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# Neurotransmitter and tryptophan metabolite concentration changes in the complete Freund's adjuvant model of orofacial pain

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## Abstract

**Background:** The neurochemical background of the evolution of headache disorders, still remains partially undiscovered. Accordingly, our aim was to further explore the neurochemical profile of Complete Freund's adjuvant (CFA)-induced orofacial pain, involving finding the shift point regarding small molecule neurotransmitter concentrations changes vs. that of the previously characterized headache-related neuropeptides. The investigated neurotransmitters consisted of glutamate,  $\gamma$ -aminobutyric acid, noradrenalin and serotonin. Furthermore, in light of its influence on glutamatergic neurotransmission, we measured the level of kynurenic acid (KYNA) and its precursors in the kynurenine (KYN) pathway (KP) of tryptophan metabolism.

**Methods:** The effect of CFA was evaluated in male Sprague Dawley rats. Animals were injected with CFA (1 mg/ml, 50  $\mu$ l/animal) into the right whisker pad. We applied high-performance liquid chromatography to determine the concentrations of the above-mentioned compounds from the trigeminal nucleus caudalis (TNC) and somatosensory cortex (ssCX) of rats. Furthermore, we measured some of these metabolites from the cerebrospinal fluid and plasma as well. Afterwards, we carried out permutation t-tests as post hoc analysis for pairwise comparison.

**Results:** Our results demonstrated that 24 h after CFA treatment, the level of glutamate, KYNA and that of its precursor, KYN was still elevated in the TNC, all diminishing by 48 h. In the ssCX, significant concentration increases of KYNA and serotonin were found.

**Conclusion:** This is the first study assessing neurotransmitter changes in the TNC and ssCX following CFA treatment, confirming the dominant role of glutamate in early pain processing and a compensatory elevation of KYNA with anti-glutamatergic properties. Furthermore, the current findings draw attention to the limited time interval where medications can target the glutamatergic pathways.

**Keywords:** Migraine, CFA model, Orofacial pain, Glutamate, Kynurenic acid

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## Background

Although the pathomechanism of orofacial pain and headache disorders, is not fully understood [1], the activation and sensitization of the trigeminovascular system (TS) probably takes part in the evolution of symptoms [2–4]. The pathomechanism of these disorders may be further investigated by using animal models with the activation of nociceptive pathways of the TS [1, 3, 5]. The administration of inflammation-inducing substances to the orofacial area can evoke the above-described activation/sensitization of the primary and secondary trigeminal neurons during pain processing [6, 7]. For the induction of this peripheral inflammation, the application of Complete Freund's adjuvant (CFA) into the whisker pad or the dural parietal surface is a widely used method [6, 8, 9] as it is able to enhance local reaction at the injection site and then to evoke the release of inflammatory cytokines, alongside with hyperalgesia/allodynia on the face via the activation/sensitization of the TS [7]. Regarding the delay of the development of peripheral and central sensitization, indirect data from studies with CFA injection to the paw demonstrated that pain hypersensitivities were observed 24 h after the injection [9–13], whereas data from studies with orofacial CFA model, more precisely from the temporomandibular joint induced inflammation model, suggest that both thermal and mechanical allodynia peak at 24 h as well [14]. The orofacial CFA model has been thoroughly studied regarding gene expression characteristics [6, 15–20]. Recently, in relation to two migraine-related biomarkers, the pituitary adenylate cyclase-activating peptide (PACAP) and calcitonin gene-related peptide (CGRP), their increasing levels were detected starting even 24 h after the administration of CFA in the trigeminal nucleus caudalis (TNC) [9]. However, there are no studies which aimed at the investigation of the small molecule neurotransmitters and neuromodulators and some of their precursors (glutamate (Glu),  $\gamma$ -aminobutyric acid (GABA), serotonin (5-hydroxy-tryptamine; 5-HT), noradrenaline (NA), tryptophan (TRP), kynurenine (KYN), kynurenic acid (KYNA)) in this model with established or presumed role in the development of peripheral and central sensitization during headache. Therefore, there are no data about how the concentration changes of these substances affect the evolution of peripheral and central sensitization. Accordingly, finding the transition point where the dominance of small molecule mediated neurotransmission shifts to that of the PACAP and CGRP mentioned earlier may have significant therapeutic consequences in view of the different targeted approaches.

The primary excitatory neurotransmitter Glu plays an important role in the primary sensory neurotransmission and trigeminal nociception [15, 21, 22].

Accordingly, the alteration of Glu levels in migraine has been widely studied and data consistently show elevated Glu levels in the CSF samples of patients with chronic migraine [23], or migraine with and without aura [24], whereas in plasma samples, the results were not consistent across studies [25–27]. Moreover, similar importance has to be attributed to the changes of the concentration of GABA, the main inhibitory neurotransmitter of the central nervous system (CNS), which is capable of modulating the excitatory pathways [28]. Recently, mainly in light of its influence on glutamatergic neurotransmission, special attention was dedicated to the investigation of the effect of KYNA, a compound of the KYN pathway (KP) of the TRP metabolism [29–34]. KYNA can influence glutamatergic neurotransmission in a complex way [35], i.e., it acts as a competitive antagonist at the N-methyl-D-aspartate (NMDA) receptor [36] and has weak antagonistic effects at the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors as well [37]. 5-HT, another well-known TRP metabolite, released from serotonergic neurons of the raphe nuclei, exerts modulating effect on TS activation [38–40]. Noradrenaline (NA) may be of interest as well, as noradrenergic neurons project to TNC and may have a role in cluster headache, another primary headache disorder [41, 42].

Based on the observed gradually increasing levels of PACAP and CGRP from 24 h following CFA injection in our previous experiment [9], the aim of the current study was to find the shift point of concentration changes of small molecule neurotransmitters and neuromodulators and the above-mentioned peptides. This may yield substantial information for the selection between different therapeutic paradigms regarding diseases involving the activation of the TS, such as primary headache disorders, including migraine.

## Materials and methods

### Animal experiments and sample collection

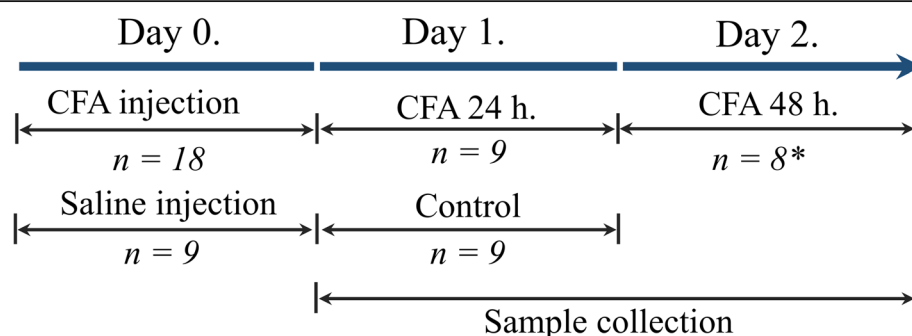
Twenty-seven young adult (10–12 weeks old, 250–300 g) male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA), were used for the experiments. The animals were bred and maintained under standard laboratory conditions with 12 h–12 h light/dark cycle at  $24 \pm 1$  °C and 50% relative humidity, 3 animals per each home cage in the Laboratory Animal House of the Department of Neurology, University of Szeged. The rats had free access to standard rat chow and water. The experiment was not pre-registered. All experimental procedures performed in this study complied fully with the guidelines of Act 1998/XXVIII of the Hungarian Parliament on Animal Experiments (243/1988) and with the recommendations of the International Association for

the Study of Pain and European Communities Council (86/609/ECC). The studies were in harmony with the Ethical Codex of Animal Experiments and were approved by the Ethics Committee of the Faculty of Medicine, University of Szeged, with a permission number of XI./1102/2018. CFA (killed mycobacteria suspended in paraffin oil, 1 mg/ml) was obtained from Sigma-Aldrich (product number: F5881; St. Louis, MO, USA), and 50  $\mu$ l was administered per animal. We tried to minimize the use of animals by adopting the key aspects of the 3Rs (Replacement, Reduction and Refinement) [43]. Therefore, the experimental groups were added in a sequential manner, starting from 24 h following CFA administration with 24 h steps till the time point where the proposed alterations diminish. Therefore, no randomization was performed to allocate subjects in the study. By the end of the experiments we had three groups, one control (CO) and two with CFA treatment (Fig. 1). Similar to the previous experiment on PACAP and CGRP in the same model [9], only sham-injected rats processed 24 h following the injection were used as CO, as a pilot study conducted on naïve and sham-injected (processed 24 and 48 h following injection) rats demonstrated that there is no difference in the level of the metabolites of interest, in neither TNC, nor ssCX ( $n = 3$  in each group, data not shown). The rats were anesthetized with intraperitoneal 4% chloral hydrate solution mainly based on its safe application (CAS ID: 302-17-0, Sigma-Aldrich, St. Louis, MO, USA; 10 ml/kg body weight dose) in the morning and 50  $\mu$ l of CFA was injected into the right whisker pad. No other analgesic was applied, otherwise the activation/sensitization phenomena during pain processing, an essential characteristic of the CFA model as well, would have been influenced. Control rats were injected with an equal volume of saline. Cerebrospinal fluid (CSF) was taken from the suboccipital cistern, including the control group ( $n = 9$ ), 24 ( $n = 9$ ) and 48 h ( $n = 9$  initially, finally  $n = 8$  as one animal died during the experiment) after injection applying the above-described anesthetic procedure, and

following that the animals were perfused transcardially with 200 ml phosphate-buffered saline (PBS). The spinal tap procedures were unsuccessful in 5 occasions and 7 of the CSF samples were excluded from analysis due to contamination with blood. Accordingly, 5–5 samples remained in the CO and CFA 24 h groups, and 4 in the CFA 48 h group for analysis. Therefore, this part of the study focusing at that secondary endpoint was only exploratory due to the low statistical power. Also as a secondary endpoint, blood samples were taken from the left ventricle into ice-cold glass tubes containing disodium ethylenediaminetetraacetate dihydrate ( $\text{Na}_2\text{EDTA}$ ; CAS ID: 194491-31-1 Lach-Ner s.r.o, Neratovice, Czech Republic) and the plasma was separated by centrifugation (1170 g for 10 min at 4 °C). Following decapitation two different brain structures, the TNC and the somatosensory cortex (ssCX) were dissected for the assessment of the targeted primary endpoints. In each case both right and left sided samples were separately removed on ice and stored at  $-80^\circ\text{C}$  until further use.

#### Instruments and chromatographic conditions

Validated high performance liquid chromatography (HPLC) measurements were performed by an Agilent 1100 HPLC system (Santa Clara, CA, USA), coupled with UV detector (UVD), fluorescence detector (FLD) and electrochemical detector (ECD). The chromatographic separations were carried out with validated methods comprehensively described elsewhere [44–46]. Prior to all measurements, during the tissue weighting or plasma/CSF precipitation process, all samples were relabeled, and a blind study was conducted, i.e., the experimenter who did the HPLC measurements was not aware of which samples were part of CO or 24 h groups. Moreover, Eppendorf tubes were randomly assigned for measurements and when the 48 h group was measured, the same systematic randomization was applied. The purity of all standards and solutions were analytical grade or HPLC grade and they were acquired from Sigma-Aldrich, St. Louis, MO, USA, except the



**Fig. 1** Time-line of the experimental procedure applied in this study. CFA Complete Freund's adjuvant.  $n$  number of the animals per group. \*One animal died in cage after CFA injection

fluorescent internal standard used for the TRP method which was synthesized at the Department of Pharmaceutical Chemistry, University of Szeged, as detailed elsewhere [44]. Briefly, the brain regions were homogenized in 0.5 M perchloric acid (PCA), at 1:5 w/v containing internal standards (ISs, 3-nitro-L-tyrosine and 4-hydroxyquinazoline-2-carboxylic acid, the latter custom-made material will be shared upon reasonable request) applied in the measurement of TRP metabolites [44] utilizing both UVD and FLD. After centrifugation the supernatant was collected and first used for the TRP metabolite measurement. The remaining supernatant was aliquoted in two further parts and were kept at  $-80^{\circ}\text{C}$  until further analyses. 150  $\mu\text{l}$  from it was applied for the determination of NA concentration by ECD [45], with addition of 10  $\mu\text{l}$  solution of the corresponding IS, 2,3-dihydroxybenzoic acid. For the measurement of Glu and GABA, another 100  $\mu\text{l}$  was diluted to 1:100 v/v with distilled water and 100  $\mu\text{l}$  of this dilution was derivatized with 100  $\mu\text{l}$  solution containing o-phthalaldehyde and 3-mercaptopropionic acid in borate buffer and further diluted with 50  $\mu\text{l}$  distilled water containing the corresponding IS, homoserine, used for this method applying FLD [46].

For the measurement of the TRP metabolites from the CSF, the method described before [44] was applied, with a slight modification. Briefly, during sample preparation, we used a dilution of 5:6 v/v, with the final concentration of PCA at 0.5 M, with the above described ISs, but only 35  $\mu\text{L}$  of the sample was injected. Furthermore, a

linearity study was conducted for rat CSF samples to determine limit of detection (LOD) and limit of quantitation (LOQ) values, because the cited article contains data only for human CSF. Accordingly, the LOD and LOQ values for rat CSF were 31.1 and 102 nM for TRP, 107 and 702 nM for KYN and 1.04 and 3.45 nM for KYNA, respectively, whereas 5-HT was undetectable in each case. Regarding Glu and GABA, the initial amount of mobile phase A applied for the brain samples was 95%, but for CSF samples it was changed to 93%, as co-elution was observed under the initial circumstances. The ratios applied for the CSF sample preparation (1:1:0.5 = sample: derivatization solution: IS) remained the same, similar to brain supernatants [46]. Due to low sample amount we omitted the determination of NA levels from CSF.

With regard to plasma samples we measured the levels of TRP metabolites as described in [44]. Glu, GABA and NA concentrations from plasma samples were not assessed because we were only interested in their role as a neurotransmitter.

As for the plasma samples, the LOD and LOQ values were 0.102  $\mu\text{M}$  and 0.308  $\mu\text{M}$  for TRP, 0.027 and 0.083  $\mu\text{M}$  for KYN and 1.23 and 3.72 nM for KYNA, respectively. In each case, the 5-HT levels from plasma samples were undetectable.

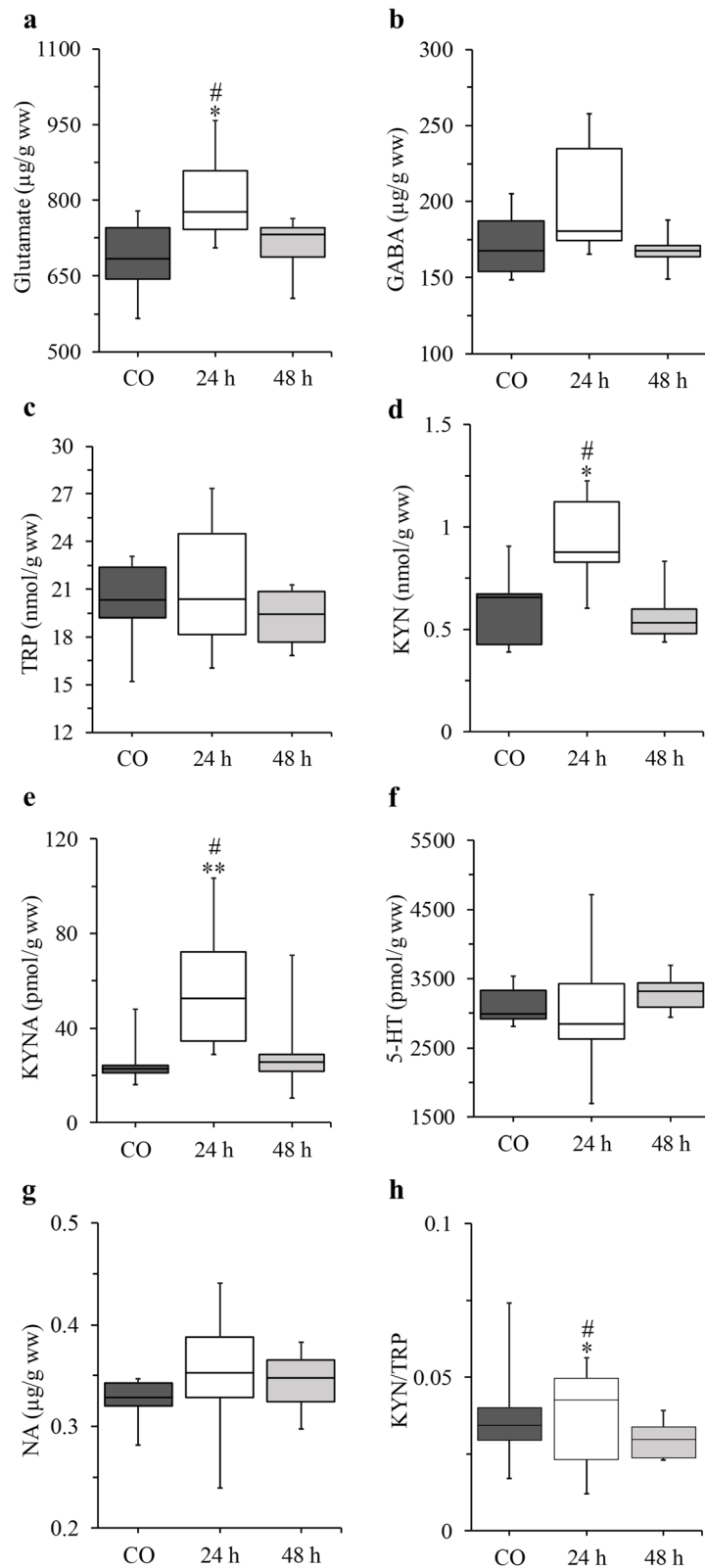
### Statistical analyses

All statistical calculations were performed with the use of the freely available R software 3.5.3 (R Development

**Table 1** Concentration levels of the measured metabolites in the analyzed brain regions

	Control group (n = 9)	CFA 24 h (n = 9)	CFA 48 h (n = 8 <sup>†</sup> )
Trigeminal nucleus caudalis (TNC)			
Glu ( $\mu\text{g/g ww}$ )	684 (644–746)	772 <sup>*,#</sup> (742–859)	731 (687–745)
GABA ( $\mu\text{g/g ww}$ )	167 (154–187)	180 (174–235)	167 (164–171)
TRP (nmol/g ww)	20.3 (19.2–22.4)	20.3 (18.2–24.5)	19.4 (17.7–20.8)
KYN (nmol/g ww)	0.656 (0.428–0.671)	0.876 <sup>*,#</sup> (0.830–1.13)	0.532 (0.480–0.597)
KYNA (pmol/g ww)	22.8 (21.2–24.2)	52.6 <sup>**,#</sup> (34.6–72.3)	25.8 (21.9–28.8)
5-HT (pmol/g ww)	2991 (2917–3333)	2841 (2629–3425)	3315 (3088–3438)
NA ( $\mu\text{g/g ww}$ )	0.328 (0.320–0.343)	0.352 (0.328–0.388)	0.348 (0.324–0.366)
Somatosensory cortex (ssCX)			
Glu ( $\mu\text{g/g ww}$ )	1178 (1082–1290)	1269 (1206–1397)	1152 (1052–1287)
GABA ( $\mu\text{g/g ww}$ )	215 (207–218)	230 (217–251)	199 (178–211)
TRP (nmol/g ww)	20.6 (17.8–23.5)	22.6 (21.5–23.7)	21.6 (20.9–22.7)
KYN (nmol/g ww)	0.824 (0.743–0.970)	0.974 (0.714–1.15)	0.616 (0.552–0.663)
KYNA (pmol/g ww)	16.2 (9.70–18.8)	27.3 <sup>*,#</sup> (17.3–39.3)	9.73 (7.01–12.8)
5-HT (pmol/g ww)	2547 (1665–2677)	2271 <sup>#</sup> (2166–2527)	2885 (2653–3172)
NA ( $\mu\text{g/g ww}$ )	0.840 (0.192–0.853)	0.754 (0.142–0.934)	0.886 (0.556–0.974)

Results are shown as median (1st–3rd quartile). <sup>†</sup>One animal died in cage after CFA injection. \*  $p < 0.05$  vs. CO, \*\*  $p < 0.01$  vs. CO, #  $p < 0.05$  vs. 48 h, 5-HT serotonin, CFA Complete Freund's adjuvant, GABA gamma-aminobutyric acid, Glu glutamate, KYN kynurenine, KYNA kynurenic acid, n number of the animals per group, NA noradrenaline, TRP tryptophan, ww wet weight



**Fig. 2** (See legend on next page.)

(See figure on previous page.)

**Fig. 2** Concentration changes in glutamate (a),  $\gamma$ -aminobutyric acid (b), tryptophan (c), kynurenine (d), kynurenic acid (e), serotonin (f), noradrenaline (g) and changes in kynurenine/tryptophan ratio (h) in the TNC. \*  $p < 0.05$  vs. CO, \*\*  $p < 0.01$  vs. CO, #  $p < 0.05$  vs. 48 h.  $n = 9$  in the control and 24 h groups and  $n = 8$  in the 48 h group. The boxplots are displayed as the intervals between the 1st and 3rd quartiles presenting the median values as well. 24 and 48 h CFA treated groups, 5-HT serotonin, CO control, GABA  $\gamma$ -aminobutyric acid, KYN kynurenine, KYNA kynurenic acid,  $n$  number of the animals per group, NA noradrenaline, TRP tryptophan, TNC trigeminal nucleus caudalis,  $ww$  wet weight

Core Team). The distribution of our data population was not determined as the applied statistical tests do not need assumptions regarding the distribution of underlying data. Accordingly, first we performed the Levene test to assess the homogeneity of variances. As the variances were equal, we performed a general independence test for two sets of variables measured on arbitrary scales, where the reference distribution was approximate based on the Monte-Carlo method. Afterwards, we carried out permutation t-tests as post hoc analysis for pairwise comparison. Permutations were applied via the Monte-Carlo method (10,000 random permutations) and Type I errors from multiple comparisons were controlled with false discovery rate. No test for outliers was conducted. With the key aspects of 3Rs in mind [43] we tried to keep the sample size as low as we can based on experiences from previous experiments ([47]: 8 and 12/group; [48]: 6/group; [49]: 6/group; [50]: 6/group; [51]: 8/group; [52]: 6 and 7/group; [53]: 6/group). For every statistically significant result, we calculated the corresponding effect size (Cohen's  $d$  in this case) and based on its value, we decided whether the increase of sample size is necessary or not. The manuscript contains the final effect sizes.

## Results

### Concentration levels of the assessed compounds in the TNC and ssCX

First of all, both contralateral and ipsilateral CNS regions were measured separately, but we did not find significant differences in concentrations of any of the metabolites between the two sides, so the coherent data were pooled for further analysis. Therefore, the concentration values presented in Table 1 demonstrate the mean values of the two analyzed sides of each CNS regions.

Regarding TNC, pairwise permutation t-tests following the independence tests revealed a significant elevation in the concentration of Glu ( $p = 0.0319$ , Cohen's  $d = 1.49$ ), KYN ( $p = 0.0123$ , Cohen's  $d = 1.58$ ) and KYNA ( $p = 0.0098$ , Cohen's  $d = 1.92$ ) 24 h following CFA injection compared to the controls and a significant decrease could be observed in Glu ( $p = 0.0357$ , Cohen's  $d = 1.29$ ), KYN ( $p = 0.0123$ , Cohen's  $d = 1.85$ ) and KYNA ( $p = 0.0263$ , Cohen's  $d = 1.39$ ) levels by 48 h compared to the 24 h group, whereas there was no difference between the control and 48 h groups (Table 1, Fig. 2).

Regarding ssCX samples, an elevation in KYNA concentration ( $p = 0.0237$ , Cohen's  $d = 1.36$ ) could be observed 24 h following CFA administration, followed by a significant decrease by 48 h ( $p = 0.0173$ , Cohen's  $d = 1.80$ ) and there was no difference between control and 48 h groups. Furthermore, in the ssCX, there was a significant increase in 5-HT levels in the 48 h group compared to the controls ( $p = 0.0479$ , Cohen's  $d = 1.21$ ) and to the 24 h group ( $p = 0.0479$ , Cohen's  $d = 1.20$ ; Table 1, Fig. 3).

We calculated the KYN/TRP and KYNA/KYN ratios as well. The KYN/TRP ratio was significantly elevated in the 24 h group compared to the controls ( $p = 0.0419$ , Cohen's  $d = 1.19$ ) or to the 48 h group ( $p = 0.0419$ , Cohen's  $d = 1.35$ ; Table 1, Fig. 2). With regard to the KYNA/KYN ratio, there was no difference in any of the investigated biological matrices (data not shown).

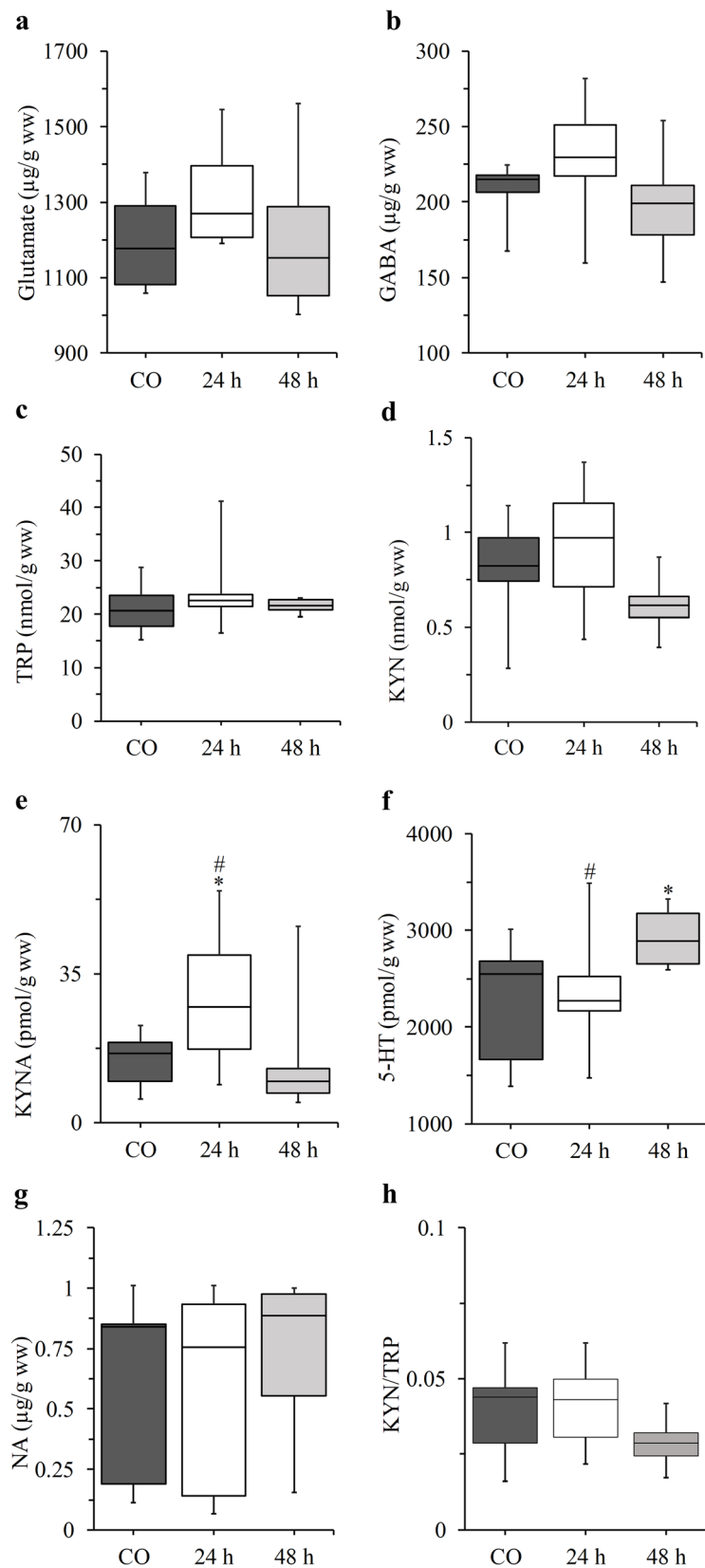
### CSF and plasma samples

Regarding CSF samples, TRP metabolites, Glu and GABA were measured. We found no significant alterations in the CSF, however, the power of the statistical tests in this case is low due to low case number ( $n = 5$ , 5, 4 for control, 24 h and 48 h groups, respectively) and the concentration values of KYN in the control and CFA treated 48 h groups were below LOD ( $0.107 \mu\text{M}$ ), except one case from each group (for more details, see Additional file 1, Table S1; due to the low amount of 5-HT in the CSF samples, we could not quantify it, as the values were below LOD,  $\text{LOD} = 0.0274 \mu\text{M}$ ). In case of plasma samples, only the TRP metabolites were measured, and no significant differences were observed (for more details, see Additional file 2, Table S2).

## Discussion

Headache is one of the most common neurological disorders and it is one of the leading causes of health-related problems worldwide. In 2010, tension type headache and migraine were the second and third most prevalent conditions in the world, respectively, according to the Global Burden of Disease (GBD) study [54, 55]. Furthermore, the GBD study in 2015 established that headache is responsible [56] for more disability adjusted life years than all other neurological disorders in combination.

The treatment of primary headache disorders is challenging, requiring both acute and preventive therapeutic



**Fig. 3** (See legend on next page.)

(See figure on previous page.)

**Fig. 3** Concentration changes in glutamate (a),  $\gamma$ -aminobutyric acid (b), tryptophan (c), kynurenine (d), kynurenic acid (e), serotonin (f), noradrenaline (g) and changes in kynurenine/tryptophan ratio (h) in the somatosensory cortex. \*  $p < 0.05$  vs. CO, #  $p < 0.05$  vs. 48 h.  $n = 9$  in the control and 24 h groups and  $n = 8$  in the 48 h group. The boxplots are displayed as the intervals between the 1st and 3rd quartiles presenting the median values as well. 24 and 48 h CFA treated groups, 5-HT serotonin, CO control, GABA  $\gamma$ -aminobutyric acid, KYN kynurenine, KYNA kynurenic acid,  $n$  number of the animals per group, NA noradrenaline, TRP tryptophan, TNC trigeminal nucleus caudalis,  $ww$  wet weight

measures [57, 58]. The preventive treatment aims to reduce the frequency, severity and duration of headaches, and to avoid medication-overuse headache. The efficacy of the currently applied drugs is not always satisfactory and the contraindications and side-effects often limit the options of the physician [59, 60]. Therefore, there is a constant need to study and develop new molecules.

### Glutamate and pain

Peripheral and central sensitization manifest mainly in forms of hyperalgesia and allodynia. The activation of the peripheral terminals of the nociceptors is responsible for Glu release at central sites with the activation of ionotropic and metabotropic Glu receptors [61]. This process was demonstrated not only in preclinical studies [62–64], but in patients with headache as well [23, 24]. Accordingly, the role of glutamatergic pathways in association with different types of pain is well established [65] and several antagonists of ionotropic glutamate receptors were investigated and found to be effective to decrease nociceptive transmission [66]. However, they had severe side effects, and therefore, the interest in this direction of research diminished [67, 68]. Nevertheless, ketamine, an NMDA receptor antagonist, is so far the only promising option in the treatment of severe or long-lasting migraine aura [69], and tezampanel, which acts on the AMPA and kainate subtypes of ionotropic Glu receptors [70], has also shown promising results in acute migraine therapy [71].

### Tryptophan metabolism and pain

It has been already demonstrated that the level of KYNA and some other KP metabolites are altered in migraine and cluster headache patients as well: there are significant reductions in the serum levels of KYN, KYNA, 3-hydroxy-kynurenine, 3-hydroxy-anthranilic acid and quinolinic acid, whereas concentrations of TRP and anthranilic acid were significantly increased [72, 73]. KYNA as an endogenous NMDA receptor antagonist, is a molecule of interest for CNS drug development in case of several neurological conditions [74], but due to its poor ability to cross the blood-brain barrier (BBB) and its rapid clearance from the body [75], its application for most CNS-related alterations is limited, and therefore several KYNA analogs were synthesized [76–79]. However, the first order neuron of pain

processing is located outside the BBB [80], so KYNA itself may have therapeutic potential as well. Accordingly, the antinociceptive properties of KYNA were proved in animal models of pain [29, 81]. Furthermore, some of the developed analogs also displayed promising results in different animal models of headache [31, 82–85]. In an earlier study we investigated two KYNA analogs where both of them proved to be effective in the formalin model of trigeminal pain [84]. However, one of them was more effective than the other and according to our analyses the better performing compound caused a more pronounced elevation of KYNA concentration on the periphery, whereas in the CNS the concentrations of KYNA were similar. Based on these results we hypothesized that the peripheral elevation of KYNA may be enough to exert beneficial effects on pain processing and targeting this component could provide an option to pharmaceutical drug design without the obligation of good penetration through the BBB.

Elevated Glu concentration in the TNC of CFA-treated rats, demonstrated by the current study, is accompanied by increased KYN and KYNA levels, which may serve as a feedback mechanism to the sensitization process caused by Glu. This hypothesis is supported by the above-mentioned findings [72, 73] that decreased KP metabolite levels are associated with those headache disorders, where increased NMDA receptor activation may play a crucial role. These results may have a great importance especially in light of the finding that the slightly, but not significantly elevated GABA level may not be enough to counterbalance the effects of increased Glu levels. With regard to 5-HT, its cortical elevation by 48 h may serve as a feedback inhibitory response as well to ameliorate the activation of the trigeminovascular pathway [86].

The current study draws attention to the limited time interval for therapies targeting glutamatergic pathways as well, as based on our previous experiments, a clear shift to dominantly peptide-mediated pain processing can be seen even from 24 h after CFA application [9]. This time point corresponds to the onset of peripheral and central sensitization of the TS as well in this model [10, 11, 14]. At this stage, mainly novel antibody-based therapies may come into account [87–90]. With regard to these novel therapies, the focus of attention is on



monoclonal antibodies targeting the CGRP pathway for the prophylactic treatment of migraine. Currently, four of these antibodies are in clinical trials (eptinezumab, galcanezumab, fremanezumab, erenumab) with promising results. However, the cost of these therapies is considerably higher than that of acute phase treatments.

## Conclusion

This is the first study assessing small molecule neurotransmitter changes in the TNC and ssCX following CFA treatment, confirming a dominant role of glutamate in early pain processing and a compensatory elevation of KYNA with anti-glutamatergic properties. The time interval for the intervention targeting the glutamatergic system is presumed to be limited to the first 24 h. The results of our previous therapeutic studies with KYNA or with its analogs strongly support this theory.

## Supplementary information

**Supplementary information** accompanies this paper at <https://doi.org/10.1186/s10194-020-01105-6>.

**Additional file 1: Table S1.** Concentration levels of the measured metabolites in the cerebrospinal fluid.

**Additional file 2: Table S2.** Concentration levels of the measured metabolites in the plasma samples.

## Abbreviations

5-HT: 5-hydroxy tryptamine (serotonin); AMPA:  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CFA: Complete Freund's adjuvant; CNS: Central nervous system; CGRP: Calcitonin gene-related peptide; CO: Control group; CSF: Cerebrospinal fluid; ECD: Electrochemical detector; FLD: Fluorescence detector; ssCX: Somatosensory cortex; GABA:  $\gamma$ -aminobutyric acid; GBD: Global Burden of Disease; Glu: Glutamate; HPLC: High performance liquid chromatography; IS: Internal standard; KYN: Kynurenine; KYNA: Kynurenic acid; LOD: Limit of detection; LOQ: Limit of quantitation; NA: Noradrenaline; Na<sub>2</sub>EDTA: Disodium ethylenediaminetetraacetic acid; NMDA: N-methyl-D-aspartate; PACAP: Pituitary adenylate cyclase-activating peptide; PCA: Perchloric acid; TNC: Trigeminal nucleus caudalis; TRP: Tryptophan; TS: Trigeminovascular system; TTH: Tension-type headache; UVD: Ultraviolet detector

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## Authors' contributions

EKCS, GV and NN designed the study, performed the HPLC experiments and analyzed the data and wrote the manuscript. TK, HP performed the animal experiments. As a corresponding author, ZD designed the study, wrote the manuscript and had final responsibility for the decision to submit for publication. JT, AP, KP, LV made further critical manuscript revisions. All authors read and commented on the final manuscript, furthermore approved the final version to be published.

## Authors' information

Edina K Cseh and Gábor Veres contributed equally to this work.

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## Availability of data and materials

The authors made available all of their data and materials on request.

## Ethics approval and consent to participate

Our experiments complied fully with the guidelines of Act 1998/XXVIII of the Hungarian Parliament on Animal Experiments (243/1988) and with the recommendations of the International Association for the Study of Pain and European Communities Council (86/609/ECC). The studies were in harmony with the Ethical Codex of Animal Experiments and were approved by the Ethics Committee of the Faculty of Medicine, University of Szeged, with a permission number of XI/1102/2018.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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