MECHANISMS OF TRIGEMINAL ACTIVATION AND SENSITISATION: IMPLICATIONS FOR MIGRAINE PATHOPHYSIOLOGY

DOCTORAL (PhD) THESIS

Timea Aczél PharmD

Science of Pharmacology Doctoral School
Neuropharmacology Program

Program Director: Erika Pintér MD, PhD, DSc

Supervisor: Kata Bölcskei MD, PhD

UNIVERSITY OF PÉCS, MEDICAL SCHOOL
DEPARTMENT OF PHARMACOLOGY AND PHARMACOTHERAPY

PÉCS
2022

INTRODUCTION

Headache disorders, migraine

Although activation of the trigeminal system is at the pathophysiological basis of all orofacial and headache disorders, location, attack frequency, duration, accompanying symptoms, and treatment response can be a guide to classify craniofacial pains.[1] Migraine–associated phenomena might include moderate-to-severe unilateral pain of pulsating or throbbing quality and accompanying symptoms such as nausea/emesis or photo-/phonophobia and allodynia.[2] It is a complex spectrum disorder caused by various genetic and environmental aetiological factors occurring in several clinical manifestations with or without aura.[3] As a primary headache disorder, migraine is a common disabling brain disorder affecting up to 15–20% of the general population. According to the *Global Burden of Disease (GBD)* study, headache disorders were enlisted second among females aged 15-49 years.[4]

The pharmacological therapy is not a resolved issue since the frequent use of combined analgesics or the specific antimigraine drugs, triptans, can paradoxically exacerbate the condition (medication overuse headache), and treatment used as prophylaxis may only offer partial relief at the cost of potentially unpleasant adverse effects.[5,6] Therefore, it is evident that identifying potential novel drug targets is needed to advance antimigraine drug development.

The trigeminovascular system (TVS) provides a critical pain-transmission link between the vascular and neuronal elements. This is the major afferent pain pathway between the cranial vessels and the nuclei in the brainstem.[7] A wide variety of triggers initiates migraine, and there is a vast variation between the aetiological factors, clinical manifestations, and severity. Therefore, for decades, there has been a great debate on the predominant importance of the vascular and neurogenic inflammatory mechanisms and peripheral versus central sensitisation processes. Furthermore, the interactions between genetic predisposal and environmental factors also seem critical.[8–10] Among the mechanisms of migraine neuro-vascular alterations, sensory neuropeptide (e.g. calcitonin gene-related peptide – CGRP and substance P – SP) and serotonin (5-hydroxytryptamine- 5-HT) release, neurogenic inflammation, plasma protein extravasation, peripheral and central sensitisation are enlisted. However, the precise pathophysiological mechanisms are still unclear.[11,12] More recently, the CGRP receptor antagonists, 5-HT_{1F} receptor agonists and anti-CGRP monoclonal antibodies have been developed. Fortunately, several of these have been approved for use.[13] Although the newly developed monoclonal antibodies against CGRP or its receptor seem to be effective and safe, the risks of long-term CGRP blockade in migraine patients still needs to be elucidated. Also, little is known about the efficacy and safety during pregnancy or in adolescents.[13] Therefore, understanding the

pathophysiological mechanisms through precise clinical and translational research approaches is crucial to identify key mediators and determine novel therapeutic targets.

Overview of the trigeminovascular system

Cell bodies of the pseudounipolar primary afferents lie in the trigeminal ganglion (TG), the first centre of the pain processing course, through which stimuli from extra and intracranial structures are transmitted further to the trigeminocervical complex (TCC). TNC and C1-C2 regions of the cervical spinal cord together form the TCC. The trigeminal nerve enters the brainstem at the pontine level, from where orofacial nociceptive stimuli are transmitted to second-order neurons. Afterwards, information is transferred to the third-order thalamocortical neurons.[14,15] Sensitisation of this system may be responsible for developing various orofacial pains and headaches and is also responsible for the development of facial allodynia. Activation of primary trigeminal afferents causes the release of vasoactive neuropeptides (e.g. CGRP and SP), thus causing vasodilatation, protein extravasation, and neurogenic inflammation.

Sensory ganglia contain the cell bodies of primary afferents that transmit sensory information from the periphery into the central nervous system (CNS). TG is localised at the base of the skull, outside the blood-brain barrier and their sensory neurons innervate the facial region.[16] The TG mainly includes soma and axons of primary afferent neurons, enveloped by satellite glial cells (SGC).[17] A significant percentage of trigeminal ganglia nociceptive sensory neurons are peptidergic, thus expressing various neuropeptides like tachykinins (SP and neurokinins) and CGRP in response to activation.[18–20] These neuropeptides constitute the main focus of primary headaches, like migraine research. Neuropeptides can be released from primary sensory neurons' peripheral and central endings, contributing to inflammatory processes and pain transmission.[21,22]

Sensitisation, trigeminovascular activation

The terminology of sensitisation is described by the International Association for the Study of Pain (IASP) as follows: "Increased responsiveness of nociceptive neurons to their normal input, and/or recruitment of a response to normally subthreshold inputs". Clinically, this may stand behind hyperalgesia, when increased pain might be felt due to a painful stimulus, and allodynia, when pain is given rise by a stimulus that does not normally provoke pain. Peripheral and central sensitisation exists depending on which type of neuron is involved. Cutaneous allodynia reported in chronic migraineurs, orofacial inflammation, or nerve injury manifests due to trigeminal sensitisation. In this process, the sensitisation of primary afferents and the second-order or third-order thalamic neurons develop. As a background for central sensitisation in chronic pain, synaptic plasticity, an imbalance

between excitatory and inhibitory neurotransmitters, and lately glia–neuron interaction have been enlisted.[23,24]

Role of glial cells in sensitisation

Due to inflammatory processes, the hyperactivation of TG neurons occurs, followed by activation of non-neuronal glial cells and macrophages and cytokine production. Glial mediators further promote neuronal sensitisation. Thus, understanding neuron-glia interaction and crosstalk is relevant in orofacial pain and headache development.[23,25]

Glial cells are non-neuronal cells of the peripheral and central nervous system (PNS, CNS) that regulate the neuronal microenvironment, taking part in the nutrition, structural maintenance of neuronal networks, and modulation of neuronal excitability. Oligodendrocytes, astrocytes, ependymal cells, and microglia are part of the CNS, while peripheral ganglions contain Schwann cells, SGCs and resident microglia-like macrophages.[26,27]

SGCs share most of the characteristics of astrocytes, for example, contributing to neuronal nutrition and functioning and maintaining the environment around neurons. Similar to astrocytes under a normal state, SGCs contains low levels of Glial Fibrillary Acidic Protein (GFAP). Still, inflammation and nerve injury cause massive expression of GFAP, observed in various orofacial pain models[14,28], including migraine.[29] Among various mediators, CGRP has been suggested to have a role in the crosstalk between neurons and glial cells.[30] Enhanced P2X7 signalling in SGCs and elevated intracellular Ca^{2+} concentration may be in the background of orofacial allodynia following peripheral nerve injury and inflammation. The increased intracellular Ca^{2+} concentration led to the synthesis and release of proinflammatory cytokines: tumour necrosis factor-alpha (TNFα) or interleukin 1-beta (IL-1β) from SGCs in the trigeminal nerve injury model. Further, IL-1β binds to its receptor in small-diameter TG neurons, increasing neuronal excitability.[30,31]

Microglia represents the principal resident immunocompetent cells in the brain sharing monocytes and macrophages' phenotypic markers and features.[32] Immune cells seem to contribute to pain hypersensitivity[33]; therefore, activation of microglial cells also occurs during orofacial inflammation. Activated microglia can release various reactive molecules and regulate neuronal excitability, such as pronociceptive cytokines: TNFα, interleukins (IL-1β and IL-18), and brain-derived growth factor (BDNF), thereby modulating pain processes.[34] Proteins, like ionised calcium-binding protein (Iba1) and CD11b, are typically used as markers of proliferation and morphologic activation of microglial cells/macrophages.[32] Extensive macrophage infiltration in the TG beside resident microglia-like macrophage might occur due to trigeminal nerve injury and inflammation.[23,26]

The tachykinin family

Tachykinins are among the neuropeptides contained in peptidergic primary afferent neurons with relevant roles in neurogenic inflammation and nociceptive transmission.[22] Important tachykinin members are SP and neurokinin A (NKA), derived from *Tac1*, neurokinin B (NKB), coded by *Tac3*, and hemokinin-1 (HK-1) by *Tac4*. The three tachykinin proteins act through the G-protein coupled neurokinin NK-1, NK-2, and NK-3 receptors.[35,36] NK-1 receptor antagonists were proved to reduce neuropathic mechanical hyperalgesia and inflammatory pain in animal models.[37,38] Nevertheless, human clinical studies could not prove the analgesic effect of these compounds, either in migraine[39] or other conditions like post-operative dental pain[40] or neuropathic pain.[41]

Hemokinin-1

The discovery of HK-1 encoded by the *Tac4* gene[42] raised new questions in tachykinin research. HK-1 might be a novel essential molecule in pain and inflammatory processes.[43] There is a growing amount of information regarding the mRNA expression of the *Tac4* gene both in the PNS and CNS. In contrast with other tachykinin members, relatively high expression of the *Tac4* gene can be found in peripheral non-nervous tissues, such as the lung, spleen, adrenal gland and immune cells like B and T lymphocytes, macrophages, and dendritic cells.[42,44] *Tac4* gene predicts one homologous transcript in mice and rats, but several peptide isoforms can be found in humans. HK-1 binds to all tachykinin receptors, presenting the highest affinity to the NK-1 receptor.[45] However, HK-1 also has distinct, NK-1-independent actions.[46] This could be explained by the activation of different signalling pathways by HK-1 compared to SP or by the suspected existence of a specific receptor for HK-1.[46] Therefore, since the receptor affinities of HK-1 are not precisely known, antagonists cannot be used to validate the target.

HK-1 produced a pronociceptive effect after intrathecal or intracerebroventricular administration in rats/mice, causing pain and scratching behaviour without influencing the withdrawal latency to a noxious heat stimulus.[47,48] However, an analgesic effect was shown in other studies using similar concentrations upon intracerebroventricular injection in mice.[49,50] Upregulation of *Tac4* mRNA expression in microglia upon lipopolysaccharide stimulation[51] and in the dorsal spinal cord of rats after complete Freund's adjuvant (CFA)-induced paw inflammation[52] has been described, suggesting a possible role in neurodegenerative and neuroinflammatory disorders. However, little is known about the *Tac4* gene / HK-1 in the trigeminovascular system.

Animal models in trigeminal sensitisation

Several reviews describe validated animal models of pain relevant for headache. Direct electrical stimulation of trigeminal neurons, administration of inflammatory, algogenic substances (inflammatory soup) to the meninges, exogenous administration (e.g. nitroglycerin, PACAP) chemicals, cortical spreading depression induction, medication overuse models of headache models present a wide range of animal models to choose from. Behavioural assays are performed besides electrophysiology, flowmetry, and various marker detection using immunohistochemistry. These reflect migraine-like phenomena such as mechanical allodynia, sensitivity to light, and altered overall spontaneous response activity.[53,54]

A frequently used inflammatory animal model applies CFA.[55,56] CFA injected in the whisker pad of rodents results in inflammation and mechanical hyperalgesia/allodynia on the orofacial region.[57] Although it is not considered a typical migraine model, it can be used as a trigeminal activation model, having the advantage of being reliable and highly reproducible. Besides the enlisted methodologies, transcriptomic analysis allows an unbiased approach to reveal critical pathways responsible for the pathophysiological changes.[58] Microarray analysis after CFA injection in the whisker pad[59] or masseter muscle[60] had been performed.

Peripheral blood mononuclear cells (PBMCs) isolated from peripheral blood consist of lymphocytes (T cells, B cells, natural killer cells) and monocytes. Due to non-invasive sampling and relatively simple isolation, PBMCs became attractive biological marker candidates in clinical practice. They are considered biological materials capable of reflecting pathophysiological changes in the CNS in various diseases. Neuroinflammatory processes can be characterised in a specific way using PBMCs. In addition, it has provided new opportunities for biomarker research.[61,62]

AIMS

1. We aimed to follow the temporal changes of facial mechanonociceptive thresholds and gene expression in TG, TNC, and PBMCs in parallel after CFA inflammation in rats using microarray and RT-qPCR analysis to get a better insight into the pathomechanism of trigeminal pain disorders.
2. To identify disease- and headache-specific pathophysiological pathways and possible therapeutic targets, we aimed to analyse the transcriptome of PBMCs of migraineurs in a self-controlled manner during and between attacks.
3. Since the role of HK-1/*Tac4* in the trigeminovascular system and facial pain have been poorly investigated, we aimed to explore the potential role of HK-1 in the trigeminal environment. We aimed to detect the changes of expression of *Tac4* in the TG in a rat inflammatory orofacial pain model and investigate behavioural alterations and gene expression variations of selected markers of neuronal sensitisation and neuroinflammation in the same model in mice, comparing wild-type and *Tac4*-deficient (*Tac4*$^{-/-}$) mice.
4. We aimed to describe the expression of the *Tac4* gene on glial cells in an in vitro set-up and the effect of HK-1 in mixed glial cell culture to get a better insight into the potential impact of HK-1 on neuron-glia communication and its molecular pathways.

MATERIALS AND METHODS

Animals

Experiments were performed on male Wistar rats (Toxicoop, Hungary) weighing 200–300 g and on male, 8-12 weeks old C57Bl/6 and *Tac4* gene-deleted (*Tac4*$^{-/-}$) mice weighing 20–25 g. Animals were kept under standard conditions. Standard diet and water were provided *ad libitum* in the animal house of the Pécs University Department of Pharmacology and Pharmacotherapy.

All experiments were approved by the National Ethics Committee on Animal Research (license No.: BA02/2000-9/2011 and BA02/2000-7/2018) and were carried out according to the European legislation (Directive 2010/63/EU) and Hungarian Government regulation (40/2013., II. 14.) on the protection of animals used for scientific purposes.

Orofacial inflammatory pain model

Induction of the local inflammatory state was achieved by unilateral s.c. injection of 50 µl complete Freund's adjuvant (CFA; Sigma-Aldrich, Saint Louis, USA; killed Mycobacteria suspended in paraffin oil; 1 mg/ml) into the whisker pad of male rats and bilateral s.c. injection of 10-10 µl CFA into the whisker pad of the mice under ketamine (rats: 72 mg/kg, mice: 100 mg/kg) and xylazine (rats: 8 mg/kg, mice: 5 mg/kg) anaesthesia. The same volume of saline injection was given in the case of control groups, both mice and rats.

Microarray analysis

Gene expression was analysed using Agilent microarray platforms. Rat TG tissue samples were collected from animals seven days after receiving s.c. CFA injection. Contralateral sides of CFA-injected rats were controls. Total RNA was isolated from snap-frozen samples using the RNeasy Mini Kit (Qiagen, Carlsbad, CA), and high-quality samples (RIN>8.0) were further used for expression analysis. Sample labelling, array hybridisation and primary data analysis were performed by ArrayStar Inc. (Rockville, MD, USA).

Mechanonociception measurement

A set of nylon von Frey monofilaments (Stoelting, Wood Dale, Illinois, U.S.A) was used to perform mechanical pain thresholds measurements on the orofacial region on days 0 (control day) and one, three, seven days after CFA or saline administration. Rodents were lightly restrained using a soft cotton glove to allow easier habituation. Out of five stimulations, the mechanonociceptive threshold was determined as the lowest force evoking at least two withdrawal responses (face stroking with the forepaw or head shaking).

Spontaneous motor activity measurement

The open field test was used to assay mice's spontaneous activity and anxiety levels. The mice were placed into the same area of a brightly lit observation box (60 cm x 40 cm), then the behaviour of mice was recorded for 10 min. Recorded videos were evaluated using Ethovision software (Noldus Information Technology, Wageningen, Netherlands).

Sample collection and handling

For RT-qPCR analysis, TG and TNC tissue samples were collected from animals one, three, seven days after receiving s.c. CFA injection, following behavioural experiments. Animals were anaesthetised using pentobarbital (rats: 50 mg/kg and mice 70 mg/kg: i.p.). Blood was drawn by cardiac puncture and further processed as follows in the *Isolation of peripheral blood mononuclear cells* section. After exsanguination, animals were decapitated, and TGs and TNCs were excised, immediately snap-frozen in liquid nitrogen, and stored at -80°C.

For the RNAscope method, animals were transcardially perfused with 0.01 M phosphate-buffered saline (PBS; pH 7.6) followed by 4% paraformaldehyde on day three after CFA or saline injections. 5 µm sections were cut using a sliding microtome (HM 430 Thermo Fisher Scientific, USA).

TG samples for Nanostring analysis were snap-frozen on day three and stored at −80°C until use. RNAscope and Nanostring analysis were performed on tissues from animals not involved in behavioural studies.

Peripheral blood mononuclear cell isolation

PBMCs were purified from fresh peripheral blood according to the Ficoll-PaquePREMIUM manufacturer's instructions (GE Healthcare, Budapest, Hungary). The obtained cells were resuspended with 1 ml of TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and stored at −80°C until further process.

Quantitative Real-Time PCR (RT-qPCR)

According to the TRI Reagent manufacturer's protocol, total RNA isolation was carried out. RNA was purified using the Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA). The quantity and quality of the RNA samples were checked on Nanodrop ND-1000 Spectrophotometer V3.5 (Nano-Drop Technologies, Inc., Wilmington, DE, USA). Total RNA was reverse transcribed using Maxima First Strand cDNA Synthesis Kit (ThermoScientific, Santa Clara, CA, USA). PCR amplification was performed with SensiFast SYBR Lo-ROX Kit (Bioline, Taunton, USA). The best-suited reference genes or combinations for normalisation were selected using geNorm: *Ppia* and

Hprt1 for PBMCs and *β2m, Hprt1* for TG and TNC rat; *Ppia* for TG and *Ppia*, *Gapdh* for PBMCs and TNC mice samples.

RNAscope in situ hybridisation (ISH)

RNAscope technique was performed on 5 µm thick longitudinal rat and mice TG sections processing with RNAscope Multiplex Fluorescent Reagent Kit v2 (ACD, Hayward, CA, USA). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted with ProLong Glass Antifade Mountant for confocal imaging. Fluorescent images were taken using an Olympus Fluoview FV-1000 laser scanning confocal microscope (Olympus, Tokyo, Japan) and Fluo-View FV-1000S-IX81 image acquisition software system.

RNAscope was performed on air-dried cytospins prepared from Mixed Glial Cell Cultures (MGCs), according to the manufacturer's protocol (320538 Tech Note).

NanoString nCounter technology

Mouse neuroinflammation panel of 770 genes included for research of immunity and inflammation, neurobiology and neuropathology were described using NanoString nCounter technology (NanoString Technologies, Seattle, WA), according to the manufacturer's instructions. The RNA samples (25 ng of each) were processed using Mus musculus Neuroinflammation panel v1.0 according to the manufacturer's instructions (manual MAN-10023-11) on the NanoString SPRINT Profiler instrument.

Mixed Glial Cell Culture

Primary cell cultures composed of astrocytes, oligodendrocytes, and microglia, free of neurons, meningeal cells, and fibroblasts, were prepared using neonatal mice cortical tissue. After removal of bulbus olfactorius and cerebellum, the whole brains of one-three-day old C57Bl6 mice pups were enzymatically dissociated using Neural Tissue Dissociation Kit (P), following the manufacturer's instructions (Miltenyi Biotec Inc, Auburn, USA). In situ hybridisation by Advanced Cell Diagnostics RNAscope was performed on air-dried cytospins prepared from MGCs. Cultures were treated with mouse HK-1 (500 nM, 1 µM, 5 µM) for the radioactive $^{45}Ca^{2+}$-uptake technique. Inflammatory cytokine measurement was also performed using MILLIPLEX®MAP Kit (Merck KgaA, Darmstadt, Germany) from the supernatants of HK-1 (500 nM, 1 µM, 5 µM) treated glial cells.

Human study

The protocol of the human study was authorised by the National Public Health Center, Ministry of Human Capacities of Hungary (28324–5/2019/EÜIG). All subjects gave their written informed consent according to the Declaration of Helsinki.

Study subjects

Episodic migraineurs with (n=3) or without aura (n=21) between 20 and 65 years were recruited. Migraine patients were included according to the criteria of the third edition of the International Classification of Headache Disorders. Patients with chronic inflammatory diseases and depression were not included in the study. Thirty-six female and 1 male subject were included in the study: 24 episodic migraine patients with or without aura and 13 healthy controls.

Protocol for sample collection

Blood samples were collected from cubital veins of participants into glass tubes containing ethylenediaminetetraacetic acid (EDTA) from migraine sufferers in an attack-free (interictal) period and during an attack (ictal). The attack-free (interictal) sample was drawn if the patient had no headache for at least 24 hours. For ictal samples, no painkiller was taken by patients until the blood sampling. There were no restrictions on fluids and food intake. Features of migraine were assessed using a detailed questionnaire filled out by participants. The PBMCs were isolated using Ficoll-Paque PREMIUM (GE Healthcare, Budapest, Hungary).

RNA extraction procedure and quality control

Extraction and purification of total RNA were performed as previously described (Quantitative Real-Time PCR), adding on-column DNase digestion during purification. RNA concentrations were determined using Qubit 3.0 (Invitrogen, Carlsbad, CA, USA), and quality control of RNA was carried out on TapeStation 4200, using RNA ScreenTape (Agilent Technologies, Santa Clara, CA, USA).

Illumina library preparation and sequencing

The library for Illumina sequencing was prepared using NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB, Ipswitch, MA, USA). Illumina sequencing was performed on the NextSeq550 instrument (Illumina, San Diego, CA, USA) with a 1x76 run configuration.

Chapter 1. Transcriptional alterations in the trigeminal ganglia, nucleus and peripheral blood mononuclear cells in a rat orofacial pain model

RESULTS AND DISCUSSION

CFA induced gene expression changes between ipsi- and contralateral sides in TG on day seven

512 DE (319 up- and 191 downregulated) transcripts were identified between the control (contralateral) and CFA (ipsilateral) TG samples ($p \leq 0.05$; fold change $|FC| > 2$), using microarray analysis. At the top of the list, a lncRNA (MRAK049104; FC 5.20) takes place; however, its function is unknown. *Neurod2,* involved in neurogenic differentiation, was the most downregulated (FC -9.20).

Several biological processes and pathways were shown to be involved in CFA-induced inflammation

Functional enrichment analysis was performed, including the DE genes ($|FC| > 2$, p-value ≤ 0.001) found in contralateral and ipsilateral comparisons, to gain information regarding the biological processes and pathways involved. Steroid and carbohydrate metabolic processes, sensory perception and olfactory transduction were enlisted among terms.

Inflammation caused decreased orofacial mechanonociceptive threshold in rats

The facial mechanonociceptive threshold of the CFA-injected side of rats was significantly decreased compared to the contralateral side starting from day one after injection. The threshold reached its minimum on day three and reversed on day seven.

Inflammation induced alterations in the expression of various genes in rat tissues

Validation of differentially expressed mRNAs in TG

Using quantitative real-time RT-qPCR, the transcription levels of four differentially expressed genes were further determined to validate microarray results: *Lkaaear1,* G-protein coupled receptor 39 (*Gpr39*) (FC 3.04 and 4.01), kisspeptin (*Kiss1*) and kisspeptin-1 receptor (*Kiss1r*), *Neurod2* (-1.74, -2.63 and -9.2). On day seven, *Gpr39* and *Kiss1r* expression changes were similar to the microarray data. However, PCR results could not confirm microarray data on *Lkaaear1, Neurod2* and *Kiss1*. Besides, we could detect *Neurod2* in TNC but not in TG using our RT-qPCR protocol. Although *FosB,* Allograft Inflammatory Factor 1 (*Aif1,* encoding Ionized Calcium-binding Adapter Molecule 1 - Iba1 protein*),* Glial Fibrillary Acidic Protein *(Gfap)* and Calcitonin Related Polypeptide Alpha (*Calca*, encoding CGRP*)* were not enlisted among DE genes, we also analysed the variation of these mRNA levels. On day seven, no significant differences were detected with the PCR method related to these markers, results being in line with microarray data.

Gene expression alterations reached their maximum on day three in TG samples

Using the RT-qPCR method, we measured temporal changes of mRNA levels of eight genes in TG tissues after CFA injection. CFA caused a significant elevation in mRNA levels of *Kiss1r*, neuronal (*FosB*), glial (*Aif1*), and astrocyte (*Gfap*) activation markers compared to the saline treatment on day one. By day three, *Gpr39* (9.18), *Lkaaear1 (*9.97), *Kiss1 (*9.51), *Kiss1r* (14.31), *Calca* (117.82), *FosB* (7.40) and *Gfap* (27.80) reached their maximum. *Aif1* reached a 3.6-fold peak on day one. mRNA levels of *Lkaaear1*, *Kiss1r*, *Aif1* gradually decreased at the last time point until reaching a non-significant level compared to the saline-treated control side.

Some of the genes presented alterations in TNC

Similar trends were seen in TNC samples. Almost all genes showed significantly altered temporal change due to CFA compared to CFA control and saline control groups, presenting a maximum at day three. There was no significant difference case of *Kiss1r* at different time points.

Gene expression alterations were reflected in PBMC samples

Alterations of *Lkaaear1* and *Kiss1r* gene mRNA levels in PBMC were low but significant when CFA was compared to saline. *Lkaaear1* showed a similar pattern to *Kiss1r*, where *Lkaaear1* presented a maximum of 2.33 and *Kiss1r* a 3.86-fold change at day one. There were no significant differences in *Gpr39* expression changes. Interestingly, *FosB* and *Aif1* showed significantly increased levels at each time point due to CFA treatment, while *Gfap* only on day seven.

To our knowledge, these data represent the first comprehensive study on the CFA-induced orofacial inflammatory rat model that includes transcriptional changes in the TG, TNC and peripheral blood mononuclear cells in correlation with behavioural observations. We detected up- and downregulation of several genes, with possible involvement in sensitisation of both primary and secondary trigeminal nerves. The advantage of PBMCs, as an easily accessible material of immune cells, is increasingly recognised in recent literature [62]. Yet, no study has evaluated TG gene expression changes by combining data with peripheral blood mononuclear cell sample measurement and behavioural studies. It is clear that the measurement of *FosB, Aif-1, Gfap, Lkaaear1* changes in the PBMCs does not have a diagnostic value at this stage, and it is too early to conclude. Although, it would be interesting to perform further studies to see whether it could have a predictive value regarding orofacial pain and headache disorders. In addition, it would be worth considering using peripheral blood mononuclear cell isolation in animal models as a translational tool for human studies.

Chapter 2. Identification of disease- and headache-specific mediators and pathways in migraine using blood transcriptomic analysis

RESULTS AND DISCUSSION

Clinical characteristics

Interictal, ictal and healthy groups were without any between-group differences in any demographic and clinical characteristics. Interictal blood samples were collected from all twenty-four migraineurs, while during the attack, eight samples were gathered in a self-control manner.

DE genes derived from interictal vs healthy comparison

Twenty interictal samples were included in transcriptomic analysis.

When interictal was compared to the healthy group, 163 DE genes were found (fold change threshold of 1.5, p-value threshold of 0.05). Among them, the interleukin *IL-1β* gene (*IL1B*), cyclooxygenase 2 (*COX2*), tumor necrosis factor (*TNF*), and numerous chemokines, such as IL-8 (*IL8*) were enlisted.

DE genes derived from ictal vs interictal comparison

In ictal - interictal comparison, 144 DE genes were detected (fold change: 1.3, p-value: 0.05): 64 were upregulated, 80 were downregulated. Heterogeneous nuclear ribonucleoprotein C like 1 (*HNRNPCL1*), olfactory receptor family 10 subfamily G member 2 (*OR10G2*) and interleukin 20 receptor subunit alpha (*IL20RA*) can be found among the top DE gene list. After false discovery rate (FDR) correction, two genes remained on the list, with the adjusted p-values below 0.25: the heterogeneous nuclear ribonucleoprotein C like 1 (*HNRNPCL1*) and the cornichon family AMPA receptor auxiliary protein 3 (*CNIH3*).

DE genes derived from ictal vs healthy comparison

In ictal samples compared to healthy ones, 131 genes were differentially expressed (fold change: 1.5, p-value: 0.05), 118 were upregulated, 13 were downregulated. The *IL1B* gene was also implicated in this comparison, among others, like *PTGS2*, *TNF*, and *IL8*.

Mitochondrial dysfunction was suggested by analysis of migraineurs' samples

Functional enrichment analysis of DE genes and ranked list enrichment of all genes was performed to better insight into functions, networks and biological processes involved in migraine. When interictal was compared to the healthy group, cytokine and chemokine receptor binding, interleukin-10 (IL-10) signalling, and oxidative phosphorylation in the mitochondria were significantly altered. When the ictal group was compared to interictal, hormone and cytokine activity,

oxidative phosphorylation and chemosensory receptors were implicated. Furthermore, the ictal-healthy comparison included IL-4, IL-10 and IL-13, and chemokine, growth factor and neuroactive ligand-receptor interactions. The ranked list enrichment analysis of all genes statistically significantly implicated the metabolic pathway of oxidative phosphorylation in interictal-healthy and ictal-interictal comparison of the PBMC samples. Mitochondrial functioning was affected in both comparisons.

To our knowledge, this is the first study to perform transcriptome analysis of PBMCs isolated from migraine patients, including samples taken in an attack-free period and during headaches. Comparing these groups with healthy controls made possible the identification of both disease-specific and headache-specific alterations and revealed the importance of inflammatory pathways and the potential contribution of various cytokines to migraine susceptibility. Furthermore, our results suggest the possible implication of mitochondrial dysfunction, oxidative stress, the importance of cytokine, chemokine, inflammatory, and immune activity in migraine.

Chapter 3. Hemokinin-1 gene expression is upregulated in trigeminal ganglia in an inflammatory orofacial pain model: potential role in peripheral sensitisation

RESULTS AND DISCUSSION

Tac4 mRNA levels changed in parallel with orofacial allodynia after CFA-induced inflammation in rat TG

The mechanonociceptive threshold of the whisker pad area of CFA-injected rats was significantly decreased compared to the saline-injected rats in all three days, reaching its minimum on day three. *Tac4* mRNA expression levels in TG correlated with the shift in von Frey thresholds, reaching its maximum on day 3.

CFA induced upregulation Tac4 mRNA in both primary sensory neurons and SGCs of the rat TG

To investigate the basal and altered level of *Tac4* mRNA expression in rat TG after saline or CFA injection, we performed fluorescent RNAscope *in situ* hybridisation that provides cellular resolution and tissue context. *Tac4* transcripts were localised primarily on SGCs and sensory neurons in saline-treated samples. CFA treatment caused significant upregulation in both cell types. *Tac4* positive transcripts were also detected on Schwann cells, identified only morphologically.

Behavioural tests suggested anxiety-like behaviour in Tac4$^{-/-}$ gene-deficient mice

To examine the effect of the lack of *Tac4* gene on behaviour, we tried to apply our valid orofacial inflammation model *Tac4*$^{-/-}$ mice. CFA administration caused significantly decreased orofacial mechanonociceptive threshold one and three days after injection in both WT and *Tac4* $^{-/-}$ mice. However, saline injection caused similar, although non-significant changes. No significant changes were observed between the thresholds of WT and *Tac4*$^{-/-}$ mice. However, saline and CFA treated groups seem to be better separated in WT mice. No substantial change in spontaneous behaviour was seen due to CFA injection (saline vs CFA). However, *Tac4*$^{-/-}$ mice spent less time in the centre zone of the open field box, suggesting an anxiety-like behaviour compared to their littermates.

CFA induced upregulation Tac4 mRNA in both primary sensory neurons and SGCs of the mice TG

Similar to *Tac4* expression found in rat TG, basal *Tac4* mRNA was detected both in sensory neurons and SGCs of mouse TG. Moreover, CFA-induced upregulation in mouse *Tac4* mRNA.

CFA-induced changes in neuronal and glial activation marker levels in the TG of Tac4 gene-deficient mice

In WT mice, neuronal *FosB* gene expression was significantly upregulated by day three compared to intact samples. However, both CFA injection and the saline administration increased *FosB* expression level. The differences between saline- and CFA-treated samples were significant only on day one. A significant upregulation of the *FosB* was only detected in *Tac4*$^{-/-}$ animals at a later time point, on day seven. Upregulation of the neuronal marker on days three and seven was significantly lower in the TG of *Tac4*$^{-/-}$ mice when compared to its matching WT. In intact animals, the microglia/macrophage activation marker (*Aif1*) presented lower expression levels in *Tac4*$^{-/-}$ mice compared to WT. *Aif1* expression was slightly higher in both saline- and CFA-treated *Tac4*$^{-/-}$ mice than their corresponding WT groups, significant on days one and three. Although, these changes were probably too small to be biologically meaningful. After treatment, the SGC/astrocyte activation marker was upregulated in all groups on all days compared to intact samples. CFA induced a significant elevation in WT compared to the respective saline-treated group on days one and three. Interestingly, inflammation did not cause alteration in *Gfap* levels in *Tac4*$^{-/-}$ mice. Moreover, most of the comparisons showed that *Gfap* mRNA expression levels were lower in all *Tac4*$^{-/-}$ groups than in the WT group.

Neuroinflammation-related genes were differently altered in TG of saline-or CFA-treated Tac4$^{-/-}$ and WT mice

Nanostring analysis was performed on TG samples collected on day three after CFA or saline injection in the whisker pad of WT and *Tac4*$^{-/-}$ mice. Results revealed various neuroinflammation-related differentially expressed genes and significant cell type-specific gene expressions correlations. In the saline-treated *Tac4*$^{-/-}$ group, 15 genes were differentially (9 upregulated, 6 downregulated) expressed (p-value threshold of 0.05) in comparison with saline-treated WT samples. Significant alterations in microglia/macrophage and cytotoxic cell-specific genes were enlisted when saline-treated *Tac4*$^{-/-}$ mice were compared to saline-treated WTs. In the TG of CFA-treated *Tac4*$^{-/-}$ mice, 22 genes were differentially expressed (p-value threshold of 0.05) compared to the CFA-treated WT group. 13 genes were upregulated, nine were downregulated in the *Tac4*$^{-/-}$ TG samples. Alterations in genes specific to neutrophil granulocytes were significantly correlated in CFA-treated *Tac4*$^{-/-}$ vs CFA-treated WT comparison. The treatment effect was reflected in the clustering of the samples in all cases.

In the current chapter, results confirmed the presence of *Tac4* mRNA in the trigeminal ganglion and its upregulation due to orofacial inflammation. We detected *Tac4* transcripts on neurons and all types of glial cells of the TG. In addition, significant inflammation-induced upregulation of *Tac4* was demonstrated in both neurons and satellite glial cells. Based on our orofacial mechanonociceptive threshold measurements in the rat and the qPCR results, we have shown that *Tac4* upregulation happened parallel to the development of allodynia which suggests a potential role in the sensitisation process. In conclusion, our present findings support the importance of HK-1 in the inflammatory processes and nociceptive sensitisation underlying orofacial pain. We have also revealed that HK-1 participates in neuron-glia interactions both under physiological conditions and after inflammation. Although we provide evidence for expression changes at the mRNA level only which is an explicit limitation of the study, the concomitant behavioural alterations suggest that the protein products of the examined mRNAs were also affected.

Chapter 4. Hemokinin-1 expression and effect on a mixed glial cell culture

RESULTS AND DISCUSSION

Tac4 transcripts were co-localised with Aif1 (Iba1), Gfap, Olig2-expressing cells of mouse MGCs

To investigate the basal level of *Tac4* mRNA expression in mouse MGCs, we performed fluorescent RNAscope *in situ* hybridisation that provides cellular resolution. *Tac4* transcripts localised uniformly throughout the cell culture. All glial cell types show *Tac4* transcripts, both in nucleus and cytoplasm, although it appears that co-expression is higher in oligodendrocytes and astrocytes than with microglia.

Hemokinin-1 treatment induced radioactive $^{45}Ca^{2+}$ uptake in mouse MGCs

Incubation of MGCs with HK-1 resulted in concentration-dependent radioactive $^{45}Ca^{2+}$ uptake. Whereas the 1 μM HK-1 could increase the $^{45}Ca^{2+}$ uptake in the cells compared to ECS, the 5 μM HK-1 treatment generated a significant influx.

Hemokinin-1 treatment increased the inflammatory cytokine production in mouse MGCs

After 24-hour treatment of MGCs with mouse HK-1, IL-1β presented nondetectable levels in supernatants of treated MGC samples. In the case of MCP-1 and TNFα, significant differences were seen in concentrations when MGSs were treated with 5 μM, but not lower HK-1 concentrations, compared with the control (ECS) treatment. Although different HK-1 treatments did not significantly affect RANTES, KC and IL-6 levels, the observed tendencies were similar to the previously mentioned cytokines.

In the present chapter, we show the presence of *Tac4* mRNA in several glial cells derived from cerebral tissue. Ours is the first study to assess the expression of *Tac4* on the mRNA level, including localisation of the transcripts on a cellular level. We showed that several proinflammatory molecules are involved in HK-1 mediated signalling for the first time. Thus, our finding supports that glial cell activation is of great importance in the context of inflammatory progression. It should be highlighted that results originate from cell cultures, where the lack of neuron and natural composition exists; thus, in vivo validation is necessary.

NOVELTY AND RELEVANCE OF OUR FINDINGS

- We have described some up-and downregulated genes at the levels of the trigeminovascular system's primary and secondary sensory neurons that might play essential roles in peripheral and central sensitisation mechanisms.
- We are the first to present transcriptomic alterations in the PBMCs similar to the changes detected in the neuronal tissues. These results open new perspectives and initiate further investigations in the research of trigeminal pain disorders.
- These are the first data revealed from transcriptome analysis of PBMCs isolated from both ictal and interictal samples of migraineurs. Comparing these groups with healthy controls made possible the identification of both disease-specific and headache-specific alterations and revealed the importance of inflammatory pathways and the potential contribution of various cytokines to migraine susceptibility.
- Furthermore, our results suggest the possible implication of mitochondrial dysfunction, oxidative stress, cytokine and immune activity in migraine.
- We have confirmed the presence of *Tac4* mRNA in the trigeminal ganglion and established the upregulation of the gene after orofacial inflammation, this being the first study to assess the changes of *Tac4* expression under pathological conditions.
- We have shown *Tac4* mRNA expression and localisation on the cellular level on sensory neurons and all types of glial cells of the trigeminal ganglion. Furthermore, significant inflammation-induced upregulation of *Tac4* was shown in both neurons and satellite glial cells. Our findings support the importance of HK-1 in the inflammatory processes and nociceptive sensitisation underlying orofacial pain.
- We have confirmed the presence of *Tac4* mRNA in several glial cells derived from cerebral tissue. Our work is the first to assess the expression of *Tac4* on mRNA level, including localisation of the transcripts on the cellular level, in all glial cell types driven from central nervous tissue.
- We have shown that inflammatory molecules, mostly MCP-1 and TNFα and eventually RANTES, KC, IL-6, are involved in HK-1 mediated signalling.

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor, Kata Bölcskei, for tutoring me through all these years. I am most grateful for the guidance, support, and knowledge that she shared with me and the friendship she showed towards me.

I want to express my deep gratitude to Prof. Erika Pintér, the leader of the Neuropharmacology doctoral program and head of the institute, for giving me the chance to be part of the Department of Pharmacology and Pharmacotherapy. I especially thank her for her ongoing support throughout the years.

I want to express my special thanks to Prof. Zsuzsanna Helyes for giving me the opportunity to work on her projects. Her enthusiasm, positive thinking and dedication to the research served as an example to follow.

I wish to express my gratitude to Prof. János Szolcsányi, the founder of this research group; I feel honoured to have known him.

I would also like to thank my roommates, ex-roommates, PhD fellows and members of the Department of Pharmacology and Pharmacotherapy for creating a cheerful atmosphere, sharing their friendship, meanwhile constantly advising and helping me professionally through all these years.

Many thanks to our collaborators: colleagues from the Szentágothai Research Centre Bioinformatics Research Group who assured my work a professional statistical and analysis background and colleagues from the University of Szeged, who, with their expertise, made it possible to conduct the human migraine study successfully.

Finally, I cannot be grateful enough to my family for standing beside me and my husband for encouraging and supporting me all the time with his patience and love.

REFERENCES

1. Sharav, Y., Katsarava, Z. & Charles, A. Facial presentations of primary headache disorders. *Cephalalgia* **37**, 714–719 (2017).
2. Goadsby, P. J. *et al.* Pathophysiology of Migraine: A Disorder of Sensory Processing. *Physiol. Rev.* **97**, 553–622 (2017).
3. Mulder, E., Van Baal, C., Gaist, D., Kallela, M., & Palotie, A. Genetic and Environmental Influences on Migraine: A Twin Study Across Six Countries. *Twin Res.* **6(5), 422-431.**, (2003).
4. Steiner, T. J. *et al.* Migraine remains second among the world's causes of disability, and first among young women: findings from GBD2019. *J. Headache Pain* **21**, 137 (2020).
5. Tajti, J., Majláth, Z., Szok, D., Csáti, A. & Vécsei, L. Drug safety in acute migraine treatment. *Expert Opin. Drug Saf.* **14**, 891–909 (2015).
6. Weatherall, M. W. The diagnosis and treatment of chronic migraine. *Ther. Adv. Chronic Dis.* **6**, 115–123 (2015).
7. Tajti, J. *et al.* Migraine is a neuronal disease. *J. Neural Transm.* **118**, 511–524 (2011).
8. L Kelman. The Triggers or Precipitants of the Acute Migraine Attack. *Cephalalgia* **27**, 394–402 (2007).
9. Martin, V. T. & Behbehani, M. M. TOWARD A RATIONAL UNDERSTANDING OF MIGRAINE TRIGGER FACTORS. *Med. Clin. North Am.* **85**, 911–941 (2001).
10. Sauro, K. M. & Becker, W. J. The stress and migraine interaction. *Headache* **49**, 1378–1386 (2009).
11. Edvinsson, L., Villalón, C. M. & MaassenVanDenBrink, A. Basic mechanisms of migraine and its acute treatment. *Pharmacol. Ther.* **136**, 319–333 (2012).
12. Pietrobon, D. & Moskowitz, M. A. Pathophysiology of Migraine. *Annu. Rev. Physiol.* **75**, 365–391 (2013).
13. de Vries, T., Villalón, C. M. & MaassenVanDenBrink, A. Pharmacological treatment of migraine: CGRP and 5-HT beyond the triptans. *Pharmacol. Ther.* 107528 (2020) doi:10.1016/j.pharmthera.2020.107528.
14. Fan, W. *et al.* The role of satellite glial cells in orofacial pain. *J. Neurosci. Res.* **97**, 393–401 (2019).
15. Goadsby, P. J. The vascular theory of migraine—a great story wrecked by the facts. *Brain* **132**, 6–7 (2009).
16. Lopes, D. M., Denk, F. & McMahon, S. B. The Molecular Fingerprint of Dorsal Root and Trigeminal Ganglion Neurons. *Front. Mol. Neurosci.* **10**, (2017).
17. Durham, P. L. & Garrett, F. G. Development of functional units within trigeminal ganglia correlates with increased expression of proteins involved in neuron–glia interactions. *Neuron Glia Biol.* **6**, 171–181 (2010).
18. Lee, Y. *et al.* Coexistence of calcitonin gene-related peptide and substance P-like peptide in single cells of the trigeminal ganglion of the rat: immunohistochemical analysis. *Brain Res.* **330**, 194–196 (1985).
19. Ma, Q.-P., Hill, R. & Sirinathsinghji, D. Colocalization of CGRP with 5-HT1B/1D receptors and substance P in trigeminal ganglion neurons in rats. *Eur. J. Neurosci.* **13**, 2099–2104 (2001).
20. Schueler, M., Neuhuber, W. L., De Col, R. & Messlinger, K. Innervation of Rat and Human Dura Mater and Pericranial Tissues in the Parieto-Temporal Region by Meningeal Afferents. *Headache J. Head Face Pain* **54**, 996–1009 (2014).
21. Fernandes, E. S., Schmidhuber, S. M. & Brain, S. D. Sensory-nerve-derived neuropeptides: possible therapeutic targets. *Handb. Exp. Pharmacol.* 393–416 (2009) doi:10.1007/978-3-540-79090-7_11.
22. Seybold, V. S. The role of peptides in central sensitisation. *Handb. Exp. Pharmacol.* 451–491 (2009) doi:10.1007/978-3-540-79090-7_13.
23. Iwata, K. & Shinoda, M. Role of neuron and non-neuronal cell communication in persistent orofacial pain. *J. Dent. Anesth. Pain Med.* **19**, 77–82 (2019).
24. Su, M. & Yu, S. Chronic migraine: A process of dysmodulation and sensitisation. *Mol. Pain* **14**, 174480691876769 (2018).
25. Aczél, T. *et al.* Identification of disease- and headache-specific mediators and pathways in migraine using blood transcriptomic and metabolomic analysis. *J. Headache Pain* **22**, 117 (2021).
26. Messlinger, K., Balcziak, L. K. & Russo, A. F. Cross-talk signaling in the trigeminal ganglion: role of neuropeptides and other mediators. *J. Neural Transm.* (2020) doi:10.1007/s00702-020-02161-7.
27. Paramel Mohan, S. & Ramalingam, M. Neuroscience of Peripheral Nerve Regeneration. *J. Pharm. Bioallied Sci.* **13**, S913–S916 (2021).
28. Hanani, M. Satellite glial cells in sensory ganglia: from form to function. *Brain Res. Rev.* **48**, 457–476 (2005).
29. Liu, Q. *et al.* α7 Nicotinic acetylcholine receptor-mediated anti-inflammatory effect in a chronic migraine rat model via the attenuation of glial cell activation. *J. Pain Res.* **11**, 1129–1140 (2018).
30. Matsuka, Y. *et al.* The role of chemical transmitters in neuron-glia interaction and pain in sensory ganglion. *Neurosci. Biobehav. Rev.* **108**, 393–399 (2020).
31. Afroz, S. *et al.* CGRP Induces Differential Regulation of Cytokines from Satellite Glial Cells in Trigeminal Ganglia and Orofacial Nociception. *Int. J. Mol. Sci.* **20**, (2019).
32. Lynch, M. A. The Multifaceted Profile of Activated Microglia. *Mol. Neurobiol.* **40**, 139–156 (2009).
33. Carniglia, L. *et al.* Neuropeptides and Microglial Activation in Inflammation, Pain, and Neurodegenerative Diseases. *Mediators Inflamm.* **2017**, (2017).

34. Berta, T., Qadri, Y. J., Chen, G. & Ji, R. R. Microglial Signaling in Chronic Pain with a Special Focus on Caspase 6, p38 MAP Kinase, and Sex Dependence. *J. Dent. Res.* **95**, 1124–1131 (2016).
35. Brain, S. D. & Cox, H. M. Neuropeptides and their receptors: innovative science providing novel therapeutic targets. *Br. J. Pharmacol.* **147 Suppl 1**, S202-211 (2006).
36. Garcia-Recio, S. & Gascón, P. Biological and Pharmacological Aspects of the NK1-Receptor. *BioMed Res. Int.* **2015**, 495704 (2015).
37. Muñoz, M. & Coveñas, R. Involvement of substance P and the NK-1 receptor in human pathology. *Amino Acids* **46**, 1727–1750 (2014).
38. Jang, J. H., Nam, T. S., Paik, K. S. & Leem, J. W. Involvement of peripherally released substance P and calcitonin gene-related peptide in mediating mechanical hyperalgesia in a traumatic neuropathy model of the rat. *Neurosci. Lett.* **360**, 129–132 (2004).
39. Diener, H.-C. Rpr100893, A Substance-P Antagonist, is Not Effective in the Treatment of Migraine Attacks. *Cephalalgia* **23**, 183–185 (2003).
40. Reinhardt R. Comparison of neurokinin-1 antagonist, L-745,030, to placebo, acetaminophen and ibuprofen in the dental pain model. *Clin. Pharmacol. Ther.* **63**, 168 (1998).
41. Goldstein, D., Wang, O., Gitter, B. & Iyengar, S. Dose-Response Study of the Analgesic Effect of Lanepitant in Patients with Painful Diabetic Neuropathy. *Clin. Neuropharmacol.* **24**, 16–22 (2001).
42. Zhang, Y., Lu, L., Furlonger, C., Wu, G. E. & Paige, C. J. Hemokinin is a hematopoietic-specific tachykinin that regulates B lymphopoiesis. *Nat. Immunol.* **1**, 392–397 (2000).
43. Dai, L. *et al.* Hemokinin-1 Stimulates Prostaglandin E $_2$ Production in Human Colon through Activation of Cyclooxygenase-2 and Inhibition of 15-Hydroxyprostaglandin Dehydrogenase. *J. Pharmacol. Exp. Ther.* **340**, 27–36 (2012).
44. Metwali, A., Blum, A. M., Elliott, D. E., Setiawan, T. & Weinstock, J. V. Cutting Edge: Hemokinin Has Substance P-Like Function and Expression in Inflammation. *J. Immunol.* **172**, 6528–6532 (2004).
45. Morteau, O., Lu, B., Gerard, C. & Gerard, N.P. Hemokinin 1 is a full agonist at the substance P receptor. *Nat. Immunol.* **2**, 1088 (2001).
46. Borbély, É. & Helyes, Z. Role of hemokinin-1 in health and disease. *Neuropeptides* **64**, 9–17 (2017).
47. Endo, D., Ikeda, T., Ishida, Y., Yoshioka, D. & Nishimori, T. Effect of intrathecal administration of hemokinin-1 on the withdrawal response to noxious thermal stimulation of the rat hind paw. *Neurosci. Lett.* **392**, 114–117 (2006).
48. Watanabe, C., Mizoguchi, H., Bagetta, G. & Sakurada, S. Involvement of spinal glutamate in nociceptive behavior induced by intrathecal administration of hemokinin-1 in mice. *Neurosci. Lett.* **617**, 236–239 (2016).
49. Fu, C.-Y. *et al.* Hemokinin-1(4-11)-Induced Analgesia Selectively Up-Regulates δ-Opioid Receptor Expression in Mice. *PLoS ONE* **9**, e90446 (2014).
50. Xia, R.-L., Fu, C.-Y., Zhang, S.-F., Jin, Y.-T. & Zhao, F.-K. Study on the distribution sites and the molecular mechanism of analgesia after intracerebroventricular injection of rat/mouse hemokinin-1 in mice. *Peptides* **43**, 113–120 (2013).
51. Sakai, A., Takasu, K., Sawada, M. & Suzuki, H. Hemokinin-1 Gene Expression Is Upregulated in Microglia Activated by Lipopolysaccharide through NF-κB and p38 MAPK Signaling Pathways. *PLoS ONE* **7**, e32268 (2012).
52. Ando, Y. Expression of hemokinin-1 in rat spinal cord after peripheral inflammation. Kokubyo Gakkai Zasshi. **76**, 81-90. (2009).
53. Harriott, A. M., Strother, L. C., Vila-Pueyo, M. & Holland, P. R. Animal models of migraine and experimental techniques used to examine trigeminal sensory processing. *J. Headache Pain* **20**, 91 (2019).
54. Romero-Reyes, M. & Akerman, S. Update on Animal Models of Migraine. *Curr. Pain Headache Rep.* **18**, (2014).
55. Krzyzanowska, A. & Avendaño, C. Behavioral testing in rodent models of orofacial neuropathic and inflammatory pain. *Brain Behav.* **2**, 678–697 (2012).
56. Gregory, N. S. *et al.* An overview of animal models of pain: disease models and outcome measures. *J. Pain* **14**, 1255–1269 (2013).
57. Martinez-Garcia, M., Miguelanez-Medran, B. & Goicoechea, C. Animal models in the study and treatment of orofacial pain. *J. Clin. Exp. Dent.* 0–0 (2019) doi:10.4317/jced.55429.
58. Perrino, C. *et al.* Epigenomic and transcriptomic approaches in the post-genomic era: path to novel targets for diagnosis and therapy of the ischaemic heart? Position Paper of the European Society of Cardiology Working Group on Cellular Biology of the Heart. *Cardiovasc. Res.* **113**, 725–736 (2017).
59. Okumura, M. *et al.* Alternation of Gene Expression in Trigeminal Ganglion Neurons Following Complete Freund's Adjuvant or Capsaicin Injection into the Rat Face. *J. Mol. Neurosci.* **42**, 200–209 (2010).
60. Chung, M.-K., Park, J., Asgar, J. & Ro, J. Y. Transcriptome analysis of trigeminal ganglia following masseter muscle inflammation in rats. *Mol. Pain* **12**, 1 (2016).
61. Rollins, B., Martin, M. V., Morgan, L. & Vawter, M. P. Analysis of whole genome biomarker expression in blood and brain. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* **153B**, 919–936 (2010).
62. Mosallaei, M. *et al.* PBMCs: a new source of diagnostic and prognostic biomarkers. *Arch. Physiol. Biochem.* **0**, 1–7 (2020).

LIST OF PUBLICATIONS

Articles related to the thesis

Aczél, T. *, Kun, J. *, Szőke, É., Rauch, T., Junttila, S., Gyenesei, A., Bölcskei, K., & Helyes, Z. (2018). Transcriptional Alterations in the Trigeminal Ganglia, Nucleus and Peripheral Blood Mononuclear Cells in a Rat Orofacial Pain Model. Frontiers in molecular neuroscience, 11, 219. https://doi.org/10.3389/fnmol.2018.00219 (IF: 3.720)

Aczél, T., Kecskés, A., Kun, J., Szenthe, K., Bánáti, F., Szathmary, S., Herczeg, R., Urbán, P., Gyenesei, A., Gaszner, B., Helyes, Z., & Bölcskei, K. (2020). Hemokinin-1 Gene Expression Is Upregulated in Trigeminal Ganglia in an Inflammatory Orofacial Pain Model: Potential Role in Peripheral Sensitisation. International journal of molecular sciences, 21(8), 2938. https://doi.org/10.3390/ijms21082938 (IF: 5.923)

Aczél, T. *, Körtési, T. *, Kun, J. *, Urbán, P., Bauer, W., Herczeg, R., Farkas, R., Kovács, K., Vásárhelyi, B., Karvaly, G. B., Gyenesei, A., Tuka, B., Tajti, J., Vécsei, L., Bölcskei, K., & Helyes, Z. (2021). Identification of disease- and headache-specific mediators and pathways in migraine using blood transcriptomic and metabolomic analysis. The journal of headache and pain, 22(1), 117. https://doi.org/10.1186/s10194-021-01285-9 (IF: 7.277)

Cumulative impact factor (without citable abstracts): **16.92**

Articles not related to the thesis

Takács-Lovász, K., Kun, J., **Aczél, T.**, Urbán, P., Gyenesei, A., Bölcskei, K., Szőke, É., & Helyes, Z. (2022). PACAP-38 Induces Transcriptomic Changes in Rat Trigeminal Ganglion Cells Related to Neuroinflammation and Altered Mitochondrial Function Presumably via PAC1/VPAC2 Receptor-Independent Mechanism. International journal of molecular sciences, 23(4), 2120. https://doi.org/10.3390/ijms23042120 (IF: 5.923)

Nemes, B., Bölcskei, K., Kecskés, A., Kormos, V., Gaszner, B., **Aczél, T**., Hegedüs, D., Pintér, E., Helyes, Z., & Sándor, Z. (2021). Human Somatostatin SST4 Receptor Transgenic Mice: Construction and Brain Expression Pattern Characterization. International journal of molecular sciences, 22(7), 3758. https://doi.org/10.3390/ijms22073758 (IF: 5.923)

Kovács-Ábrahám, Z., **Aczél, T.**, Jancsó, G., Horváth-Szalai, Z., Nagy, L., Tóth, I., Nagy, B., Molnár, T., & Szabó, P. (2021). Cerebral and Systemic Stress Parameters in Correlation with Jugulo-Arterial CO2 Gap as a Marker of Cerebral Perfusion during Carotid Endarterectomy. Journal of clinical medicine, 10(23), 5479. https://doi.org/10.3390/jcm10235479 (IF: 4.241)

Pohóczky, K., Kun, J., Szentes, N., **Aczél, T.**, Urbán, P., Gyenesei, A., Bölcskei, K., Szőke, É., Sensi, S., Dénes, Á., Goebel, A., Tékus, V., Helyes, Z. Discovery of novel targets in a Complex Regional Pain Syndrome mouse model by transcriptomics: TNF and JAK-STAT pathways. *Under revision in Pharmacological research. (IF:7.68)*

Cumulative impact factor of all publications (without citable abstracts): **33.007**

Citations MTMT: 18

Citations Google Scholar: 28

Oral presentations related to the thesis

2019- European Pain School (EPS), Siena, Italy
Investigation of gene expression changes in animal models of trigeminal sensitisation
Timea Aczél, Angéla Kecskés, Éva Szőke, József Kun, Balázs Gaszner, Zsuzsanna Helyes, Kata Bölcskei

2018 – III. Neuroscience Center PhD and TDK Conference, Pécs, Hungary
Génexpresszió-változások vizsgálata trigeminális szenzitizációban (3rd prize)
Timea Aczél, József Kun, Eva Szőke, Tibor Rauch, Sini Junttila, Attila Gyenesei, Kata Bölcskei, Zsuzsanna Helyes

2018 – Pain Mechanisms and Therapeutics Conference, Taormina, Sicily
Temporal changes of gene expression in trigeminal ganglia, trigeminal nucleus caudalis and peripheral blood mononuclear cells in a rodent orofacial pain model.
Timea Aczél, József Kun, Eva Szőke, Tibor Rauch, Sini Junttila, Attila Gyenesei, Kata Bölcskei, Zsuzsanna Helyes

Oral presentations not related to the thesis

2016 – V. Interdisciplinary Doctoral Conference, Pécs, Hungary
Cortical spreading depression-induced blood flow changes measured by Laser Speckle Contrast imaging in Transient Receptor Potential Ankyrin 1 (TRPA1) and Vanilloid 1 (TRPV1) deficient mice
Timea Aczél, Kata Bölcskei, Zsuzsanna Helyes, Erika Pintér

2014 – VI. EFIS-EJI South Eastern European Immunology School (SEEIS2014), Timisoara, Romania
Cardiomyocyte inflammation model in Sirt3$^{-/-}$ mouse
Timea Aczél, Manuel Vázquez-Carrera, Xavier Palomer, Előd Nagy

2014 – The 21st Students' Scientific Conference, Târgu Mures, Romania
Sirt3$^{-/-}$ egér szívizomsejt hipertrófia-gyulladás modellje (1st prize)
Timea Aczél, Manuel Vázquez-Carrera, Xavier Palomer, Előd Nagy

Poster presentations related to the thesis

2019 – Hungarian Pain Society's Conference, Szeged, Hungary
Tac4 szerepének vizsgálata orofaciális és dura gyulladással kiváltott trigeminális szenzitizáció állatmodelljeiben
Timea Aczél, Angéla Kecskés, Éva Szőke, József Kun, Balázs Gaszner, Zsuzsanna Helyes, Kata Bölcskei

2019 – The 7th Mediterranean Neuroscience Conference, Marrakesh, Morocco
Hemokinin-1 is involved in trigeminal sensitization
Timea Aczél, Angéla Kecskés, Éva Szőke, József Kun, Balázs Gaszner, Zsuzsanna Helyes, Kata Bölcskei

2019 – III. Gyógyszer Innovációs Kongresszus, Gárdony, Hungary
Orofaciális gyulladással kiváltott génexpressziós változások Tac4 génhiányos egerekben
Timea Aczél, Éva Szőke, József Kun, Anikó Perkecz, Zsuzsanna Helyes, Kata Bölcskei

2017 – Hungarian Physiological Society's Conference, Debrecen, Hungary
Trigeminális neuronok és perifériás leukociták génexpresszió-változásai patkány orofaciális fájdalommodellben (Society's Main Prize)
Timea Aczél, József Kun, Éva Szőke, Tibor Rauch, Kata Bölcskei, Zsuzsanna Helyes

2017 – 7th BBBB International Conference on Pharmaceutical Sciences, Balatonfüred, Hungary
Time course of gene expression changes in trigeminal neurones and peripheral blood mononuclear cells in a rat orofacial pain model
Timea Aczél, József Kun, Éva Szőke, Tibor Rauch, Kata Bölcskei, Zsuzsanna Helyes

2017 – Federation of European Neuroscience Societies FENS Regional Meeting, Pécs, Hungary
Gene expression analysis of trigeminal ganglia and peripheral blood mononuclear cells in a rat orofacial pain model
Timea Aczél, József Kun, Éva Szőke, Tibor Rauch, Kata Bölcskei, Zsuzsanna Helyes

Poster presentations not related to the thesis

2018 – Association of Medical Schools in Europe Conference, Pécs, Hungary
Role of sirtuin 1 activation in trigeminal sensitization
Timea Aczél, Maja Payrits, Éva Szőke, József Kun, Zsuzsanna Helyes, Kata Bölcskei

2018 – Hungarian Physiological Society's Conference, Szeged, Hungary
A sirtuin 1 aktiváció szerepe trigeminalis neuronok szenzitizációjában
Timea Aczél, Maja Payrits, Éva Szőke, József Kun, Zsuzsanna Helyes, Kata Bölcskei

2016 – Hungarian Physiological Society's Conference, Pécs, Hungary
Agykérgi kúszó depolarizáció által kiváltott perfúzióváltozások és a Tranziens Receptor Potenciál Ankyrin 1 (TRPA1) és Vanilloid 1 (TRPV1) ioncsatornák szerepének vizsgálata egérmodellben
Timea Aczél, Kata Bölcskei, Zsuzsanna Helyes, Erika Pintér