration of the interaction of CB1 and TRPV1 by cannabinoids. **A** ACEA activates both TRPV1 and CB1 in a native setting, leading to no observed inhibition. **B** when TRPV1 is inhibited, activation of CB1 leads to an attenuation of the CGRP release

evidence from previous studies of the involvement of CB1 in CGRP release, particularly in relation to TRPV1 activation. This leads to a discussion of the interaction between the cannabinoid and TRPV1 systems. Several

arachidonic acid metabolites, including anandamide, are viewed as putative endogenous TRPV1 ligands, termed endovanilloids [44]. Interestingly, many of the transient receptor potential (TRP) channels have been considered



“ionotropic cannabinoid receptors” [45], and anandamide has been shown to stimulate CGRP release through additional activation of TRPV1 [31]. Somewhat paradoxical, eCBs would therefore be able to: i) stimulate CGRP release through activation of TRPV1, and ii) inhibit CGRP release through activation of  $G_{i/o}$ -coupled CB1 receptors or by homologous and heterologous desensitization of TRPV1 and TRPA1, respectively [30, 31]. Indeed, Akerman and colleagues have suggested that in the TCC or TNC, anandamide inhibits neurons with C-fibre input through activation of CB1, an inhibition which is increased after blockade of TRPV1, [29] and Messlinger and colleagues have indicated opposing dual actions of eCBs on CGRP release in the dura [33]. This contrasts with the actions of anandamide in the periphery where the primary effect is postsynaptic inhibition of dural blood vessel dilatation, and only partial inhibition of CGRP release [28]. Current knowledge on how and which cannabinoids target TRP channels is still scarce [46]. It appears that ACEA adds to the eCB analogues that previously have been shown to activate TRPV1 [47], and in our setup can cause CGRP release.

In comparison to other systems, several neurons in the dorsal periaqueductal grey (dPAG) co-express CB1 and TRPV1, making it possible that an endogenous substance, i.e., anandamide, may exert both panicolytic and panicogenic effects via its actions at CB1 and TRPV1, respectively [48]. It has been postulated by Tognetto and colleagues that lower anandamide levels could act mainly on CB1, whereas TRPV1-mediated actions may prevail over CB1 at higher concentrations [49]. We are therefore not the first to suggest that if large amounts of endogenous anandamide could be produced, that could activate, rather than inhibit, nociceptive sensory neurons [49]. In support of this, it has previously been shown that anandamide application results in excitation of peripheral terminals of capsaicin-sensitive primary sensory neurons via cannabinoid receptor independent mechanisms, most likely TRPV1 activation, and causes CGRP release [50]. Further, Tognetto and colleagues showed that application of higher concentrations of anandamide to capsaicin-sensitive dorsal root ganglia (DRG) neurons leads to neuropeptide release [49]. It has long been known that the endogenous ligand anandamide behaves as an agonist of TRPV1 [51]. One major contrast between the cannabinoid- and capsaicin-evoked currents for TRPV1 is the lack of apparent pore dilation (a characteristic of a two state channel) in response to cannabinoids, even when large currents are driven by high doses of cannabinoids or long induction times [52]. However, the *in vivo* effects of this are not well understood. Interestingly, this cross activation of TRPV1 also seems to apply to the more CB1-specific ACEA, as we in the current study were not

able to find a window of pure inhibition *per se*. Thus, we conclude that cannabinoid signalling in the TG shows similarities to other systems and appears to be complexly regulated.

What effect does CB1 activation exert when TRPV1 is blocked? AMG9810, which is a potent and competitive TRPV1-selective antagonist [53], was administered prior to ACEA in order to determine whether the ACEA-induced increase in CGRP levels without stimulation was a result of TRPV1 activation. Unlike capsaizepine, which only inhibits the capsaicin-induced  $Ca^{2+}$  influx, AMG9810 inhibits capsaicin-, proton-, heat- and endogenous agonist-induced activation of TRPV1 [53], thus presenting a broader spectrum of inhibition. When TRPV1 was blocked, CB1 activation had inhibitory effects both in the dura and in the TG (Fig. 6). It has been well established in both human and rodent assays that  $G_{i/o}$ -coupled CB1 receptors are involved in retrograde suppression of neurotransmitter release [24, 25]. We therefore conclude that CB1 activation leading to cannabinoid-mediated inhibition of CGRP release only occurs when TRPV1 is blocked. This supports the findings by Akerman et al., in which anandamide inhibited A-fibre inputs to the TCC only in the presence of capsaizepine [29], as well as the findings by Fischer et al., showing the dual actions of eCBs on heat activated CGRP release [33], highlighting the dual agonistic properties of cannabinoids throughout the TVS and with various stimuli.

In the current context, a study by Summ et al. demonstrated that application of TRPV1 antagonist A-993610 did not *per se* inhibit C- and A $\delta$ -fibre activity in the TCC in response to electrical stimulation of the middle meningeal artery or after spreading depression [54]. Therefore it does not appear that TRPV1 antagonism in itself is a sufficient migraine target, but the data presented here show that it could potentially be combined with other drugs targeting the eCB system. Finding the right dose combination, which stimulates CB1 without activating TRPV1, would need more complex pharmacokinetics. Nevertheless, our study confirms and further support the hypotheses [10, 55] that receptors which lower the cAMP levels are potential pharmacological targets for treating migraine. Our study highlights the need to preclinically test for any off-target effects.

## Conclusion

In conclusion, application of ACEA alone significantly stimulated CGRP release from the dura, indicating that ACEA does not exhibit antimigraine potential, at least based on data from the current setup. Cannabinoid signalling pathways appear to be very complex with many factors, such as functional selectivity, agonist concentration and regional differences in protein distribution,

capable of modulating the response. We show that CB1 could have antimigraine potential when TRPV1 is blocked, but that the clinical applicability of this finding might be limited. Although the physiological activation of TRPV1 most likely is heat activation, we suggest that future studies should consider that TRPV1 channel activation could be due to a pathological response during overactive cannabinoid signalling.

#### Abbreviations

ADP: Adenosine Diphosphate; AC: Adenylate Cyclase; Anandamide: N-arachidonylethanolamine; ACEA: Arachidonyl-2'-chloroethylamide; CGRP: Calcitonin Gene-Related Peptide; CB1: Cannabinoid Receptor Type 1; CB2: Cannabinoid Receptor Type 2; cAMP: Cyclic Adenosine Monophosphate; CNS: Central Nervous System; dPAG: Dorsal Periaqueductal Gray; DRG: Dorsal Root Ganglia; eCB: Endocannabinoid; ECS: Endocannabinoid system; ELISA: Enzyme-Linked Immunosorbent Assay; GPCR: G-Protein Coupled Receptor; GTN: Nitro-glycerine; IHC: Immunohistochemistry; MMA: Middle Meningeal Artery; NO: Nitric Oxide; PBS: Phosphate Buffered Saline; PNS: Peripheral Nervous system; RAMP1: Receptor Activity Modifying Protein 1; RT: Room Temperature; SGC: Satellite Glial Cell; SIF: Synthetic Interstitial Fluid; SP: Substance P; TRP: Transient Receptor Potential; TRPA1: Transient Receptor Potential Ankyrin 1; TRPV1: Transient Receptor Potential Vanilloid 1; TG: Trigeminal Ganglion; TNC: Trigeminal Nucleus Caudalis; TCC: Trigemino-cervical Complex; TVS: Trigemino-vascular System; TRP: Transient Receptor Potential; 2-AG: 2-arachidonoylglycerol; 5-HT: 5-hydroxytryptamine.

#### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s10194-022-01399-8>.

**Additional file 1: Supplementary Fig. 1.** Negative control for CB1 and CB2. The negative control for the FITC antibody used for CB1 and CB2 IHC.

**Additional file 2: Supplementary Fig. 2.** mRNA expression in the trigeminal ganglion. The average RNA counts of 10 rats in the TG. RNA counts are depicted on a logarithmic scale relative to 5-HT<sub>1B</sub> and are shown for 5-HT<sub>1B</sub>, 5-HT<sub>1D</sub>, CB1, CB2, TRPV1, RAMP1 and CGRP.

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#### Authors' contributions

IMC and KAH designed the study; IMC, JCAE, PVR and KAH performed experiments; LE provided tools and reagents; IMC, JCAE, PVR, LE and KAH analysed the data; IMC and KAH wrote the manuscript; JCAE, PVR and LE made further critical manuscript revisions. All authors read and commented on the final manuscript. The authors read and approved the final manuscript.

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#### Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

#### Declarations

#### Ethics approval and consent to participate

The animal part of the study followed the guidelines of the European Communities Council (86/609/ECC) and was approved by the Regional Ethical

Committee on Animal Research, Malmö/Lund, Sweden (M43–07) and by an auspices of the Swedish Department of Agriculture (M8–09, M126–12) and the Danish Animal Experimentation Inspectorate. All animal experiments were therefore performed in accordance with the European Community Council Directive on 'The Protection of Animals Used for Scientific Purposes' (2010/63/EU).

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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