

**ROLE OF PITUITARY ADENYLATE CYCLASE-
ACTIVATING POLYPEPTIDE (PACAP)
IN THE TRIGEMINOVASCULAR SYSTEM:
PRECLINICAL AND CLINICAL RESULTS**

PhD thesis summary

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I. CONTENTS

II. INTRODUCTION	4
III. AIMS	6
IV. MATERIALS AND METHODS	7
V. RESULTS	11
VI. DISCUSSION	15
VII. CONSLUSIONS	16
VIII. ACKNOWLEDGEMENTS	18
IX. REFERENCES	19
X. SCIENTOMETRICS	21

II. INTRODUCTION

The main concept of this study was to explore the functions of PACAP, which may possibly be involved in the activation of the trigeminovascular system and hence in the pathomechanism of migraine. The findings may potentially contribute to the development of new solutions in the therapy of headache diseases.

A. *Migraine*

Migraine is a common, paroxysmal, highly disabling primary headache disease with high socio-economic and personal impacts on the quality of life. It is a complex neurovascular disorder, which involves head pain and a wide spectrum of concomitant clinical symptoms. In spite of that it affects more than 12% of the general population, the genetic background of migraine has not been established in detail. Moreover appropriate prophylactic and clinical therapy for the treatment of attacks is not available [1].

In recent years there has been considerable interest in the pathomechanism of migraine. The exact details of the processes are unknown, but the activation of the trigeminovascular system (TS) [2] and the releases of different neuropeptides and neuroactive molecules (calcitonin gene-related peptide (CGRP), substance-P, vasoactive intestinal peptide, nitrogen monoxide, Ca_{2+} /calmodulin-dependent kinase, serotonin) have been confirmed in the processes of migraine. The pituitary adenylate cyclase-activating polypeptide (PACAP), which is present in the perivascular nerve fibres, the trigeminal sensory neurones and which has an important role in nociceptive mechanisms, was recently found to be a potential regulator molecule during migraine attacks [3].

B. PACAP

PACAP is a member of the VIP/secretin/glucagon neuropeptide superfamily and is considered to be a “brain-gut peptide“, by virtue of its widespread expression and functions in the human organism [4, 5]. The peptide exists in two biologically active forms: PACAP-27 and predominantly, PACAP-38. PACAP is widely distributed in the body [6-8], thereby functioning as a pleiotropic peptide [9]. The effects of PACAP are mediated through G-protein linked receptors: VPAC₁, VPAC₂ and PAC₁; the latter has 1000-fold higher specific affinity for both forms of PACAP than for VIP [10].

PACAP-38 has a vasodilating effect [11], and a broad range of data suggest that it is an integrator of nociceptive and sensitization processes, besides being involved in neurogenic inflammation [12, 13]. PACAP is present in the primary sensory neurones of the TRG [14], the parasympathetic otic and the sphenopalatine ganglia [15]. Moreover, PACAP-38 is found in the cell bodies and nerve fibres of the human TNC and the upper regions of the cervical spinal cord, which suggests that PACAP may be closely related to the TS [6]. A clinical study has revealed that the intravenous administration of PACAP-38 causes headache in healthy subjects, and migraine-like attacks in migraine patients without aura, 6 h on average after the start of the infusion [16]. In addition, it was accompanied by the decrease of the mean blood flow velocity in the middle meningeal artery and the increase of the diameter of superficial temporal artery. It is assumed that PACAP may be one of the mediators involved in the mechanisms of TS activation. However, no clinical data are available on endogenous alterations in PACAP levels in relation to migraine.

III. AIMS

The aim of our study was to determine whether there are any alterations in the concentration of PACAP in the blood plasma and the trigeminal system in the case of TS activation and migraine disorder.

1) *Preclinical animal experiments* were conducted by the stimulation of the TS in rats:

- NTG-induced chemical stimulation
- Electrical stimulation of the TRG.

PACAP-27-like immunoreactivity (LI) and PACAP-38-LI were measured following the development of the models in a time-dependent manner. The cerebrospinal fluid (CSF), the venous blood plasma, the area of the second-order sensory neurones (trigeminal nucleus caudalis = TNC), the lower cervical spinal cord (C₃-C₄) and the TRG were examined in order to determine the central and peripheral concentration changes of these peptides.

2) *Clinical human investigations* were carried out to confirm the specificity and relevance of PACAP in migraine.

PACAP-38-LI was measured in the peripheral blood plasma during the ictal and interictal periods in migraine patients in comparison with healthy control subjects.

The clinical features of the disease, the plasma CGRP-LI and the PACAP-38-LI were compared to explore possible correlations.

IV. MATERIALS AND METHODS

1) *Preclinical animal experiments*

Animals, ethics

Fifty-nine young adult Sprague-Dawley rats of either sex (8-12 weeks old, 250-350 g body weight) were used in these studies:

- 28 rats were involved in the nitroglycerine (NTG-Nitrolingual Pumpspray, Pohl-Boskamp GmbH, Germany, in a dose of 10 mg/kg)-induced chemical TS activation model.
- 20 rats were involved in the electrical TRG-stimulation model (duration of stimulation: 30 min; stimulation rate: 10 Hz; duration of impulse: 5 ms; current: 1 mA; stimulation mode: continuous).
- 11 rats were intact in the control group.

All experimental procedures performed in this study complied fully with the guidelines of Acts, Decrees and Ethical Codex of Animal Experiments.

Models

During the experiments the animals were anaesthetized with i.p. chloral hydrate solution. Blood sampling followed immediately in the intact group, but only 90 min or 180 min after TS activation (NTG administration or ES-TRG) in the two other groups. Blood samples (5 ml per animal) were taken from the right cranial vena cava into ice-cold glass tubes containing ethylenediaminetetraacetic acid (EDTA) (12 mg) and the protease inhibitor aprotinin (Gordox, 1200 IU). Samples were kept at 4 °C until the blood plasma was separated by centrifugation (5,000 rpm for 10 min at 4 °C). Before cupping, CSF (~150 µl per animal) was taken from the suboccipital

cistern, and following cupping different nerve structures (the TNC, spinal cord and TRG) were excised from the animals at the 90 or 180 min time points. Samples were stored at -80 °C until the measurement of PACAP-38-LI and PACAP-27-LI by radioimmunoassay (RIA) and determination of these peptides by mass spectrometry (MS).

Determination of PACAP-38-LI and PACAP-27-LI in the rat plasma, CSF and nerve tissues by RIA and MS

Plasma and CSF concentrations of PACAP-38 (“88111-3”) and PACAP-27 (“88123-3”) were determined with specific and sensitive RIA techniques [17]. The antisera were raised in rabbits with synthetic peptides conjugated to bovine serum albumin (BSA) or thyroglobulin with glutaraldehyde or carbodiimide. The high specificity and C-terminal sensitivity of these antibodies were confirmed by cross-reactivity studies.

Identification of PACAP-38 and PACAP-27 in the rat plasma and CSF samples in comparison with standard solutions was performed with matrix-assisted laser desorption ionization time of flight (MALDI TOF) MS. The quasimolecular ions of the PACAP-38 Na⁺ adduct (MW: 4558.7) and PACAP-27 (MW: 3147.6) or its [M+Na]⁺ were determined.

Statistical analysis

Data are presented as mean+S.E.M. of the results on n=11-28 animals. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Tukey’s *post-hoc* test with GraphPad Prism 5.0 software. Levels of probability p<0.05 were considered significant.

2) *Clinical human investigations*

Participants, ethics

87 migraine patients with or without aura and 40 healthy control subjects were enrolled in this study. The migraineurs were selected in accordance with the criteria of the Headache Classification Committee of the International Headache Society 2004 [1]. The study groups were age-matched. A detailed questionnaire was used to compile a homogeneous group of migraineurs as concerns the features of their migraine disease (duration, attack frequency, allodynia, severity of pain, menstrual cycle, other non-migraine, chronic pain disorder, depression). Healthy volunteers serving as controls were screened for non-reported/non-treated headaches.

Study design and procedures

The study was approved by the Ethics Committee of the Faculty of Medicine, University of Szeged (87/2009).

There were no restrictions as regards food and drink intake. Blood samples were drawn from migraineurs during an attack and/or in an attack-free period. Affected patients were asked not to start their usual attack treatment until blood samples had been taken. Accordingly, 80 interictal and 28 ictal samples were collected. From among the 87 patients, blood samples could be collected in both periods from 21 migraineurs. A single blood sample was taken from each control. Blood samples (6 ml per subject) were taken in a sitting position during rest from the cubital vein and collected in ice-cold glass tubes containing the anticoagulant (EDTA, 12 mg) and the protease inhibitor aprotinin (Gordox, 1200 IU), and kept at 4 °C until centrifugation (2000 rpm for 10 min at 4 °C). Plasma samples

were stored at -80°C until the PACAP-38-LI- and CGRP-LI were measured by RIA.

RIA measurements and data acquisition

Plasma concentrations of PACAP-38 were determined with specific and sensitive RIA techniques [17]. The PACAP-38 antiserum “88111-3” was raised in rabbits with synthetic peptides conjugated to bovine serum albumin (BSA) or thyroglobulin with glutaraldehyde or carbodiimide. The high specificity and C-terminal sensitivity of this antibody were confirmed by cross-reactivity studies. No cross-reactivity was found with PACAP-27 or with other related neuropeptides in either case. CGRP-LI was measured with specific and sensitive RIA techniques developed earlier.

Statistical analysis

Data expressed as means \pm SD if not stated otherwise. The normality of the data was tested with the Shapiro-Wilk test. Group comparisons were carried out with the Student’s unpaired, paired t-tests and the Wilcoxon-test with SPSS 17.0. Data were analysed with multivariate test (repeated measure ANOVA) in the case of menstruation cycle and chronic pain condition related to PACAP-38 level. Statistical significance was accepted at $p<0.05$.

V. RESULTS

1) *Preclinical animal experiments*

1. The concentration of PACAP-27/38 increased in the region of the brainstem (TNC) in response to both chemical (**Fig.1/A**) and electrical activation (**Fig. 2/A**) of the TS in the rat.

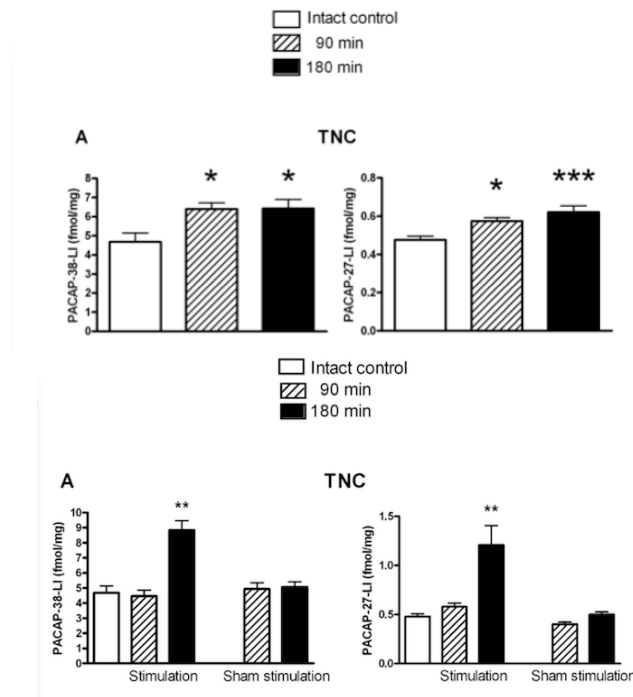


Figure 1/A.

Significant PACAP-38- and PACAP-27-LI increases were detected in the TNC in NTG-model (* $p < 0.05$, *** $p < 0.001$) and ES-TRG model (** $p < 0.01$) **Figure 2/A.**

2. The concentration of PACAP-38 was elevated in the venous blood flow after electrical stimulation of the trigeminal ganglion (**Fig. 3**).

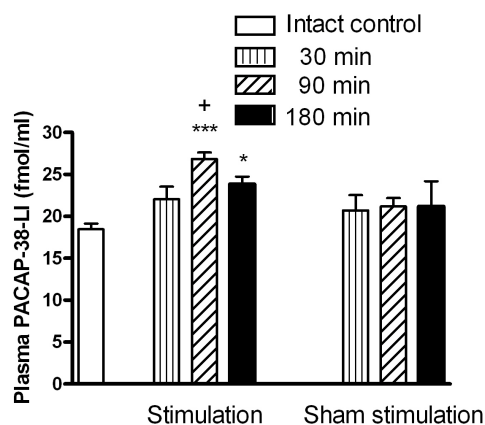


Figure 3.

Significantly elevated plasma PACAP-38 levels were found in the ES-TRG model (* $p < 0.05$, *** $p < 0.001$).

PACAP-27 was not present in the plasma.

Neither PACAP form could be identified in the

2) Clinical human investigations

3. Significantly lower blood plasma PACAP-38 concentration was revealed in the interictal phase of migraineurs as compared with healthy controls and elevated PACAP-38-LI was found during migraine attacks relative to the attack-free period (**Fig. 4**).

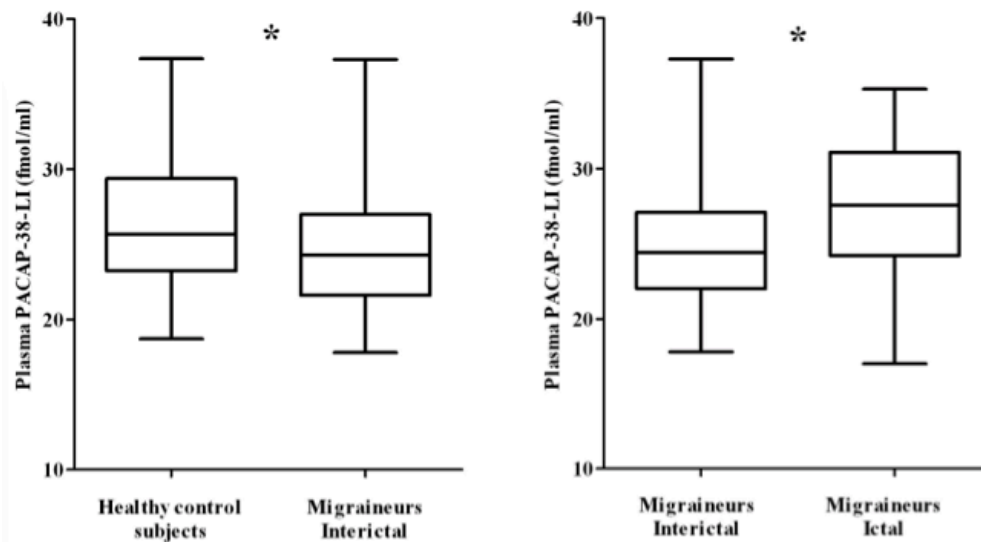


Figure 4. Significant plasma PACAP-38-LI changes were detected in the interictal and ictal phases of migraineurs (* $p < 0.05$).

4. The lower interictal plasma PACAP-38 concentration is associated with the duration of migraine disease (**Fig. 5**).

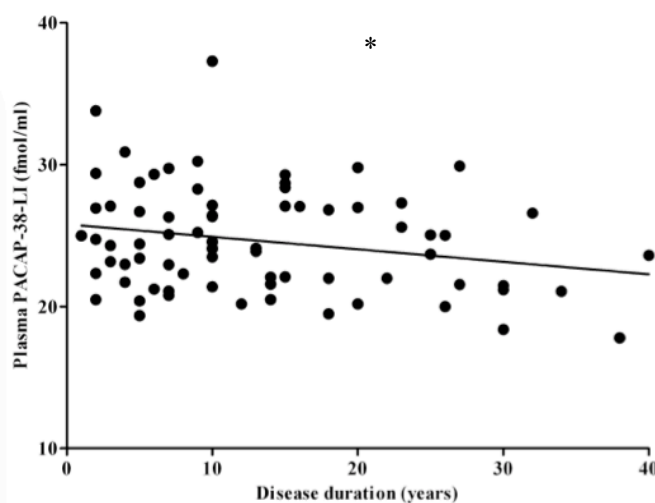


Figure 5. A mild negative correlation was observed between the interictal PACAP-38-LI and the disease duration (* $p < 0.05$).

5. Significantly higher blood plasma PACAP-38 and CGRP concentrations were observed in the ictal phase of migraineurs as compared with the attack-free period (**Fig. 6**).

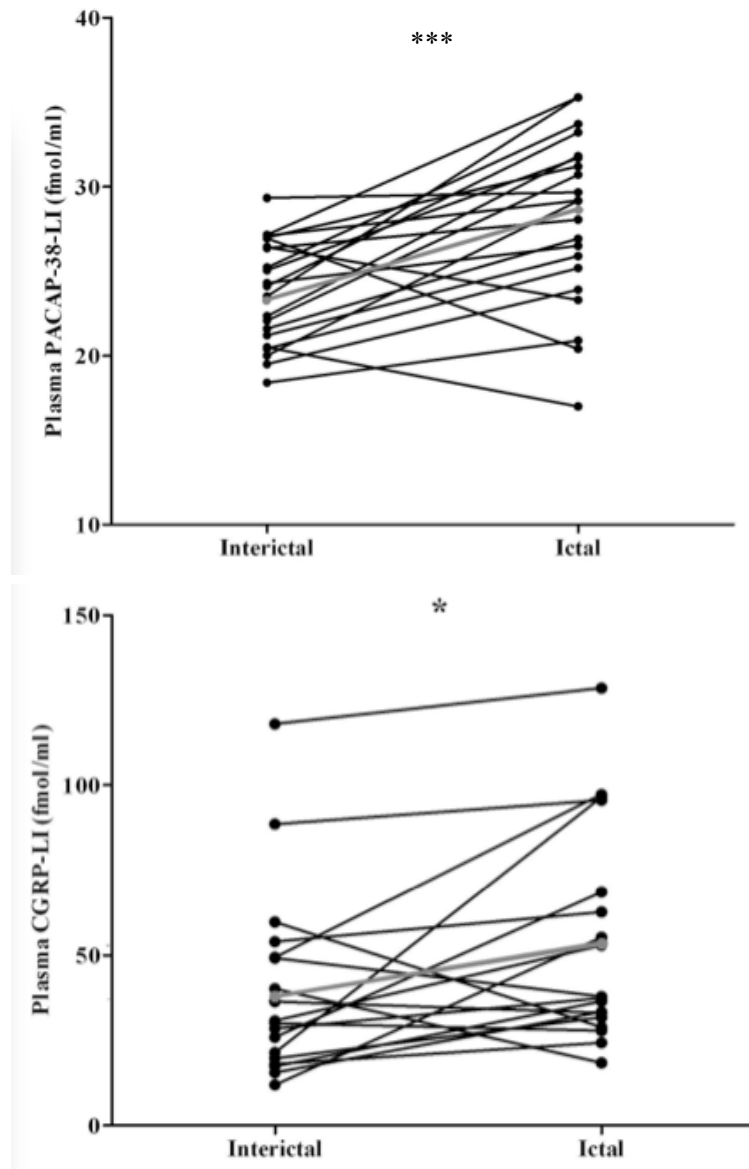


Figure 6. Elevated PACAP-38 and CGRP levels were measured during migraine attacks relative to the attack-free period in the 21 migraineurs ($***p_{\text{PACAP-38}} < 0.001$; $*p_{\text{CGRP}} < 0.05$).

6. The concentration of plasma PACAP-38 was significantly elevated in those groups whose headache is not related to the menstrual cycle or who not represented chronic pain conditions (low-back pain, lumbago, knee- and hip-joint arthrosis) (**Fig. 7**).

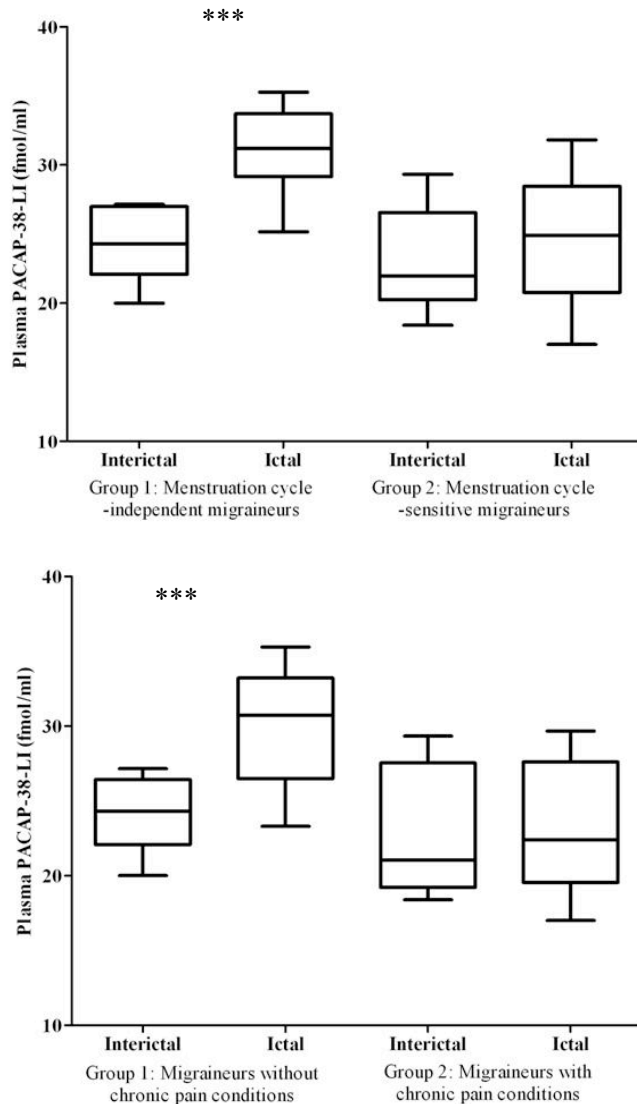


Figure 7. Significant elevations of the plasma PACAP-38-LI were detected in the ictal phases of menstruation cycle-independent ($n=11$) and other chronic pain-free ($n=15$) subgroups of migraineurs ($***p<0.001$).

Plasma PACAP-38-LI did not correlate with the age, attack frequency, allodynia and the VAS-score (ANOVA, linear regression, $p>0.05$) or differences were not found regarding the gender, hormonal changes and pain (Student's unpaired t-test, $p>0.05$).

VI. DISCUSSION

1) *Preclinical animal experiments*

Our results support the neuropeptide theory of the development of activated TS. Both peripheral and central sensitization processes were accompanied by PACAP concentration changes in our models, suggesting the complexity of this neuro-vascular system. The slightly divergent results observed in the two models can be explained by the differences in the activation mechanisms.

The selective and significant increases of PACAP-27/38 in the TNC in the rat suggest, that the trigeminovascular trigger induces a marked release of PACAPs from the central terminals of the primary sensory neurones [12].

It seems that ES of the TRG also generates a massive TS activation. In response, PACAP may be released from the peripheral branches of the TRG, but the correct source of this peptide is unknown. Then the PACAP may enter the circulatory system, present in elevated concentration in the blood and exert its vascular [18], neuronal [6, 19] and mast cell effects [20] related to migraine.

2) *Clinical human investigations*

We presume that similar mechanisms occur in migraineurs. In consequence of the trigeminal trigger, the systemic level of PACAP-38 increases. The PACAP, similar to the CGRP [21]) exerts its vasodilating, sensitizing effects and can contribute to the development and aggravation of headache.

The correlation between the disease duration and the lower PACAP-38 concentration during the attack-free period may be a consequence of the higher PACAP-38 releases in the ictal phase,

which may progressively deplete the PACAP-containing terminals. This reduction might be explained indirectly in terms of different brain energy deficits [22, 23] or it might be associated with degenerative changes [24] affecting the PACAP-releasing circuitries. In addition, the ictally elevated PACAP-38-LI seems to be migraine specific alteration, while it did not show significance in the case of menstruation cycle-dependent migraine and the presence of other chronic pain conditions.

There are assumptions that the integrity of blood-brain barrier (BBB) is disrupted in migraineurs [25]. The enhanced BBB permeability in migraine may facilitate the PACAP to penetrate into the brain parenchymal elements and exert its central effects. From the opposite aspect, the PACAP released in the brain can also penetrate through the BBB, and hence may be detected in the plasma [5].

VII. CONSLUSIONS

Our results suggest that PACAP is a special modulator of the TS. The fact that this peptide has an important role in the central sensitization involved in migraine-like headache is confirmed by the clear association between the migraine phases and the alterations in plasma PACAP-38 concentration in human observations.

These data facilitate the understanding of the mechanisms of activated TS. In addition our results indicate the need for further investigations of the role of plasma PACAP-38 as a putative biomarker of migraine, which might provide new perspectives and targets in the therapy of migraine.

ORIGINAL STATEMENTS OF THE THESIS

Preclinical animal experiments

1. The concentrations of PACAP-27/38 increased in the region of the brainstem (trigeminal nucleus caudalis) in response to both chemical and electrical stimulation of the trigeminovascular system in the rat.
2. The concentration of PACAP-38 was elevated in the venous blood flow after electrical activation of the trigeminal ganglion in the rat.

Clinical human investigations

3. A significantly lower blood plasma PACAP-38 concentration was revealed in the interictal phase of migraineurs as compared with healthy controls.
4. The lower interictal plasma PACAP-38 concentration is associated with the duration of migraine disease.
5. A significantly higher blood plasma PACAP-38 concentration was observed in the ictal phase of migraineurs as compared with the attack-free period.
6. The concentration of plasma PACAP-38 was significantly elevated during migraine attacks in those groups whose headache is not related to the menstrual cycle or who did not represent chronic pain conditions (low-back pain, lumbago and knee- and hip-joint arthrosis).

VIII. ACKNOWLEDGEMENTS

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X. SCIENTOMETRICS

ORIGINAL PAPERS RELATED TO THE THESIS

Tuka B, Helyes Z, Markovics A, Bagoly T, Szolcsányi J, Szabó N, Tóth E, Kincses ZT, Vécsei L, Tajti J. Alterations in PACAP-38-like immunoreactivity in the plasma during ictal and interictal periods of migraine patients. *Cephalalgia*. 2013;33(13):1085-95. **IF: 3,485**

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Csati A, Tajti J, **Tuka B**, Edvinsson L, Warfvinge K. Calcitonin gene-related peptide and its receptor components in the human sphenopalatine ganglion -- interaction with the sensory system. *Brain Res*. 2012;1435:29-39. **IF:2,879**

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Cumulative impact factor of the publications related to the thesis: 17.411

Number of independent citations: 17

Σ Publications: 11

Σ Presentations (oral and poster): 22

Cumulative impact factor of all papers: 32.113

Number of total citations: 69

Number of total independent citations: 44

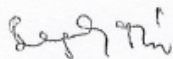
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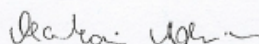
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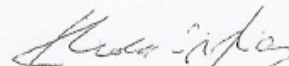
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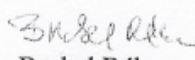
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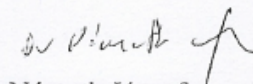
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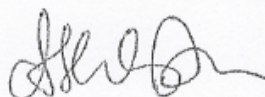

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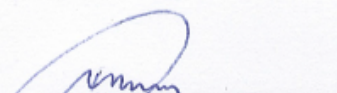

Németh József


Tajti János

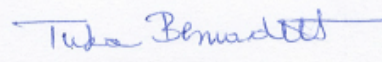

Helyes Zsuzsanna

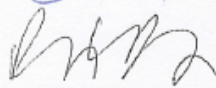

Párdutz Árpád



Tóth Eszter


Kincses Zsigmond Tamás


Reglődi Dóra


Tuka Bernadett


Márk László


Szabó Nikoletta


Vécsei László

Szeged, 2013. 11. 18.