TRIGEMINAL SENSITIZATION MECHANISMS INVOLVED OROFACIAL PAIN AND MIGRAINE DETERMINED BY TRANSCRIPTOMICS AND METABOLOMICS IN CELL CULTURES, ANIMAL AND HUMAN STUDIES

DOCTORAL (PhD) THESIS

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INTRODUCTION

Orofacial pain (OFP) and migraine

OFP and headache conditions represent some of the most severe pain challenges worldwide, significantly impacting 25 % of population.[1] The pathophysiological mechanisms beyond OFP and migraine may vary, however the activation and sensitization of trigeminal primary afferents is evident. Dysfunction in the trigeminovascular system is thought to contribute to the development of pain in these conditions, yet the precise pathophysiology remains elusive. Trigeminal nerve consists of ophthalmic (V1), maxillary (V2) and mandibular (V3) branches. V1 branch is considered the most important in primary headache disorders, like migraine, V2 and V3 might also innervate the dura mater, not only the maxillary and mandibular regions of the head, meaning an overlap between regions.[2]

Molecular component of trigeminovascular system

Trigeminal sensory neurones are transporters of sensory transduction to the central nervous system. Nociceptive afferents mainly consist of thinly myelinated and unmyelinated A and C fibres.[2] The trigeminal ganglion (TG) primarily consists of the soma and axons of primary afferent, peptidergic neurons, along with glial cells. The cell bodies of trigeminal neurons are surrounded by satellite glial cells (SGCs).[3] Mast cells (MCs) are highly adaptable immune cells capable of responding to a wide range of stimuli acting as mediators between external and internal environments. The primary function of MCs is their involvement in the immune response through activation and degranulation. During this process, they release stored vasoactive compounds that increase vascular permeability, promote fluid accumulation, and recruit other immune cells like eosinophils, natural killer (NK) cells, monocytes, macrophages, and neutrophils, amplifying the inflammatory response.[4–7] Meningeal MCs are in a close relationship with the nociceptors in the dura, and histamine and cytokines released by them are increased in migraineurs. [8,9]

Pituitary adenylate cyclase-activating peptide (PACAP)

PACAP is a neuropeptid, member of the VIP/glucagon/growth hormone releasing factor/secretin superfamily, existing in in 27- and the predominant in mammals 38-amino acid-containing forms (PACAP-27 and PACAP-38). Three main PACAP receptors have been described: $VPAC_1$, $VPAC_2$ and PAC_1, all coupled to G-proteins.[10] Mas-related G-protein coupled

receptor (Mrgpr) activation by PACAP-38 or its truncated derivatives, PACAP(6-38) or PACAP(6-27), was described on mast cells. [11] PACAP(6-38) is an antagonist of PAC1 receptor in various neuronal cell lines, however our previous results clearly showed that PACAP(6-38) treatment did not inhibit PACAP-38, but produced identical effects by itself in rat primary sensory neurons. Both PACAP-38 and PACAP(6-38) could inhibit neuropeptide release from sensory nerve terminals of isolated trachea [12] and induce Ca^{2+}-influx in primary cultures of trigeminal ganglion cells. [13] The headache-inducing effect of PACAP was first identified in a study investigating cerebral blood flow in healthy volunteers. In this study, 10 out of 12 participants reported experiencing mild to moderate headaches following PACAP infusion, suggesting a link between PACAP and headache generation.[14]

Hemokinin-1 (HK-1)

Tachykinins are neuropeptides found in peptidergic primary afferent neurons, playing important roles in neurogenic inflammation and nociceptive transmission. These neuropeptides contribute to pain signaling and inflammation by activating receptors on target cells, further amplifying the pain response.[15,16] Important members of the tachykinin family include SP and neurokinin A, both derived from the *Tac1* gene, neurokinin B from the *Tac3* gene, and HK-1 from the *Tac4* gene. These tachykinins exert their effects through G-protein coupled receptors: NK-1, NK-2, and NK-3 receptors, which play critical roles in pain signaling and inflammation.[17,18]Unlike other tachykinin family members, *Tac4* shows relatively high expression in peripheral non-nervous tissues, including the lung, spleen, adrenal gland, and various immune cells such as B and T lymphocytes, macrophages, and dendritic cells. This suggests a broader role for *Tac4* in both immune response and peripheral organ function, potentially linking it to inflammation and pain mechanisms.[19–22]

Model systems in trigeminal sensitization

While most studies focus on using primary sensory neurons harvested from naive or injured animals, permanent cell lines are also available, offering a consistent and controlled environment for investigating pain signaling pathways.[23,24] One advantage of primary sensory neurons can be the heterogenity enabling the investigation of different interactions among the diverse cell types. Sensory neurons isolated from mouse or rat TG have been the primary *in vitro* model for preclinical pain research.

Several reviews highlight validated animal models for pain relevant to headache research. One of the major advantage of animal model is being able to study separately different tissues or organs limited in human studies. These models include direct electrical stimulation of trigeminal neurons, administration of inflammatory or algogenic substances ("inflammatory soup" like bradykinin, serotonin, histamine, and prostaglandins) to the meninges, and exogenous chemicals like nitroglycerin and PACAP. These models are complemented by behavioral assays, electrophysiology, flowmetry, and immunohistochemical marker detection. They help reflect migraine-like phenomena such as mechanical allodynia (e.g., using von Frey filaments on the whisker pad or periorbital areas to measure withdrawal responses), light sensitivity (e.g., place or light avoidance tests), and changes in spontaneous response activity. [25–27]

Injecting Complete Freund's Adjuvant (CFA) into the whisker pad of rodents induces localized inflammation and results in mechanical hyperalgesia and allodynia in the orofacial region. [28]This model is commonly used to study pain mechanisms and the effects of inflammatory mediators in facial pain conditions. [29–32]Although the CFA injection model is not traditionally used for migraine studies, it serves as an effective trigeminal activation model, offering the advantages of reliability and high reproducibility. [33]

Transcriptomics and metabolomics in clinical studies

Transcriptomics allows scientists to examine the expression of various biomarkers by analyzing RNA transcripts, helping them understand how these markers influence biological processes and identify their underlying mechanisms. However, it also has limitations/challenges: large data and complexity, lack of functional correlation, costs and time. One should compare transcriptomic results directly with functional outcomes, as increased mRNA levels do not always equate to higher protein levels or activity.

Metabolomics focuses on the study of metabolites, providing insight into the metabolic pathways that are active during specific pathological or physiological conditions. It can give insights into functional biology, however, there is a limitation in coverage of metabolites, as not all can be detected. Together, these approaches offer a detailed view of the molecular changes occurring in response to disease or treatment.[34] However, there are more challenges during data analysis in multi-omics. Challenges come from data collection – different types of data, integrative analysis – inefficient computation, diverse signal/noise ratio of different data, poor biological interpretation and community distribution. [35]

It has been particularly valuable in research on neurological diseases, where it helps to identify novel biomarkers and therapeutic targets, contributing to the understanding of disease progression and potential interventions.[36–39] A multi-omics approach offers a promising solution for identifying and validating biomarkers, which can be particularly useful in complex scenarios.[40] Peripheral blood mononuclear cells (PBMCs), which include lymphocytes (T cells, B cells, natural killer cells) and monocytes, are commonly isolated from peripheral blood due to the minimally invasive nature of the procedure and the straightforward isolation process. As a result, PBMCs have become a promising source for biological marker candidates in clinical practice. These cells are valuable for reflecting pathophysiological changes occurring in the CNS across various diseases, particularly in neuroinflammatory processes, providing new avenues for biomarker research and enhancing our understanding of disease mechanisms.[41–43] Combining PBMC transcriptomics with blood metabolomics may provide deeper insights into complex biological scenarios.

AIMS

1. Investigating the effects and mechanisms of action of PACAP-38 and the tachykinin HK-1 on cultured TG primary sensory neurons to identify potential targets, explore signalling pathways.

2. Analysing the transcriptomic profile of the TG and metabolomics of the plasma in the rat Complete Freund's adjuvant-induced orofacial inflammatory pain model to identify pathophysiological pathways and targets using an unbiased multi-omics approach and bioinformatic tools.

3. Determining headache- and disease-specific metabolomic profiles of the plasma of migraineurs during (ictal) and (interictal) periods in comparison with healthy volunteers, and analysing the data together with PBMC transcriptomic alterations to identify key pathophysiological mediators and novel pharmacological targets.

MATERIALS AND METHODS

Cell culture study

TG neurons of 1–4-day-old Wistar rat pups were used for primary cell cultures. For the experiment, the cell cultures were treated with either 1 μM PACAP-38 or PACAP6-38, with untreated cultures used as controls. Six hours after the administration of PACAP-38 or PACAP6-38, the samples were collected for RNA isolation. HK-1 was given in two concentrations: 500 nM (no evoked calcium influx) and 1 μM evoked calcium influx. [44] Untreated cultures were used as controls. After 6 h and 24 h of HK-1 administration, samples were collected for RNA isolation.[45] Total RNA isolation and purification, sequencing, alignment to the *Rattus norvegicus* reference genome, readmapping to protein-coding genes were performed, and gene-specific read counts were obtained using the HTSeq library (v0.11.1). [45,46] Gene count data normalization was performed via the trimmed mean of M values (TMM) method from the edgeR R/Bioconductor package (v3.28), and then log-transformed using the voom approach for statistical analysis with the limma package.[45,46] To validate the upregulated, downregulated, and unaltered genes identified through RNA sequencing, we used RT-PCR. [47] Fold changes (FC) and p-values from a moderated t-test were calculated as part of the limma linear modeling process. Differentially expressed (DE) genes were identified using thresholds of FC ≥ 1.2 and p-value ≤ 0.05 for comparisons between HK-1 1 μM (24 h) and HK-1 500 nM (6 h) treatments versus untreated control, FC ≥ 1.3 and p-value ≤ 0.05 for HK-1 1 μM (6 h) vs. control, and FC ≥ 2.0 and p-value ≤ 0.05 for HK-1 500 nM (24 h) vs. control. For PACAP treatments, thresholds were set to FC ≥ 2 and p-value ≤ 0.05 for PACAP-38 vs. untreated control, and FC ≥ 1.5 and p-value ≤ 0.001 for PACAP6-38 vs. control. P-values were corrected for multiple comparisons using the Benjamini-Hochberg method. Normalized gene expression was reported in transcripts per million (TPM). For functional analysis, gene annotations were sourced from the Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome databases. Functional enrichment was performed using Fisher's exact test for GO, and hypergeometric tests for KEGG and Reactome, utilizing topGO (v2.37.0), ReactomePA (v1.30.0), and gage (v2.36.0). Ranked list enrichment was assessed via non-parametric Kolmogorov-Smirnov tests (GO and KEGG), and hypergeometric tests (Reactome). KEGG pathway visualizations were generated with the pathview package (v1.26.0). The Revigo tool was employed to condense GO terms to the most relevant ones. [45,46]

Animal model

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).

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 induced by unilateral s.c. injection of 50 µL CFA (Sigma-Aldrich, Saint Louis, USA; 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. [48] Blood samples were collected from the animals *via* cardiac puncture, and collected in Anticoagulant Citrate Dextrose-A (ACD-A)-tube (BD Vacutainer) and after centrifugation (300×g for 15 min, twice at 2500×g for 15 min) plasma samples aliquotes were stored at −80 °C until metabolomic analysis.

Untargeted plasma metabolomic fingerprint was carried out at the University of Pécs (UP) and the Medical University of Bialystok (MUB). The Molecular Feature Extraction (MFE) algorithm in Mass Hunter Qualitative Analysis Software B.07.00 (Agilent, Santa Clara, California, USA) was used for cleaning the raw data of background noise and unrelated ions. 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. As statistically significant features with p(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. 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.

Targeted plasma metabolomic profiling was completed using at Semmelweis University, followed by data analysis. Samples showing signs of hemolysis were excluded. 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. The analysis was conducted using a Shimadzu Nexera XR ultra-performance liquid chromatograph (Simkon Kft, Budapest, Hungary) coupled to a Sciex Qtrap 5500 mass spectrometer equipped with an electrospray ionization unit, and operated in multiple reaction monitoring mode (Per-form Hungária Kft, Budapest, Hungary).

Total RNA extraction and purification from rat TG samples were described previously. [48]The samples were sequenced with Illumina's HiSeq2500 instrument using single-end sequencing with 50bp read length at the Next Generation Sequencing Facility of the Vienna Biocenter Core Facilities GmbH (Vienna, Austria). Data analysis was performed as discussed in cell culture study section.

For further pathway analysis, QIAGEN Ingenuity Pathway Analysis (IPA) version 122103623 was used. A core analysis was run on metabolites and genes considered as discriminants with ($p \leq 0.05$) 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. [210]

Human study

The study was performed under the approvement of the National Public Health Center, Ministry of Human Capacities of Hungary (28324–5/2019/EÜIG), and all participants provided written informed consent in line with the Declaration of Helsinki. Participants included episodic migraine patients aged 20–65 years, with or without aura, selected based on the third edition of the International Classification of Headache Disorders criteria [49]

A total of 37 participants (36 females, 1 male) were enrolled, consisting of 24 migraine patients and 13 healthy controls. One male participant was excluded due to the coherent data analysis. Blood samples (13 mL per person) were collected from the cubital vein, processed for metabolomic analysis, and plasma was stored at −80°C until aanalysis. Biocrates MetIDQ^{TM} software analyzed metabolite concentrations and performed quality control. For 42 LC-MS/MS-quantified metabolites, six-point calibration curves with linear regression (1/concentration weights) were used, except for dopamine (quadratic regression). Determination coefficients ranged from 0.9894 to 0.9999 (median: 0.9972). For 64 LC-MS/MS metabolites, peak areas were compared to internal standards. FIA-MS/MS analysis of 524 metabolites was automated via MetIDQTM using Biocrates MxP® Quant 500 kit algorithms. No data filtering/correction was applied. Metabolites with >80% detection post-QC and RSD <15% were selected. Metabolomic profiles of controls and migraine patients (interictal/ictal) were compared using ANOVA with Tukey's post-hoc test; paired t-tests analyzed ictal vs. interictal samples. FDR correction (Benjamini-Hochberg) was set at 0.2 for control-interictal comparisons and $p<0.05$ for paired analyses. Randomization was applied for control-interictal comparisons. All statistical analyses were conducted using R (version 4.3.3). Following package were used in R: dplyr (version 1.1.4), tidyr (version 1.3.1), ggplot2 (version 3.5.1), multcomp (version 1.4-26), readxl (version 1.4.3).

Significant metabolites collected with published PBMC transcriptomic [50]data were core analysed in IPA software.

RESULTS

In 1) 2) 3) and partially 4) chapters results are published, but changed and formatted to the thesis.

1) Transcriptional alterations in the TG induced by PACAP-38 and PACAP(6-38)

Expression of potential targets for PACAP in TG cell culture

The PAC1 (Adcyap1r1) and VPAC2 receptors, as well as several Mrgpr receptors were detected in most samples.

DE genes induced by PACAP-38- and PACAP(6-38) in TG

Sample collection 6 h after the treatment yielded 200 common differentially expressed genes for PACAP-38 and PACAP6-38. For PACAP-38, 70 other DE genes, for PACAP6-38 132 other DE genes were found at the 6 h samplings. Common DE genes potentially involved in neurological pathophysiology following treatments with PACAP-38 and PACAP(6-38) in comparison to untreated control cell cultures. Key findings from the DE gene list include the upregulation of Cenpb, Gnal, Hsp90aa1, Hmga1, Tomm70, Gnai1, and Tomm34 in both PACAP-38- and PACAP(6-38)-treated TG cell cultures. Notably, in both treatments, Ndufb6 (NADH oxidoreductase subunit B6) was significantly downregulated compared to the control group (with fold change values of −50.7 and −80.9 for PACAP-38 and PACAP(6-38) treatments, respectively), while Trpm8 was upregulated in both cases. Additionally, Fbl (Fibrillarin), Fhl2 (four and a half LIM domains 2), Slc25a5 (solute carrier family 25 member 5), and Tomm6 (translocase of outer mitochondrial membrane 6) were markedly downregulated in both treatments.

Pathway analysis with shared results

Reactome analysis identified key intracellular pathways involving the DE genes in PACAP-38- and PACAP(6-38)-treated cultures. Common pathways were: upregulation of CREB1 phosphorylation via adenylate cyclase, PKA activation in glucagon signaling, and post-NMDA receptor activation events. Calcium-dependent processes were upregulated, while Complex I biogenesis was downregulated, suggesting potential mitochondrial dysfunction. Both GO (term: Inner mitochondrial membrane protein complex, mitochondrial membrane part, inner membrane)and Reactome analyses support the peptides inhibitory effects on mitochondrial functions. KEGG analysis identified shared DE genes in the calcium signaling pathway for both PACAP-38 and PACAP(6-38) treatments. Upregulated genes included GnaI and Prkacb, while F2R and Slc25a5 were downregulated, indicating potential mitochondrial involvement.

Discussion

Both PACAP-38 and PACAP(6-38) were shown to elevate intracellular Ca^{2+} levels in the same TG cell cultures[13] supported by the current findings, where alterations in calcium signaling

pathways were observed in response to both treatments. The stimulating effect of PACAP(6-38) was unexpected, given that it is traditionally recognized as an antagonist of PAC1, and VPAC1/2 receptors, as demonstrated in studies using CHO, Cos7 cells, and *Xenopus oocytes*[11,13,51]. Our previous and current findings suggest that PACAP-induced trigeminovascular activation [14]may be involved in migraine in PAC1, VPAC1/2 independent way where mast cells might be involved[5], the MrgB3 receptor was thought as a potential target of both peptides to induce rat meningeal mast cell activation.[11] Elevated intracellular calcium levels can impact mitochondrial function, as indicated by functional enrichment analysis showing mitochondrial alterations linked to dysfunction in the electron transport chain. Notably, the B6 subunit of NADH oxidoreductase (Complex I) was significantly downregulated by both PACAP-38 and PACAP(6-38) treatments. This finding is particularly intriguing as it suggests a potential link between PACAP's effects and migraine, considering that similar metabolic and mitochondrial dysfunctions, such as reduced activity of Complexes I, III, IV, and citrate synthase, have been observed in migraine patients.[52,53] This finding is in line with previously shown result with transcriptome of peripheral blood mononuclear cells of migraine patients also revealed that the mitochondrial electron transport chain was significantly affected in ictal and interictal periods.[50]Mitochondria and the endoplasmic reticulum (ER) are crucial regulators of intracellular calcium homeostasis, which influences neuronal excitability. Mitochondrial dysfunction can increase reactive oxygen species (ROS) production, contributing to nociceptor sensitization via various pathways [54]. Inhibition of mitochondrial complex III in airway C fibers has been shown to enhance excitability through TRP channel and protein kinase C activation.[55,56]Additionally, ROS and mitochondrial DNA release can induce inflammation, further sensitizing nociceptors. Another significant pain-related gene in the differentially expressed list is the upregulation of TRPM8, a menthol- and cold-sensitive ion channel found in dorsal root and trigeminal ganglion cells. [57,58] TRPM8 is expressed in both nociceptive and non-nociceptive sensory neurons and co-expressed with TRPV1 in nociceptive cells [59–61]. Physiologically, TRPM8 detects both innocuous and noxious cold temperatures [62–64], and can reduce nociceptor activation, explaining why cooling or menthol relieves pain. However, TRPM8 has also been implicated in cold allodynia in chronic pain models [65]. Notably, genome-wide association studies have identified polymorphisms near TRPM8 that reduce migraine risk [66–68], and topical menthol application has been shown to alleviate migraine headaches. [69]

2) Transcriptional alterations in the TG induced by HK-1

After 24 hours of exposure to 1 μM HK-1, previously shown to induce calcium influx in trigeminal sensory neurons, Asic3, Grin1, and Ccl7 were downregulated. At the earlier 6-hour timepoint, Slc25a5 was downregulated, while Mag was upregulated. The top genes upregulated at 6 hours, independent of concentration, included Mag, Itga4, and Lbb. In contrast, 500 nM HK-1, which did not induce calcium influx in prior studies, downregulated Slc25a5 at 24 hours and upregulated Ndufb6. Mt-nd6 was downregulated at 6 hours with 500 nM HK-1 treatment. Over time, altered DE genes (at 6 h and 24 h) in response to the same HK-1 concentration highlight critical mechanisms. The 500 nM HK-1 treatment at 24 hours revealed the most DE genes, with Nr4a1, Slc25a5, F2r, Ndufb6, and Gnb2 all upregulated, confirmed by qPCR (FC: 1.726-; 1.783; 2.205; 2.105; 1.445). At 6 hours post 1 μM HK-1 treatment, Itga4, Fgf5, and Gnai1 were upregulated, while Ndufb6, Gnb2, and F2r were downregulated, confirmed by both sequencing and qPCR (FC: 2.942; 3.652; 1.14; −1.15; −1.01; −1.08). Fgfr1 and Gnai1 remained unchanged based on RNA sequencing and qPCR (FC: 1.385; 1.365) after 24 hours with 1 μM HK-1. However, in the 24-hour 500 nM HK-1 group, Fgfr1 was downregulated according to RNA sequencing but upregulated by qPCR (FC: 1.18). In response to 1 μM HK-1, Itga4, Antxr2, and Tenm3 displayed similar expression changes. Only one DE gene, Cxcl9, was downregulated after 500 nM HK-1. The 24-hour results suggest a stronger concentration-dependent effect of HK-1, with fewer common DE genes across groups at different timepoints: 30 upregulated and 6 downregulated genes at 6 hours, and 3 upregulated and 5 downregulated at 24 hours. At 6 hours, Antxr2 and Itga4 were upregulated regardless of concentration, along with Prss12, Mal, and Mag, while Scn4b was downregulated. At 24 hours, concentration-independent upregulation included Fzd1 and Hacd2, while Rph3a, Gabra2, Ryr2, Mag, and Scn1a were downregulated. At 24 hours with 1 μM HK-1, Kcnip4 and Mbp were downregulated, while Gpr108 was upregulated. At 6 hours, 1 μM HK-1 upregulated Fgf9, Ndufb6, Myef2, Mpzc, and GDNF. Interestingly, 500 nM HK-1 downregulated PACAP and upregulated Pmp2 and GDNF at 6 hours, as well as Tmem128 and Itgav at 24 hours.

Potential Targets for HK-1

To explore potential target molecules, we identified receptors associated with neural and inflammatory mechanisms using gene databases. One notable receptor, the MAS-related G protein-coupled receptor B5 (Mrgprb5)**,** was present in all groups, although its TPM was below 2.

The receptor expression levels were consistent across both timepoints. Rack1**,** Ngfr, and Ednrb had high TPM values at both sampling times, and Tnfrsf12a**,** Adipor1**,** and Adipor2 were expressed at both concentrations. Noteworthy receptors like Ntrk1**,** P2rx3**,** and F2r were also expressed, suggesting a role in pain transmission and calcium ion regulation. Cxcr4 was detected only at 6 hours. Adipor2 was downregulated at 500 nM after 24 hours, while F2r was upregulated at 1 μM for 6 hours and again at 500 nM for 24 hours. In addition to F2r**,** Egfr was also upregulated at 1 μM after 6 hours. A suprising finding was the change in expression of Trpm3**,** Trpm7, and Trpm8 cation channels at 500 nM after 24 hours.

Signaling Pathways affected by HK-1 Treatments

Analysis of pathways significantly altered 6 hours after treatment with 500 nM and 1 μM HK-1, based on the KEGG database, revealed that the most impacted pathways were predominantly linked to calcium signaling and Wnt signaling Among the receptors, F2r showed downregulation, while Egfr was upregulated. On the transcriptomic level, genes such as Slc25a5 and Prkaca (protein kinase CAMP-activated catalytic subunit alpha) exhibited negative changes, whereas Gna11 (G protein subunit Alpha 11) and Prkacb (protein kinase cAMP-activated catalytic subunit beta) were positively regulated. For HK-1 1 μM, 6 h, GO terms were protein kinase inhibitor activity, protein kinase A regulatory subunit binding, and thyroid hormone receptor binding the most interesting. The synaptic cleft was a significant cellular component, independent of HK-1 concentration. In the case of HK-1 500 nM, 6 h, key biological processes were adenylate cyclase-activating GPCR signaling, positive regulation of T cell-mediated immunity, neutrophil chemotaxis, and leukocyte adhesion to vascular endothelial cells, suggesting potential immunological effects. Cellular components included the synaptic cleft, T-tubule, and myelin sheath, while molecular functions like adrenergic receptor binding, NADH dehydrogenase activity, and cAMP binding were notable. For HK-1 1 μM, 24 h pathways, like glutamate and insulin receptor signaling were affected, with intracellular responses to calcium ions. The synaptic vesicle was a key cellular component, and molecular functions included ligand-gated ion channel activity, postsynaptic neurotransmitter receptor activity, and sodium channel activity. For HK-1 500 nM, 24 h processes like presynapse assembly, mitochondrial ATP synthesis, Schwann cell proliferation, and dendritic spine development were regulated. Molecular functions included palmitoyltransferase activity, FGF binding, and oxidoreductase activity. Reactome pathway database, highlighting the DE genes for various HK-1 treatment groups: 1 μM at 6 h, 500 nM at 6 h, and 500 nM at 24 h. No significant findings were reported for the 1 μM HK-1 24 h group. Key

pathways for 1 μM HK-1 at 6 h include apoptosis, signal amplification, programmed cell death, G alpha (s) signaling events, chaperone-mediated autophagy, and opioid signaling. For the 500 nM HK-1 6 h group, additional important pathways were ADP signaling through P2Y purinoreceptor 12, oxidative stress-induced senescence, GABA receptor activation, and opioid signaling. Common to both 1 μM and 500 nM at 6 h was the G alpha (s) signaling events, indicating potential concentration- and calcium influx-independent effects of HK-1. Notable pathways exclusive to 500 nM HK-1 at 6 h included GPCR signaling, GPCR ligand binding, and glycosphingolipid metabolism.

Discussion

To our knowledge, we presented the first transcriptomic data on the signaling pathways of HK-1 in rat primary sensory neurons, focusing on potential HK-1 targets, mechanisms of action, and DE genes related to pain transmission and inflammation. The effects of HK-1 are both concentration- and time-dependen. This neuropeptid affects not just primary sensory neurons, but satellite glial cells also, this research provides insights into their interaction, aiming to reflect in vivo conditions. [70,71]

Cxcl9 downregulated at 500 nM at both time points. CXCL9, a chemokine, is a known mediator of nociception.[72] The CXCR3 receptor, activated by CXCL9, is involved in glia activation and pain modulation.[73,74]Upregulation of Prss12**,** Mal**,** and Mag**,** alongside downregulation of Scn4b, was observed 6 hours post-treatment with both HK-1 concentrations. Prss12 (motopsin) can activate astrocytic PAR receptors, triggering neuronal NMDA receptor activation.[75,76] Mal is primarily expressed by oligodendrocytes and Schwann cells, inhibiting peripheral nerve myelination,[77] while Scn4b, a subunit of voltage-gated sodium channels, plays a crucial role in action potential generation and chronic pain pathologies.[78]Fzd1 upregulation in both concentration at 24 h, associated with astrocyte cross-talk, may also play a role in pain processes. [79,80] The fibroblast growth factor 9 (Fgf9**)** downregulation observed in this study aligns with findings from HK-1-deficient mice.[81] Fibroblast growth factors (Fgfs) are involved in neuron–glia interactions and glial proliferation, contributing to neuroinflammatory processes [82] A protein, the integrin subunit alpha 7 (Itga7), playing a role in glial proliferation,[83] was downregulated both in the mouse model[81] and the study, where Itgav—expressed in glial cells—also showed

downregulation.[84]Kcnj9 and F2rl2 (PAR3) upregulation in the DRG under neuropathic pain conditions was found by Stevens and his colleaguges, paralleling our findings for F2r and PAR1.[85]

Interestingly, the expression of Tacr genes encoding tachykinin receptors was near the detection limit, consistent with earlier studies on human primary sensory neurons[86] made by Linnarson and co-workers on dorsal root ganglion (http://linnarssonlab.org/drg/) potentially raising the question of other possible target receptors.

3) Metabolomics and transcriptomics results of CFA induced orofacial pain in Wistar rat

CFA induces facial allodynia 3 days after the injection

CFA treatment significantly decreased the mechanonociceptive thresholds compared to sine-treated control rats on day 3. No changes in the contralateral/saline threshold were observed in the whisker pad area as previously shown.[48,81]

The untargeted analysis highlighted 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. 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 decreased in both ion modes significantly.

CFA-induced orofacial inflammation alters not just lipid, but amino acid and monoamine profile of the plasma determined by targeted metabolomic analysis

LPC 17:0, LPC 18:2, LPC 20:3 were discriminating in all three measurements: targeted, and in both ion mode in both untargeted measurement. According to nonparametric Kruskal - Wallis test, p values were $p<0,1$ for the following metabolites: Alanine, Asparagine, Histidine, Isoleucine, Leucine, Phenylalanine, Proline, Tryptophan, Asymmetric dimethylarginine, Indoleacetic acid, Cer d18:1/24:0, Cer d18:1/25:0, PC 26:0, PC 32:2, PC 32:3, PC 34:3, PC 34:4, PC 36:1, PC 36:3, PC 36:4,PC 36:5, PC 36:6, PC 38:0, PC 38:3, PC 38:4, PC 38:5, PC 38:6, PC

40:1, PC O-38:0, PC O-38:4, PC O-40:1, PC O-40:2, PC O-40:4, PC O-40:5, PC O-40:6, PC O-42:0, PC O-42:1, PC O-42:2, PC O-42:3, SM 41:2, TG 16:0_34:4, TG 18:0_36:5, TG 18:3_32:0.

Significantly altered genes in TG of rat.

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, however AABR07062758.1, AABR07026233.1, fibronectin type III and SPRY domain 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.

Pathway analysis of metabolites and TG genes in IPA software by Qiagen

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, γ-linolenate Biosynthesis II (Animals), Mitochondrial L-carnitine Shuttle Pathway, Tryptophan Degradation III (Eukaryotic), Glyoxylate metabolism and glycine degradation, Fatty Acid β-oxidation I, Nucleotide catabolism were found significantly altered.

Discussion

Our analysis identified altered tryptophan catabolism and the biosynthesis of alanine and glycine III. This supports earlier evidence of reduced amino acid metabolism and biosynthesis (including alanine, phenylalanine, aspartate, glutamate, tryptophan, tyrosine, valine, leucine, and

isoleucine), as well as decreased levels of lipids (glycerolipids, glycerophospholipids, sphingolipids) in the urine of rats with CFA-induced inflammatory pain.[87] In the kynurenine pathway metabolites with proinflammatory, anti-inflammatory, oxidative, antioxidative, neurotoxic, and neuroprotective properties are processed, with enzymes such as indoleamine 2,3-dioxygenase, tryptophan 2,3-dioxygenase, and others playing significant roles in influencing immune and inflammatory mechanisms.[88] Migraine patients have shown reduced levels of kynurenine metabolites (L-kynurenine, kynurenic acid, and others) in plasma.[89] Notably, while 5-hydroxy-indoleacetic acid levels were elevated in the plasma of migraine patients during ictal phases, [89] we observed lower indole derivatives in CFA-treated rats, highlighting their relevance in acute headache attacks rather than chronic conditions. CFA treatment also resulted in changes in lipid mediators, such as fatty acids, dihydroceramide, cholesterol esters, and lysophosphocholines. These results are consistent with alterations observed in CFA-treated rat DRG, where glycerophospholipid, retinol, linoleic acid, and arachidonic acid pathways were significantly affected.[90] Glycerophospholipid metabolism, including arachidonic acids and polyunsaturated fatty acids, is crucial for proinflammatory signaling. [91] Polyunsaturated fatty acids can form oxidized lipids that promote inflammatory pain [92] and mitochondrial dysfunction via egeneration reactive oxygen species, that is key process in migraineurs. [93] In a large cohort study, migraine was associated with altered lipid metabolism, where apolipoprotein A1, HDL, and free cholesterol were reduced.[94] Additionally, elevated non-alpha-hydroxy-sphingosine ceramides and reduced lysophosphatidylethanolamines were noted in migraineurs,[95] which align with our OFP rat model results. Conversely, higher levels of CE(20:4), CE(18:2), and others were observed in osteoarthritis pain models,[96] suggesting different mechanisms in degenerative conditions. Pathways involving tryptophan, arginine, and proline metabolism, as well as aminoacyl-tRNA biosynthesis, are known to be associated with migraine,[97] supporting our observations. Migraine patients, however, typically have low serum serotonin levels. [95] Notably, carnosine, spermine, and spermidine were found at lower levels during migraine attacks. [50]

4) Metabolomical pattern of migraineurs and integrated analysis with PBMC transcriptomics in IPA

Significant metabolites with different statistical test varies

After PCA clustering univariate statistical analysis using one-way ANOVA (FDR correction) with post-hoc Tukey HSD test was performed. PC aa C34:1, PC aa C38:3, SM C26:1, PC aa C32:0, PC aa C36:3, PC aa C42:4, SM C24:1, Arachidonic Acid (AA), DiCA(14:0) were found marginally signicant among the groups. Using t-test for interictal and healthy groups PC aa C32:0, PC aa C34:1, PC aa C36:3, PC aa C38:3, SM C26:1, PC aa C38:5, PC aa C40:6, PC aa C34:3, PC aa C38:6, SM C24:0, Hex3Cer(d18:1/16:0), DiCA(14:0), PC aa C42:4, PC ae C40:2, SM C24:1, AA, PC aa C36:4, PC aa C42:6, HexCer(d18:1/16:0) were found significant and also this result was strengenthed with randomized statistical t-test. No marginal significant difference was observed among ictal and interictal patients potentially caused by very low sample number in ictal phase.

Pathway analysis with significant metabolites and earlier PBMC transcriptomic result

Earlier published PBMC transcriptomic data[50] was used for further IPA analysis with the smarginally significant metabolites identified with t-test. The main canonical pathways from integrative analysis in IPA among the interictal and healthy group were: Interleukin-10 signaling, NGF-stimulated transcription. They were upregulated, Granulocyte adhesion and diapedesis did not change, however, IL-10 signaling, FXR/RXR activation, LXR/RXR activation and PPAR signaling pathways decreased. Significantly increased routes were Class A/1 (Rhodopsin-like receptors), S100 family signaling pathway, IL-17 Signaling.

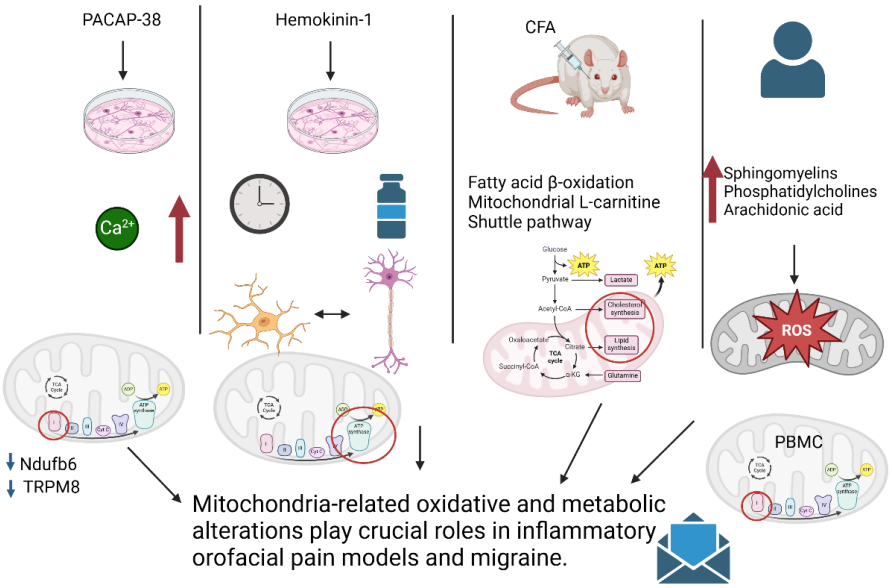
Discussion

Marginally significant metabolites were identified between groups, based t-test and were analyzed also with ANOVA FDR correction followed by Tukey post-hoc HSD, and randomization tests among interictal and healthy group. These metabolites have potential relevance in distinguishing the conditions under study. Metabolites identified as marginally significant by all test: PC aa C32:0, PC aa C34:1, PC aa C36:3, PC aa C38:3, PC aa C42:4, SM C24:1, SM C26:1 between interictal and healthy group, that is in line with adjusted sphingomyelin species SM18:0 and SM18:1 were associated with an increased migraine risk [98] Fatty acids and lipid metabolites, particularly PC and SM, are often involved in signaling, and inflammatory responses. The significance of metabolites like AA and SM C24:1 could indicate altered lipid metabolism or signaling pathways, potentially related to the physiological or pathological states of the migraine

condition. This is in line with earlier finding AA to be higher in cluster type headache patient's serum, [99,100] and with reactive oxygen species production from AA leading to mitochondrial dysfunction. [101]

The main upregulated canonical pathways from integrative analysis in IPA among the interictal and healthy group were: IL-10 signaling, NGF-stimulated transcription, Class A/1 (Rhodopsin-like receptors), S100 family signaling pathway, IL-17 Signaling, while Granulocyte adhesion and diapedesis did not change, however, IL-10 signaling, FXR/RXR activation, LXR/RXR activation and PPAR signaling pathways downregulated. These alterations cover neuroinflammation processes, energy production and other metabolisms alterations. Olfactory transduction and mitochondrial dysfunction were higlighted, that is in line with other findings [50] and with the fact odor or taste is one of the most common triggers of migraine.[102]

SUMMARY OF NEW FINDINGS



Shematic representation of new findings. Created in https://BioRender.com

— PACAP-38 elevates intracellular Ca^{2+} level and triggers related signaling events, which is likely to be independent of PAC1 and VPAC1/2 activation, since PACAP(6-38) know to be an antagonist at these targets induces similar alterations. Transcriptomic changes mainly demonstrate mitochondrial dysfunctions such as downregulated Ndufb6 and TRPM8.

— HK-1 exerts concentration-and duration-dependent effects on TG primary sensory neurons. Altered mitochondrial ATP synthesis, oxidoreductase activity, other pathways like positive regulation of T cell-mediated immunity, neutrophil chemotaxis, and leukocyte adhesion to vascular endothelial cells were detected suggesting potential immune-modulating and glia-, Schwann cells- and macrophages-related effects impacting the role of HK-1 in glia-neuron communications.

— In the inflammatory orofacial pain rat model reduced amino acid metabolism and biosynthesis, as well as decreased lipid metabolism potentially linked to decreased mitochondrial processes is observed in the plasma.

— In the interictal plasma samples PCs, SMs, arachidonic acid of migraineurs marginally significantly were increased. We could not detect any marginally significant metabolomic changes in ictal phase possible due to low sample size.

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List of Publications

Takács-Lovász K, Kun J, Aczél T, Urbán P, Gyenesei A, Bölcskei K, Szőke É, Helyes Z. PACAP-38 Induces Transcriptomic Changes in Rat Trigeminal Ganglion Cells Related to Neuroinflammation and Altered Mitochondrial Function Presumably via PAC1/VPAC2 Receptor-Independent Mechanism. Int J Mol Sci. 2022 Feb 14;23(4):2120. doi: 10.3390/ijms23042120. PMID: 35216232; PMCID: PMC8874739.

IF: 5,6 Quartile: Q1, D1

Takács-Lovász K, Aczél T, Borbély É, Szőke É, Czuni L, Urbán P, Gyenesei A, Helyes Z, Kun J, Bölcskei K. Hemokinin-1 induces transcriptomic alterations in pain-related signaling processes in rat primary sensory neurons independent of NK1 tachykinin receptor activation. Front Mol Neurosci. 2023 Oct 27;16:1186279. doi: 10.3389/fnmol.2023.1186279. PMID: 37965042; PMCID: PMC10641776.

IF:3,5 Quartile: Q2 (Q1 at the time of submission)

Takács-Lovász K, Aczél T, Mohos V, Harmath M, Pirkuliyeva J, Karvaly G, Farkas R, Ciborowski M, Godzien J, Bölcskei K, Kun J. and Zsuzsanna Helyes. Altered aminoacid and lipid metabolism in a rat orofacial inflammation model determined by omics approach: potential role in trigeminal sensitisation. In The Journal of Headache and Pain accepted for publishing on 01.04.2025.

IF: 7,3 Quartile: Q1

Cumulative IF: 16,4

Other publication

Zalai D, Hevér H**, Lovász K,** Molnár D, Wechselberger P, Hofer A, Párta L, Putics Á, Herwig C. A control strategy to investigate the relationship between specific productivity and high-mannose glycoforms in CHO cells. Appl Microbiol Biotechnol. 2016 Aug;100(16):7011-24. doi: 10.1007/s00253-016-7380-4. Epub 2016 Feb 24. PMID: 26910040; PMCID: PMC4947490.

IF:3,7 Quartile: Q1

Cumulative IF: 20,1

Number of citation (MTMT): 24

Presentations related to this thesis

Year	Abbreviation of the conference	Poster/Oral presentation	Title
2021	MEDPECS2021 Medical Conference for PhD students	Poster	Transcriptomic changes in trigeminal ganglion cells induced by pituitary adenylate cyclase-activating polypeptide (PACAP)-38 or PACAP6-38 treatment
2022	ICBEI2022 International Conference of Biomedical Engineering and Innovation	Poster	Hemokinin-1-induced transcriptomic alterations in rat trigeminal ganglion primary sensory neurons related to pain signalling
2023	FAMÉ Hungarian Society of Experimental and	Poster	Altered aminoacid, monoamine and glycerophospholipid metabolite profile in the rat plasma in an orofacial inflammatory pain model

	Clinical Pharmacology International bi-yearly conference		
2023	MOFT Magyarországi Fájdalomtársaság 2023-as évi kongresszusa	Poster	Megváltozott aminosav-, monoamin- és glicerofoszfolipid-metabolit-profil a patkányplazmában egy orofaciális gyulladásos fájdalommodellben
2024	INC2024 International Neuroscience Conference	Poster	Altered purine, fatty acid and ester, amino acid and hormone profiles in migraineurs during the ictal and interictal periods
2024	HUPHAR2024 Hungarian Society of Experimental and Clinical Pharmacology	Poster	Altered aminoacid, downregulated glycerophosho- and sphingolipid, and upregulated fatty acid metabolite profile in a rat model of inflammatory orofacial pain
2024	4th Nordic Metabolomic Conference	Poster	Metabolomic Changes and Combined Analysis with Differentially Expressed Trigeminal Genes in a Neuroinflammatory Animal Model
2024	MOFT Magyarországi Fájdalomtársaság 2024-as évi kongresszusa	Presentation	Plazma metabolomikai és trigeminus ganglion transzkriptomikai változásainak kombinált elemzése orofaciális gyulladásos fájdalom patkánymodelljében