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# Altered aminoacid and lipid metabolism in a rat orofacial inflammation model determined by omics approach: potential role in trigeminal sensitisation

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## Abstract

**Background** Trigeminal activation and sensitisation involved in chronic inflammatory orofacial pain share several similarities with headaches, including migraine. Therefore, understanding the pathophysiological mechanisms is important to determine novel therapies, in which animal models are crucial. Here we aimed to identify key mediators, mechanisms and networks using unbiased multi-omic approaches in a rat orofacial inflammatory pain model.

**Methods** Complete Freund's Adjuvant (CFA, 50 µl, 1 mg/mL) was injected into the right whisker pad of male Wistar rats ( $n = 5-11$ /group), mechanonociceptive threshold was measured by von Frey filaments. Plasma concentrations of metabolites were measured both by targeted (MxP Quant 500 kit) and untargeted mass spectrometry methods on day 3 when maximal facial allodynia developed. Next-generation sequencing of the trigeminal ganglia (TG) was performed, furthermore, transcriptomic and plasma metabolomic data were analysed together.

**Results** Plasma carnosine, serotonin and fatty acid levels significantly increased, while tryptophan, kynurenine, tyrosine, phenylalanine, asparagine, glycerolipids, and sphingolipids decreased in response to orofacial inflammation. CFA upregulated the Cxcr3 chemokine receptor and downregulated GNRHR in the TG. Bioinformatic analysis revealed altered amino acid metabolism and fatty acid beta-oxidation involved in mitochondrial energy production, neuroinflammation and immune responses.

**Conclusions** Integrated joint pathway analysis of metabolomic and transcriptomic data provides a useful approach to determine pathophysiological mechanisms of trigeminal sensitization and identify novel drug targets for orofacial pain and headaches.

**Keywords** Orofacial inflammation, Migraine, Metabolomics, Transcriptomics, Bioinformatics, Pathway analysis

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## Introduction

Orofacial pain (OFP) and headache represent complex disorders characterized by recurring and intense pain, often without effective therapeutic interventions. Their aetiologies and pathophysiological mechanisms involve genetic, environmental, and neurobiological factors. Besides some differences, they share several common features, which are also reflected in the clinical manifestations. Migraine and other headaches are more common in OFP patients [1]. Pathological activation, sensitisation and altered function of the trigeminal system are key elements of pain in all these conditions. Peripheral and later central sensitisation results in a lower pain threshold and enhanced response to normally innocuous stimuli called allodynia in the inflamed area [2–5]. Similar pain sensitivity changes occur in various temporomandibular joint disorders, dental inflammation, different neuropathies and migraine [3, 6–8]. Understanding the diverse neurovascular-immune-endocrine interactions is crucial to develop targeted therapeutic approaches addressing the underlying causes for personalised treatment of these greatly unmet medical need conditions [5, 9].

Animal models are important for revealing pathophysiological mechanisms and pathways to identify and validate novel drug targets. However, most models have several limitations due to the complexity of OFP and primary headache processes [5, 10, 11]. Complete Freund's Adjuvant (CFA) injection into the rodent whisker pad represents a frequently used, reliable and highly reproducible inflammatory OFP model [10, 12] supported by our experience [13, 14] and others [15–18]. We earlier characterised this model by transcriptomic analysis of peripheral blood mononuclear cells and identified neuroinflammatory and mitochondrial dysfunction mechanisms [14].

Similar to transcriptomics, metabolomics is rapidly evolving in biomedical research, revealing important pathophysiological pathways and processes to aid diagnosis, prognosis and drug development [19, 20]. Metabolites are small intermediate and end-product molecules of cellular processes, providing a snapshot of the homeostatic, physiological, and also pathophysiological states [19–21]. Several recent papers have discussed metabolomic changes in chronic pain [22, 23], including migraine [24–27]. Despite the obvious advantages of investigating human samples, limitations are due to patients' heterogeneity, difficulties in sampling and interventions. Using animals kept under controlled environments, lifestyle and diet, substantially reduces the heterogeneity and confounding factors, and the results of the peripheral blood can be compared to changes in the tissue samples, such as the TG. Therefore, further exploration of the pathophysiology of OFP using animal studies is necessary [28, 29].

Here we investigated the rat plasma metabolic profile in an OFP model. We applied both basic, untargeted (metabolic fingerprinting) and targeted approaches [21] together with transcriptomic analysis of the TG using complex bioinformatic tools. We demonstrate the advantages of a joint analysis platform for the plasma metabolite and primary sensory neuronal mRNA alterations to determine key pathophysiological pathways. Getting a comprehensive insight into the molecular complexity of inflammatory OFP mechanisms could contribute to the identification of novel biomarkers and drug targets.

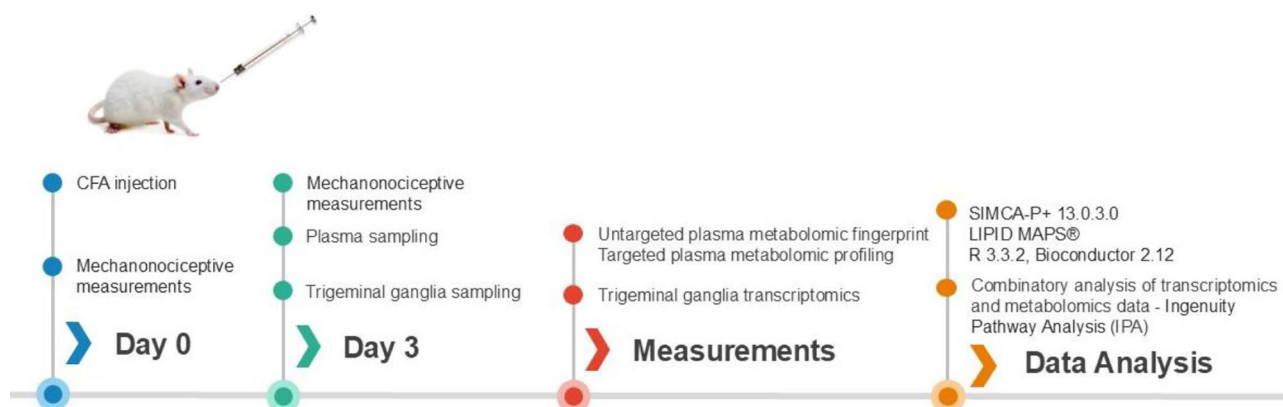
## Materials and methods

### Experimental paradigm, procedures and pain assessment

Thirty-four 200–300 g male Wistar rats (Toxicoop Zrt., Hungary) were kept in the local animal house of the University of Pécs, Medical School, Department of Pharmacology and Pharmacotherapy, under standard light-dark cycle (12-h light/dark cycle) and temperature (24–25 °C), provided with food and water *ad libitum*. Orofacial inflammation was generated by unilateral s.c. injection of 50 µL CFA (Sigma-Aldrich, Saint Louis, USA; killed mycobacteria suspended in paraffin oil; 1 mg/mL) into the right whisker pad under ketamine (72 mg/kg) and xylazine (8 mg/kg) anaesthesia. Control rats received the same volume of saline, the contralateral side remained intact. The measured mechanonociceptive threshold was further analysed statistically with two-way ANOVA followed by Tukey's multiple comparison test. Blood samples were collected on day 3 when the inflammatory allodynia was maximal based on earlier experience. The mechanical touch sensitivity of the orofacial region was measured by von Frey filaments, as previously described [14]. Blood samples were collected from the animals *via* cardiac puncture and collected in an Anticoagulant Citrate Dextrose-A (ACD-A) tube (BD Vacutainer). After centrifugation (300×g for 15 min, twice at 2500×g for 15 min), plasma sample aliquots were stored at –80 °C until metabolomic analysis. Samples showing signs of hemolysis were excluded. The experimental timeline is presented in Fig. 1.

### Measurement of plasma metabolomics

Samples were analyzed using untargeted plasma fingerprinting and a targeted assay for quantitative measurement. Untargeted analyses are prone to bias, especially when using a relatively small number of samples. For this reason, plasma fingerprinting was performed independently in two laboratories in Hungary at the UP and in Poland at the MUB, using the same methodology [30]. To minimize false positive findings, metabolic features selected *via* statistical analysis common between UP and MUB were considered discriminating and forwarded for metabolite annotation. Since most of the selected



**Fig. 1** Experimental protocol and timeline. Orofacial inflammation and pain were induced by s.c. Complete Freund's Adjuvant (CFA) injection into the right whisker pad of male Wistar rats. Mechanonociceptive thresholds in the orofacial area were measured with von Frey filaments before and 3 days after CFA treatment. Blood and trigeminal ganglia (TG) were collected on day 3, plasma and tissue samples were stored at -80 °C for further processing

molecules were lipids, a targeted assay was also performed by the MxP® Quant 500 kit to validate the findings of the untargeted analysis and to expand metabolite coverage by more polar and ionic metabolites, such as amino acids and biogenic amines.

#### Untargeted metabolomic profiling

**Plasma sample Preparation and mass spectrometry measurement** Plasma samples were treated as described previously [31]. For a full description of the method, see Supplementary Information.

**Data processing and metabolite identification** The raw data collected were cleaned of background noise and unrelated ions using the Molecular Feature Extraction (MFE) algorithm in Mass Hunter Qualitative Analysis Software B.07.00 (Agilent, Santa Clara, California, USA). The MFE lists all ions described by neutral mass, retention time (RT), and abundance. The following adduct settings were applied to identify co-eluting ions of the same molecule: +H, +Na, +K in positive ion mode and -H, +HCOO, +Cl in negative ion mode. Neutral loss of water was allowed in both polarity modes. Alignment and data filtering was performed using Mass Profiler Professional 12.6.1 (Agilent, Santa Clara, California, USA). Parameters applied for the alignment were 1% for RT and 15 ppm for the mass variation.

Multivariate analysis was performed in SIMCA 15.0 (Sartorius Stedim Biotech) and covered the use of principal component analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA). PCA was used to check the data quality, evaluate sample spread and clustering, and detect potential outliers. OPLS-DA was used to visualize between-group separation and select metabolites underlying this separation. Statistically

significant features with  $p(\text{corr})$  above 0.5 and VIP score greater than 1 were considered.

Based on the MS/MS fragmentation, metabolites selected via statistical analysis were identified [32]. MS/MS spectra were acquired in data-independent mode (DIA), using exactly the same chromatographic and spectrometric conditions as during the initial analyses. Based on the previously determined accurate mass and retention time, ions were targeted for collision-induced dissociation (CID) fragmentation on the fly. Accurate masses of features were simultaneously searched against the METLIN, KEGG, LIPIDMAPS, and HMDB databases via CEU Mass Mediator (available online search engine, <http://ceumass.eps.uspceu.es/mediator/>). The identity of metabolites was confirmed by matching the experimental MS/MS spectra to MS/MS spectra from databases. Lipids were identified based on a previously described characteristic fragmentation pattern [33].

#### Targeted plasma metabolic profiling

**Plasma sample Preparation and analysis** Plasma samples were thawed and allowed to equilibrate to room temperature [34]. The Biocrates MxP® Quant 500 Kit, purchased from Biocrates Life Sciences AG (Innsbruck, Austria), was employed for the profiling. The kit preparation was accomplished as described by the manufacturer.

**Data processing and analysis** The raw data were processed using Sciex Analyst v.1.6.3 software for instrument control and data acquisition. Peak review and analyte quantitation were conducted using the Biocrates MetIDQ™ (Nitrogen version) software supplied with the kit. Targeted data filtering was performed based on quality control samples, with metabolites showing >20% variation or measured less than twice being excluded. The data were not normalized. Metabolites were selected

using the Kruskal-Wallis test with a threshold of  $p \leq 0.05$ . Further discrimination between groups was achieved using orthogonal partial least squares discriminant analysis (oPLS-DA). Metabolites with  $p \leq 0.05$ , correlation  $> 0.5$ , and variable importance in projection (VIP) scores  $> 1$  were used for further analysis. Data analysis was performed as for the untargeted fingerprinting data set, described above in Sect. 2.3.4. GraphPad Prism was used for metabolite graphs. The parameters of the model can be found in Supplementary Information (Suppl. Figure 1).

### Transcriptomics of the TG samples

Total RNA extraction, purification and quality control from rat TG samples were described previously [14] and presented in Supplementary Information.

The samples were sequenced with Illumina's HiSeq2500 instrument using single-end sequencing with 50 bp read length at the Next Generation Sequencing Facility of the Vienna Biocenter Core Facilities GmbH (Vienna, Austria). The reads obtained from the instrument were base called using the instrument manufacturer's base calling software. The reads were aligned against the *Rattus norvegicus* reference genome (Ensembl Rnor 6.0 release) with STAR version 2.5.1b using 2-pass alignment mode. After alignment, the reads were associated with known genes and the number of reads aligned within each gene was counted using [HTSeq] [35] tool version 0.5.4p3. The data were normalised using the TMM normalisation method of the edgeR R/Bioconductor package (R version 3.3.2, Bioconductor version 2.12). For statistical testing, the data were further log transformed using the [voom] approach in the limma package. For the visualizations and result files the TMM normalised counts are represented as TPM values. Transcriptomic analysis was performed of trigeminal ganglia and combined with targeted metabolomics results in Ingenuity Pathway Analysis by Qiagen to reveal the altered pathways and links of the transcripts and metabolites [34].

### Data contextualization and bioinformatic analysis

For further pathway analysis, QIAGEN Ingenuity Pathway Analysis (IPA) version 122103623 was used. A core analysis was run on metabolites and genes considered significant with  $p$ -value ( $p \leq 0.05$ ) against the Ingenuity Knowledge Base as a reference set. The analysis identified canonical pathways, upstream regulators, causal networks, diseases and functions, and networks. For joint core analysis for both metabolites and genes, a background list was applied based on the detected molecules by our analytical platforms. Pathway analysis was performed using the LIPID MAPS® reaction explorer for lipids. Different lipid species were linked based on reactions from various sources, including scientific literature, the lipid research community, and other existing databases

such as Rhea, WikiPathways, KEGG, Ecocyc, and MetaCyc. Pathway analysis was performed based on the KEGG metabolic pathways for polar and ionic metabolites, finding the connection between detected and discriminating metabolites.

## Results

### CFA induces facial allodynia 3 days after the injection

CFA-induced orofacial inflammation significantly decreased the mechanonociceptive thresholds compared to both the contralateral side and saline-treated control rats on day 3. No changes in the contralateral/saline threshold were observed in the whisker pad area (Fig. 2), as previously shown [13, 14].

### The untargeted analysis determined altered plasma lipids in the CFA-induced orofacial inflammation

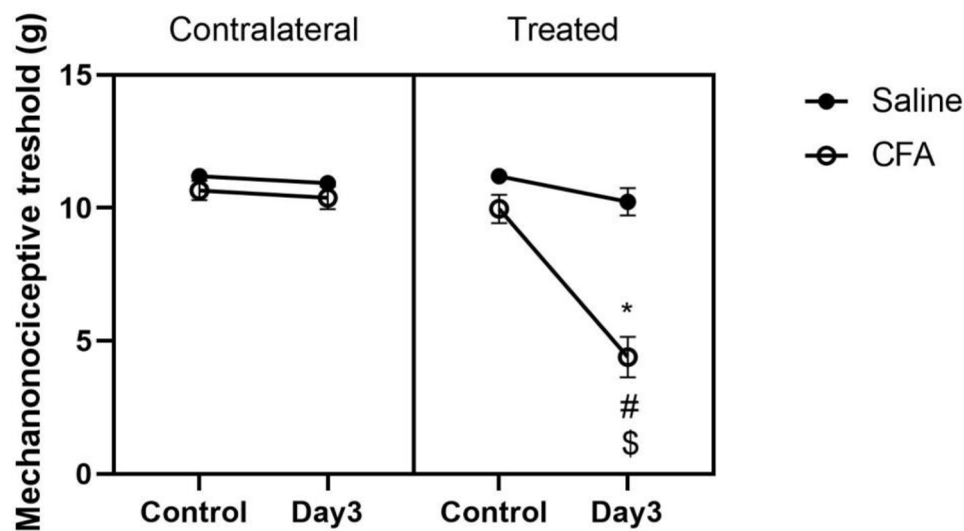
Multivariate statistical analysis of the results of the untargeted measurement revealed a good overlap between the results of the two laboratories of MUB and UP, where the untargeted measurements were parallelly executed. In both cases LPC 17:0, LPC 18:2, LPC 20:3 were discriminating in all three measurements: targeted and in both ion modes, in both untargeted measurements. LPC 16:0, LPC 18:1, LPC 18:0, PC 32:2, PC 34:4, PC 35:4, PC 36:6, PC 36:4, PC 36:5, PC 38:6, PC 38:5, PC 40:6 were found to be decreased in both ion modes significantly (See Supplementary Information, Suppl. Table 1).

### CFA-induced orofacial inflammation alters lipid, amino acid and monoamine profile of the plasma determined by targeted metabolomic analysis

The sample clustering observed in the oPLS model reflects alterations in the plasma or may arise as a result of these alterations. The detailed results for each metabolite can be found in Supplementary Information (Suppl. Table 2). The selected metabolite concentrations showed more than 25% changes in response to the CFA treatment for triacylglycerols, serotonin, carnosine, methionine-sulfoxide, kynurenine, phosphocholine, alanine, proline, and methionine, among others (Fig. 3).

### Orofacial inflammation results in altered transcriptomic fingerprint in TG

Significantly altered genes in TG of rat, like Luteinizing hormone/choriogonadotropin receptor (Lhcgr), gonadotropin-releasing hormone receptor (GNRHR), AABR07072807.1, sorting nexin 31 (SNX31), vanin 1 (VNN1), AABR07044301.1, muscleblind-like splicing regulator 3 (Mbnl3), BPI fold containing family A, member 6 (Bpifa6), AABR07024757.1, AABR07063724.1 and FOS like 2, AP-1 transcription factor subunit (FOSL2) were downregulated, meanwhile AABR07062758.1, AABR07026233.1, fibronectin type III and SPRY domain



**Fig. 2** Orofacial mechanical allodynia induced by Complete Freund's Adjuvant (CFA) injection. Facial mechanonociceptive thresholds measured with von Frey filaments before (control) and 3 days after CFA or saline injection (50  $\mu$ l s.c.) into the right whisker pad. Data points represent the means  $\pm$  S.E.M. of  $n = 11$ –11 rats (\* $p \leq 0.0001$  vs. saline; # $p \leq 0.0001$  vs. contralateral side; \$ $p \leq 0.0001$  vs. before treatment (control), determined by two-way ANOVA followed by Tukey's multiple comparison test)

containing 2 (FSD2), solute carrier family 27 member 6 (Slc27a6), C-X-C motif chemokine receptor 3 (Cxcr3), AABR07022072.2, AABR07054361.1, similar to predicted gene ICRFP703B1614Q5.5 LOC499240, microRNA 770 (Mir770), similar to protocadherin gamma B1, AABR07031734.13, myomesin 3 (Myom3), peroxisomal biogenesis factor 11 gamma (Pex11g), insulin-like growth factor binding protein, acid labile subunit (Igfals) were upregulated (see Supplementary Information, Suppl. Table 1). Raw data for transcriptomic results can be found in Supplementary Table 4.

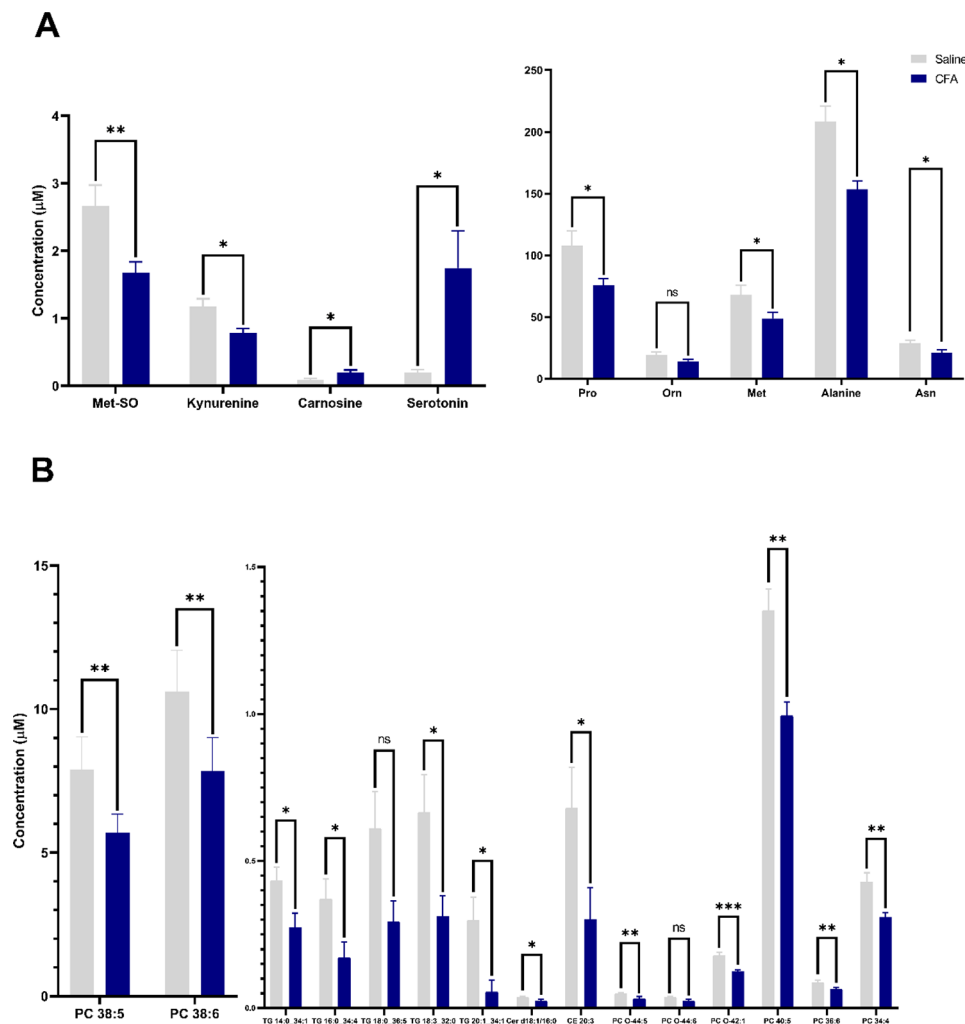
#### Pathway and network analysis of multi-omics data with IPA

IPA is a comprehensive tool that gives a valuable look for pathways affected in our dataset, and based on it, can give other molecules interacting with our targets. The affected pathways can be grouped as represented in Fig. 4A. Pathway analysis of the significantly altered metabolites and transcriptomic fingerprint in IPA software by Qiagen revealed altered Tryptophan catabolism, Alanine Biosynthesis III, Metabolism of water-soluble vitamins and cofactors, Class A/1 (Rhodopsin-like receptors), Thio-molybdenum Cofactor Biosynthesis, Glycine Biosynthesis III, Alanine metabolism, Alanine Degradation III, Alanine Biosynthesis II, Molybdenum Cofactor Biosynthesis, Pathogenesis of Multiple Sclerosis, Tryptophan Degradation to 2-amino-3-carboxymuconate Semialdehyde, Fatty Acid Activation, NAD biosynthesis II (from tryptophan), Mitochondrial iron-sulfur cluster biogenesis, Phenylalanine and tyrosine metabolism, Glutamate and glutamine metabolism, Metabolism of amine-derived hormones,  $\gamma$ -linolenate Biosynthesis II (Animals),

Mitochondrial L-carnitine Shuttle Pathway, Tryptophan Degradation III (Eukaryotic), Glyoxylate metabolism and glycine degradation, Fatty Acid  $\beta$ -oxidation I, Nucleotide catabolism (Fig. 4B).

Certain disease mechanisms and functions were also linked to Cellular function and maintenance, Amino-acid Metabolism, Cell Death and Survival, Cell-To-Cell Signaling and Interaction, Inflammatory Response, Cell Signaling, Nervous system Development and Function, Infectious Diseases, Inflammatory Disease (see Supplementary Information, Suppl. Figure 1). We detected fatty acid, dihydroceramide, 1,2-DG, Cholesterol ester, O-acyl-L-carnitines, lysophosphocholines, triacylglycerols, sphingomyelin, ceramide were altered due to the treatment. All lipid classes were downregulated except fatty acid (Fig. 5A). Figure 5B summarizes the most important non-lipidic reactions and the affected metabolites in targeted measurement. Several metabolic pathways, i.e. urea cycle, alanine, aspartate, glutamate, and tryptophan metabolism were affected (Fig. 5B).

Figure 6A, B and C show in a Network analysis the interacting genes with the discriminant metabolites found in our dataset. In these networks, N,N-dimethylarginine directly inhibits CXCR3 receptor. CXCR3, LNHGR, GNRHR are in an indirect relationship. In our experiment, N, N-dimethylarginine was downregulated, thus it is consistent with the prediction of upregulated CXCR3. The MYOM3 was upregulated, which is in interaction with miRNAs. Solute Carrier 27 A mediating long-chain fatty acid uptake is in a direct relationship with ceramide and FSD2 in an indirect relationship. These are parts of Fatty acid beta-oxidation and the



**Fig. 3** A. Amino acid and B. Lipid metabolite alterations in the plasma of CFA-treated rats in comparison with saline-treated ones. Each column represents the mean concentration  $\pm$  SEM of  $n=7$ /group and statistical evaluation was performed with the Mann-Whitney U-test pairwise (\* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ ). Abbreviations: Met-SO: methionine-sulfoxide, Pro: proline, Orn: ornithine, Met: methionine, Asn: asparagine

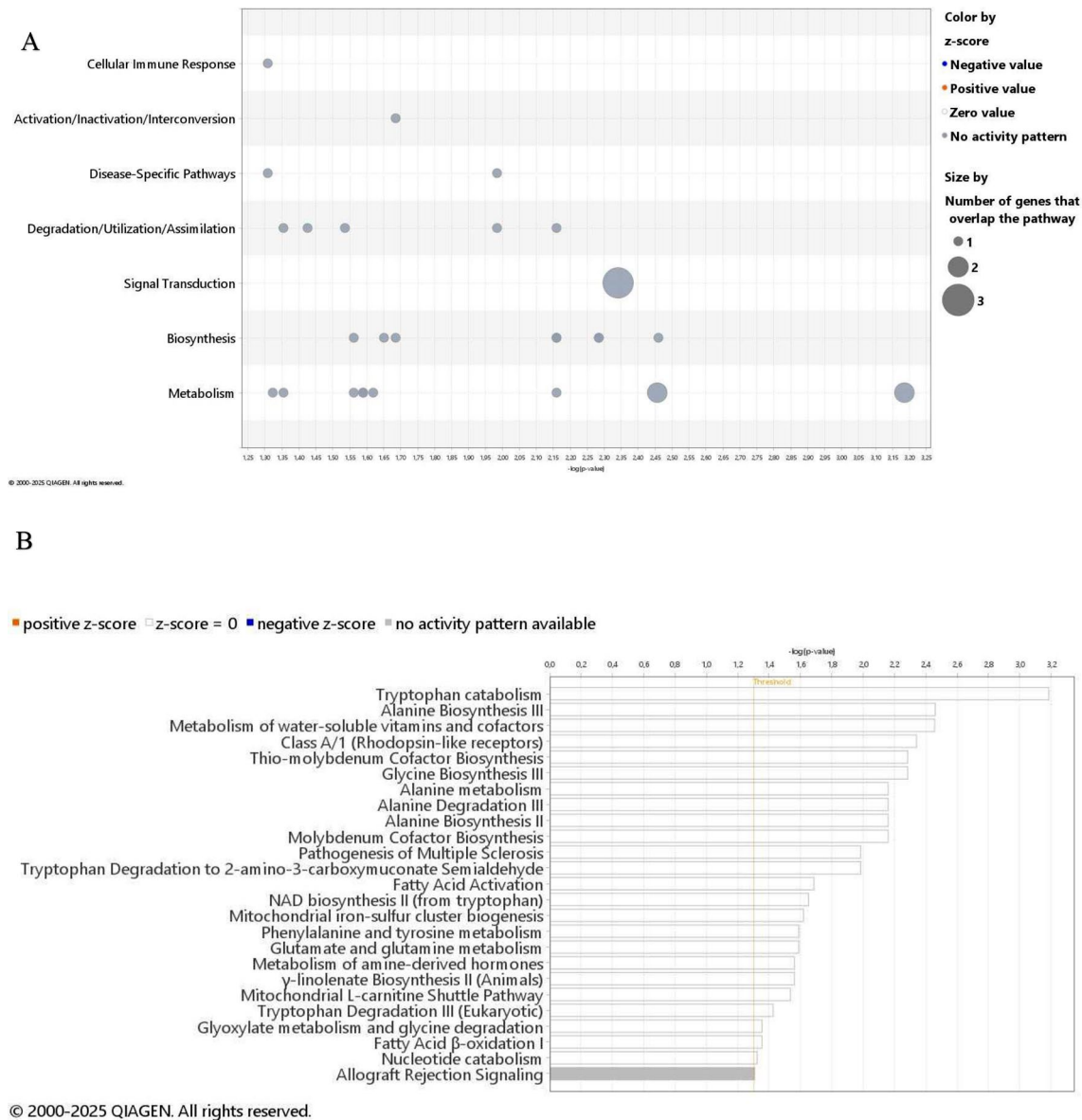
Mitochondrial L-carnitine Shuttle pathway. SNX31 was downregulated, which is in line with the prediction of inhibition. With these pathways, other molecules are in indirect relationship: MBNL3, L-alanine, N,N-dimethyl-arginine, MYOM3, PEX11G, phosphatidylcholine, Igfals, and mir770. Additional information on other potential regulators and targets can be gained from the IPA analysis predicting drugs, such as topiramate, nebivolol and dexamethasone as well as PPARG a fatty acid regulator.

## Discussion

We provide here the first data on plasma metabolomic profile alterations in a rat orofacial inflammatory pain model using both untargeted and targeted metabolomics. This is also the first approach to integratively analyse plasma targeted metabolomic results with the TG transcriptomics, which revealed altered glucocorticoid signaling and lipid transport, as well as predicted the

effect of topiramate and the involvement of GPR132. The results of this unbiased multi-omic study show altered amino acids, kynurenine, alanine, glycine biosynthesis, lipid metabolism, including fatty acid  $\beta$ -oxidation, which is involved in neuroplasticity, mitochondrial energy production, neuroinflammation and immune responses. Potential involvement of i) cell cycle, cell-to-cell signaling and interactions via IL22, G protein-coupled glucocorticoid receptors, CXCR3 fractalkine receptor, ERK and AKT signaling, ii) lipid synthesis, homeostasis and transport via mitochondrial L-carnitine shuttle pathway, phosphatidylcholine and fatty acid activation and iii) inflammatory and immune mechanisms via IL6, CXCL8, and SIRT6 are determined with the help of a highly sophisticated, curated database.

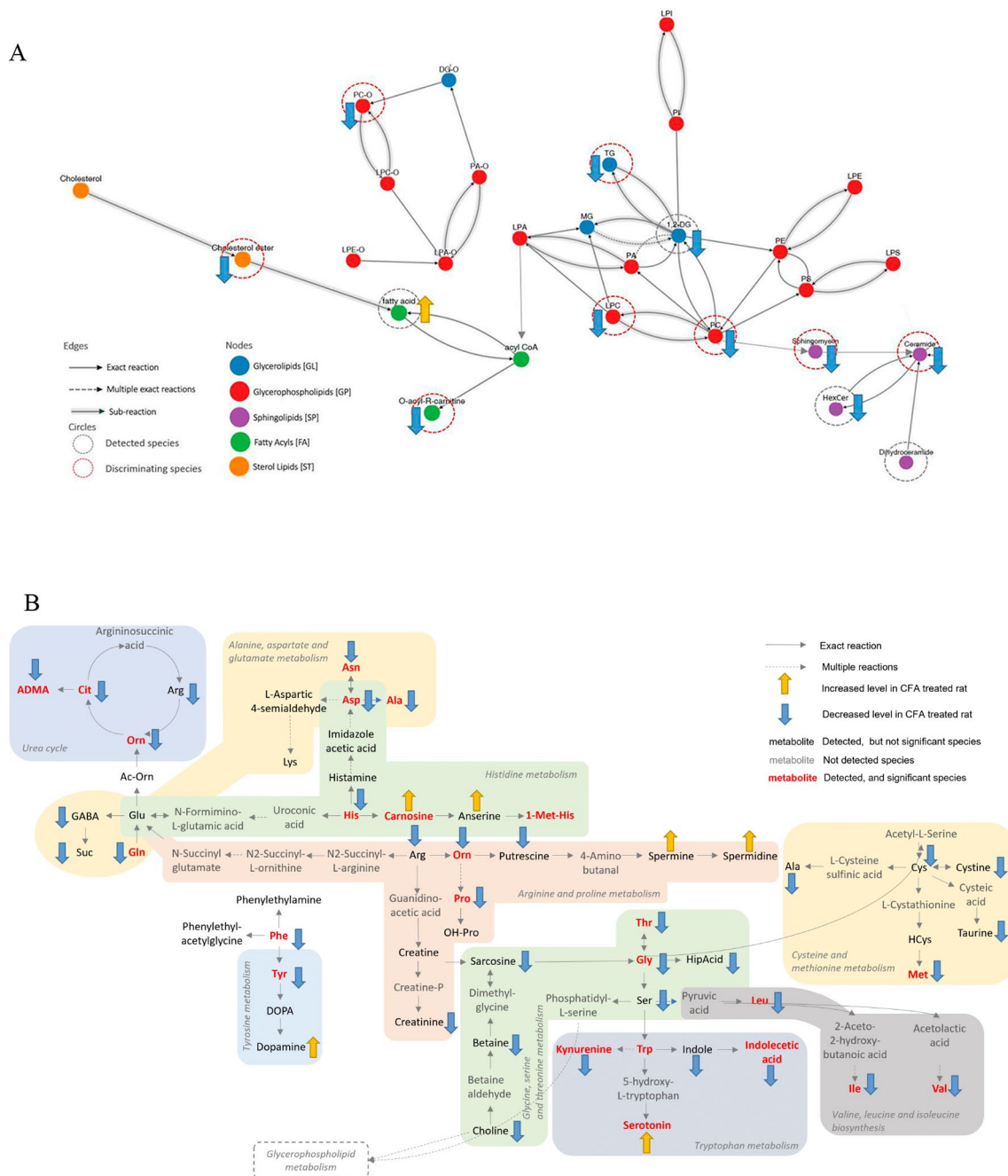
Although several *in vivo* studies focusing on pain have recently used a metabolomic approach, they were mainly neuropathy models, suggesting altered amino



**Fig. 4** Significantly altered canonical pathways **A.** Bubble chart demonstrates general pathway categories and bubble sizes depending on the number of molecules involved. **B.** Horizontal bar chart indicates pathways identified by the IPA software. The x-axis represents the negative logarithm of the p-values calculated using the right-tailed Fisher's exact test, measuring the probability of the association between the molecules in a dataset and the canonical pathways. Longer bars indicate greater significances; the grey bar shows the pathway with no current activity prediction. White bars demonstrate pathways with z-scores around zero or pathways with fewer than four associated molecules making them ineligible for directionality analysis (z-score = NaN). In Supplementary Table 4 molecules involved in each pathway are collected

acid and fatty acid pathways [36, 37]. These data support our results in the inflammatory OFP model, sensitisation might be involved in common mechanisms. Enrichment analysis revealed the downregulation of tryptophan catabolism, alanine and glycine biosynthesis III in our model. This is in agreement with earlier data demonstrating decreased metabolism and biosynthesis of aminoacids (alanine, phenylalanine, aspartate, glutamate, tryptophan, tyrosine, valine, leucine and isoleucine), vitamins (ascorbate, vitamin B6), aldarate, and

lipids (glycerolipid, glycerophospholipid, sphingolipid) in the urine samples of rats with intraplantar CFA-induced inflammatory pain [38]. The kynurenine and methoxyindole pathways are the main routes of tryptophan catabolism [39], in which different bioactive metabolites (e.g. proinflammatory, anti-inflammatory, oxidative, antioxidant, neurotoxic, neuroprotective, immunologic compounds) are formed. Therefore, reduced L-kynurenine plasma concentrations and upregulated enzymes, including kynureninase in our model, are likely to be directly

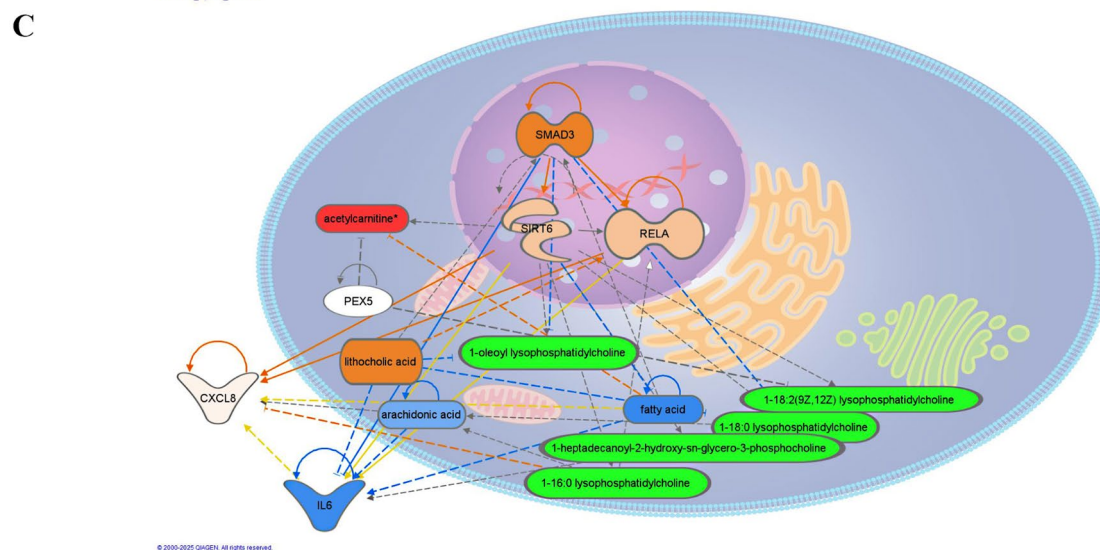


**Fig. 5** (A) Lipid- and (B) non-lipid-related pathways involved in orofacial inflammation and related pain. Images were generated with the Lipidmaps database, arrows indicate metabolite alterations in the plasma of CFA-treated rats in comparison with saline-treated controls

involved in the inflammatory and immune processes as well as neuroinflammation and related pain [39, 40]. Decreased levels of kynurenine metabolites (L-kynurenine, kynurenic acid, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, 5-hydroxyindoleacetic acid, quinolinic acid) were found in the plasma of migraine patients [41]. These results suggest the role of impaired amino acid metabolism in the pathological activation of the trigeminovascular system and support the translational relevance of

the CFA-induced orofacial inflammation model to study this pathway. Indol derivatives were significantly lower in CFA-treated rats in contrast to increased 5-hydroxyindoleacetic acid in migraine patients in the ictal phase [41], suggesting its relevance in acute headache attacks, but not chronic conditions.

CFA treatment also induced changes in several lipid mediators, such as fatty acids, dihydroceramide, 1,2-DG, cholesterol ester, O-acyl-R-carnitines,



**Fig. 6** (See legend on next page.)

(See figure on previous page.)

**Fig. 6** Combined core analysis resulted in three networks of targeted plasma metabolomic and TG transcriptomic data. **A.** Cell cycle, cell-to-cell signaling and interaction, tissue morphology; **B.** Lipid metabolism, molecular transport, small molecule biochemistry; **C.** Organismal injury and abnormalities, inflammatory disease, inflammatory response. Red color represents metabolites, genes found increased, while green decreased in our dataset. Orange represents predicted activation, blue represents predicted inhibition, and the intensity of the color means less or greater is the predicted effect. The dashed line means an indirect relationship, the solid line means a direct relationship. Red line leads to activation, blue line to inhibition, yellow means findings are inconsistent with the state of a downstream molecule, and grey means the effect has not been predicted. Abbreviations: Luteinizing hormone/choriogonadotropin receptor (Lhcgr), gonadotropin-releasing hormone receptor (GNRHr), sorting nexin 31 (SNX31), vanin 1 (VNN1), muscleblind-like splicing regulator 3 (Mbnl3), fibronectin type III and SPRY domain containing 2 (FSD2), solute carrier family 27 member 6 (Slc27a6), C-X-C motif chemokine receptor 3 (Cxcr3), microRNA 770 (Mir770), similar to protocadherin gamma B1, myomesin 3 (Myom3), peroxisomal biogenesis factor 11 gamma (Pex11g), insulin-like growth factor binding protein, acid labile subunit (Igfals). PTHR: Pth/Pthrp Receptor, KLRC4-KLRK1: killer cell lectin-like receptor subfamily C, member 4 and KLRK1 killer cell lectin-like receptor subfamily K, member 1, MCL1: myeloid cell leukemia sequence 1, NCR1: natural cytotoxicity triggering receptor 1, RT1-M1-5: RT1 class I, M1, gene 5 (member of MHC/ANTIGEN (complex)), ERK1/2: Extracellular signal-regulated protein kinases 1 and 2, PK1: protein kinase inhibitor, HOXA1: homeobox A1, IL22: interleukin 22, PLA2G4E: phospholipase A2 group IVE, LBP: lipopolysaccharide binding protein, KYNU: kynurenine, LDLR: low density lipoprotein receptor, GPR132: G protein coupled receptor 132, S1PR1: sphingosine-1-phosphate receptor 1, C11orf16: chromosome 11 open reading frame 16, MYH9: myosin heavy chain 9, AGO2: Argonaute 2, GCLM: glutamate-cysteine ligase modifier subunit, APP: Amyloid beta precursor protein, SIRT6: Sirtuin 6, SMAD3: SMAD family member 3, KRAS: KRAS proto-oncogene, GTPase, RPS6: Ribosomal Protein S6, PEX5: peroxisomal biogenesis factor 5, ACAC: Acetyl-coA carboxylase, IL6: Interleukin 6, RIN1: Ras and Rab interactor 1, predicted peroxisome proliferator-activated receptor gamma (PPARG)

lysophosphocholines, triacylglycerols, sphingomyelin and ceramide. This is in line with changes observed in the dorsal root ganglia of CFA-treated rats, showing that glycerophospholipid, retinol, linoleic acid, and arachidonic acid metabolisms were the main altered pathways [42]. Glycerophospholipid metabolism (e.g., arachidonic acids and polyunsaturated fatty acids) plays a key role in proinflammatory signaling [43]. Polyunsaturated fatty acids can result in oxidized lipids, which promote inflammatory pain [44]. In a large cohort, a significant association was found between altered lipid metabolism and migraine: in migraineurs, apolipoprotein A1, high-density lipoprotein and free cholesterol were decreased [45]. In agreement with our rat OFP results, non-alpha-hydroxy-sphingosine ceramides were significantly elevated, while lysophosphatidylethanolamines decreased in the serum of migraineurs [46]. However, unlike our findings, plasma levels of CE(20:4), CE(18:2), CE(22:6), PC(38:7), PC(18:0/18:2) and SM(d34:1) were significantly higher in mice with osteoarthritis pain [47], suggesting distinct mechanisms under degenerative conditions. Our results predicted the involvement of GPR132, which is an effector of lysophosphatidylcholine and oxidized free fatty acid action, and may have proton-sensing activity. In a mouse neuropathic pain model, GPR132 deficiency significantly reduced hypersensitivity, immune cell infiltration, and proinflammatory cytokine release, suggesting that GPR132 inhibition may offer a novel therapeutic strategy [48].

Similarly to our results, amine metabolites significantly decreased in the plasma of mice with collagen-induced arthritis [49] and CFA-induced plantar inflammation [50]. It has been described that tryptophan, arginine and proline metabolism, aminoacyl-tRNA biosynthesis, valine, leucine and isoleucine biosynthesis, valine, leucine, and isoleucine degradation are migraine-related metabolic pathways [51], which validates our findings.

In contrast, low serotonin levels were found in migraine patients' serum [51].

Combined IPA analysis of the plasma metabolomic and TG transcriptomic results suggested a potential role of the glucocorticoid receptor (GR) signalling. This receptor up- or down-regulates the transcription of several glucocorticoid-responsive genes [52]. In our study, CXCR3 expression level increased, which has been proposed to be involved in chronic pain [53] via GR signaling. The mechanisms underlying neuronal hyperexcitability are still unclear, but CXCR3 activates immune cells and releases inflammatory mediators, which activate and/or sensitize the sensory neurons [54]. Furthermore, a direct relationship was proposed between CXCR3 and topiramate, which we have recently shown to reduce CFA-induced inflammatory orofacial allodynia [55]. A similar direct relationship was found between topiramate and vanin 1 (VNN1), also involved in inflammation and oxidative stress [56], mitochondrial damage, metabolic abnormalities and consequent energy deficit, playing a role in trigeminal pain syndromes [25, 34, 57]. This is supported by our previous results, where peripheral blood mononuclear cell transcriptomics of migraineurs showed increased inflammatory and immune cell activity and oxidative stress [34].

The involvement of PPARs nuclear receptors regulating lipid, carbohydrate, and amino acid metabolism [58] was predicted in our model by the complex pathway analysis, which is in agreement with increased levels of fatty acids. PPARs are also important in apoptosis, cell differentiation, inflammation and neuroinflammation involved in neuropathic and inflammatory pain [58]. In animal models, PPAR activators inhibited neuroinflammation and allodynia by reducing inflammatory mediators (e.g., proinflammatory cytokines) and ion channels [58]. These results support potential benefits for PPAR agonists in inflammatory OFP too.

Besides the conceptual message, this paper also has an important methodological impact. We showed that untargeted metabolomics is highly reproducible in two different laboratories. Targeted metabolomic techniques can be used synergistically to identify pathophysiological pathways. We performed pathway analysis, with comprehensive network analysis of a multi-omic approach via connecting TG transcriptomics with blood metabolite profile evaluation, which adds depth to interpretation and provides an integrated database for future pain and inflammation-related drug target research. The use of various bioinformatic tools provides a useful approach to determine pathophysiological mechanisms of trigeminal sensitization and identify novel drug targets for orofacial pain and headaches, however, these findings should be functionally validated in further experiments.

### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s10194-025-02024-0>.

Supplementary Information

Supplementary Information (Tables and Figs)

Supplementary Table 3

Supplementary Table 4

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### Author contributions

MC and ZH: conceptualization. TA, GK, RF: methodology. JK, MC: software. TA: sample preparation. RF, JP, MH, VM, AT, KT-L and JK: formal analysis, data curation, and visualization. ZH: investigation and supervision. MC and ZH: resources and funding acquisition. KT-L, AT, VM: writing—original draft preparation. KT-L, TA, VM, JK, KB, JP, GK, JG, MC and ZH: writing—review and editing. All authors contributed to the article and approved the submitted version.

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### Data availability

No datasets were generated or analysed during the current study.

### Declarations

#### Ethical approval

The animal study was conducted according to the European legislation (Directive 2010/63/EU) and Hungarian Government regulation (40/2013., II. 14.) regarding the protection of animals used for scientific purposes and was in full compliance with the recommendations of the International Association for the Study of Pain. The study was approved by the Animal Welfare Committee of the University of Pécs and the National Scientific Ethical Committee on Animal Experimentation of Hungary as well as licensed by the Government Office of Baranya County (BA02/2000-75/2023).

#### Competing interests

The authors declare no competing interests.

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