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

PhD thesis

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I. CONTENTS

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II. LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
AC	adenylate cyclase
ADCYAP1	PACAP gene
ANOVA	analysis of variance
ATP	adenosine triphosphate
BBB	blood-brain barrier
BSA	bovine serum albumin
C_1-C_2	cervical$_1$-cervical$_2$ segments of the spinal cord
C_3-C_4	cervical$_3$-cervical$_4$ segments of the spinal cord
c-fos	protein of immediate early gene, general marker of neuronal activity
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine-monophosphate
CGRP	calcitonin gene-related peptide
CS	chemical stimulation
CSD	cortical spreading depression
CSF	cerebrospinal fluid
CHCA	α-cyano-4-hydroxycinnamic acid
DAG	diacyl-glycerol
DRG	dorsal root ganglia
EDTA	ethylenediaminetetraacetic acid
ES	electrical stimulation
Gi	inhibitory G-protein
Gq/11	heterotrimer G-protein subfamily
Gs	facilitating G-protein
GTP	guanosine triphosphate
HPA	hypothalamic-pituitary-adrenal axis
IHS	International Headache Society
IP_3	inositol-triphosphate
ir	immunoreactive
LI	like immunoreactivity
LC	locus coeruleus
MA	migraine with aura
MMA	middle meningeal artery
MALDI TOF	matrix-assisted laser desorption ionization time-of-flight
MLCK	myosin light-chain kinase

MO	migraine without aura
MRI	magnetic resonance imaging
MS	mass spectrometry
NMDA	N-methyl-D-aspartic acid
nNOS	neuronal nitric oxide synthase
NO	nitrogen oxide
NOS	nitric oxide synthase
NRM	nucleus raphe magnus
NTG	nitroglycerin
PAC_1	PACAP type I receptor
PACAP	pituitary adenylate cyclase-activating polypeptide
PACAP-27	pituitary adenylate cyclase-activating polypeptide-27
PACAP-38	pituitary adenylate cyclase-activating polypeptide-38
PAG	periaqueductal grey matter
PIP_2	phosphoinositol-diphosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
RhoA	Ras homolog gene family, member A; small GTPase protein
RIA	radioimmunoassay
SD	standard deviation
S.E.M.	standard error of the mean
SC	spinal cord
SP	substance P
TAT	trans-activator of transcription
TCC	trigeminocervical complex
TFA	trifluoroacetic acid
TNC	trigeminal nucleus caudalis
TRG	trigeminal ganglion
TS	trigeminovascular system
VAS	visual analogue scale
VIP	vasoactive intestinal peptide
$VPAC_1$	VIP, VIP1 or PACAP type II receptor
$VPAC_2$	VIP2 or PACAP type III receptor

III. INTRODUCTION

The main concept of this study was to explore molecules, which may be involved in the activation of the trigeminovascular system (TS) 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 [1, 2], paroxysmal primary headache disorder. Characteristically, this is a highly complex, restrictive [3-5] and extremely costly [6, 7] disease, which has high socio-economic and personal impacts on the quality of life (workdays, school performance, family and social relationships).

Pathomechanism

Although there have been extensive researches in the field of migraine, the exact details of the pathomechanism are still unknown. Several hypotheses have been proposed to explain the processes of headache diseases. Although the predisposition to the development of migraine is presumably genetically determined, certain environmental factors (alcohol, certain foods, stress, hormonal changes, etc.) can trigger the emergence of headache. Factors assumed in the background of the mechanism of migraine include neuro-vascular alterations, neuropeptide release, the presence of neurogenic inflammation, plasma protein extravasation, peripheral and central sensitization, cortical spreading depression (CSD), a brain energy deficit and lesions in the white matter, as separately or simultaneously occurring phenomena.

Since the 1990s, the central theme of migraine research has been the trigeminovascular theory, proposed by Moskowitz [8]. The TS provides an important pain-transmission link between the vascular and neuronal elements, because this is the major afferent pain pathway between the cranial vessels and the nuclei in the brainstem [9] ***(Fig. 1)*** [10]. Activation of the TS can therefore contribute to the development of migraine.

The TS consists of the primary sensory pseudounipolar neurons whose cell bodies are located in the trigeminal ganglion (TRG), its terminals and the meningeal vasculature. The peripheral branches innervate the cranial vessels and meningeal tissues (the supratentorial dura mater, the dural vasculature and the pial arteries on the surface of the brain). The central fibres project to the area of the second-order neurons in the brainstem, the trigeminal nucleus

caudalis (TNC) and more caudally in the upper regions of the spinal cord (the nucleus spinalis nervi trigemini). The third-order neurons are located in the thalamus.

The sensory trigeminal unit is controlled by the descending pathways from the monoaminergic nuclei (the nuclei raphe, the periaqueductal grey matter (PAG) and the locus coeruleus (LC)), referred to as migraine generators. Their exact functions are unknown, because the activation of these nuclei may be either a trigger or a consequence of migraine attacks. It is sure that elevated numbers of c-fos-immunoreactive (-ir) cells have been confirmed in the nucleus raphe magnus (NRM) following electrical stimulation of the TRG in rat [11].

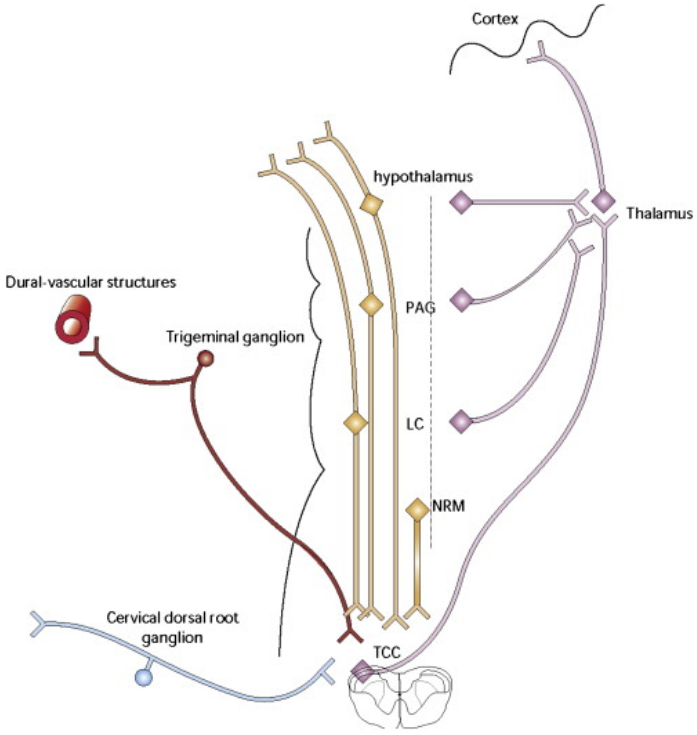

Figure 1. *Trigeminovascular system (TS)*

TCC: trigeminocervical complex; PAG: periaqueductal grey matter;

LC: locus coeruleus; NRM: nucleus raphe magnus

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Transmitters, neuropeptides and sensitization

Trigeminovascular activation produces a significant release of vasoactive molecules and various neuropeptides from the terminals of the trigeminal branches. Calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP) and substance P (SP) can induce

functional changes such as vasodilatation, protein extravasation, mast cell degranulation, neurogenic inflammation and sensitization [12]. The co-existence of SP-, CGRP- and pituitary adenylate cyclase-activating polypeptide (PACAP)-ir fibres has been demonstrated in the region of the human TNC. Numerous SP-positive fibres have been identified in the LC, the NRM and the PAG, and also a few VIP fibres in the latter two structures. CGRP-ir cells in high number and PACAP-ir cell bodies have been found in the LC. It seems that, of the above molecules, the role of CGRP is the most significant in the mechanisms of migraine [13]. Stimulation of the TRG in laboratory animals and also in humans increases the intracranial blood flow in part, presumably via CGRP release [14, 15]. During migraine attacks, an elevated plasma CGRP level has been reported in the external jugular vein [16], but these results were recently disputed [17, 18]. Furthermore, an elevated CGRP concentration was observed in the cubital vein during nitroglycerin (NTG)-provoked headache, which returned to the baseline after the cessation of the pain. A correlation was detected between the CGRP level, the timing of the attack and the severity of the pain. The influence of CGRP in migraine headache is validated by the administration of triptans, which successfully ameliorate the attacks, the level of CGRP returning to the control [19].

Epidemiology and general features

Epidemiological studies have revealed that the prevalence of migraine in the adult population in the developed countries is approximately 12%. Migraine may develop at any age and gender, but it is relatively common in young adult women. The diagnostic criteria of migraine are at least five attacks/month fulfilling the specified length of the attacks, the characteristics and the specific accompanying symptoms. Typically, the attacks are separated by shorter or longer painless intervals [20].

Types

The 2004 guidelines of the Headache Classification Committee of the International Headache Society (IHS) [20] state that there are two main forms of this disease: migraine with or without aura.

Migraine without aura (Common/simple migraine)

This is the predominant, a clinical syndrome manifesting in recurrent attacks. The throbbing headache is characterized by a unilateral location, moderate or severe intensity and a lasting nature (4-72 hours). Routine physical activity aggravates the pain, and the attacks are

accompanied by specific features and associated symptoms (autonomic, eg. nausea, vomiting, vertigo, a feeling of weakness and shivering, and sensory, e.g. osmo-, photo- and phonophobia and often allodynia). Allodynia, which means an exaggerated pain reaction to a normally innoxious stimulus, may be highlighted, as it was examined in our study. The sensitization of the peripheral (TRG) and second-order (trigeminocervical complex; TCC) trigeminovascular neurons evokes throbbing and cephalic allodynia. The sensory input converges from the cephalic vasculatures, the meninges, the scalp and the facial skin. The activation of the third-order trigeminovascular neurons (the posterior thalamic nuclei) can be associated with the formation of extracephalic allodynia, which is related to the sensitivity of the facial and body skin [21, 22]. Additionally, migraine without aura often displays a menstrual relationship [23], which was also investigated in our project.

Migraine with aura (Classic/complicated migraine)

In migraine with aura, the episodic headache attacks and associated migraine symptoms are similar to those mentioned previously. However, they are usually preceded or sometimes accompanied by the phenomenon of aura, which is a transient, focal neurological symptom, lasting from minutes to an hour. Patients report the aura as a premonitory phase, occurring hours or days before the headache, and a headache resolution phase. They experience visual (scintillating scotoma and fortification spectrum) and sensory (paresthesia) symptoms and/or with aphasia; hyperactivity, hypoactivity, depression, a craving for particular foods, repetitive yawning, fatigue and neck stiffness and/or pain may also occur.

Phases and symptoms

The period of the migraine attack can be divided into 4 sections.

a) The prodromal phase, as the early warning signs of the beginning of the attack, occurs several hours or even the day before the migraine. The patients usually notice unusual sensations and they may experience either strange energetic, excitable or depressed feelings, they may be irritable, they may feel thirsty or a craving for certain foods, they may be sleepy, with frequent yawning, or they may need to urinate more. These symptoms can facilitate the diagnosis of the problem as involving migraine.
b) The second phase is typical only of aura with migraine. In this case, the patients have mainly strange visual, auditory and skin sensations, language problems, confusion and difficulties in concentration.
c) The third phase is the headache with the accompanying symptoms mentioned above.

d) The last phase is the resolution or postdrome period, where the patient may feel tired or hung over, and they may have a mild headache, cognitive difficulties, gastrointestinal symptoms, mood changes and weakness. The patients generally note depression and malaise after an attack, though in extreme cases some people feel unusually refreshed or euphoric.

Therapy

In clinical practice, various drug treatments are available for the therapy of migraine. First and foremost, elimination of the provoking factors is the most important. Such agents include hormonal changes, foods, synthetic sweeteners, flavour enhancers, alcohol, sleep deprivation, stress, strong smells, weather changes and some drugs.

The drug treatment of the diagnosed migraine patient involves both attack and interval therapy. In milder cases, general analgesics and non-steroidal inflammatory drugs together with antiemetics are sufficient, but in more severe cases specific antimigraine drugs are indispensable. Ergot alkaloid and serotonin (5-hydroxytriptamine, 5-HT) 1B/D-receptor agonist triptans are the most effective drugs in the abolition of migraine attacks, while β-receptor and Ca^{2+}-ion channel blockers, 5-HT_2 anatgonists, tricyclic antidepressants and anticonvulsive drugs are of value as prophylactic therapy [24-26]. However, the effectiveness of these pharmaceuticals is not sufficient in all patients and the migraine-specific drugs usually have undesired side-effects. Hence, the investigation of new molecules, targets and markers involved in the pathomechanism of migraine is indispensible, with the development of effective drugs and successful therapy.

Among the numerous “migraine-related” processes and substances, recent studies have potentially implicated the vasoactive PACAP in the pathophysiology, and hence in the future therapy of migraine [27, 28].

B. PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP)

Characteristics

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 [29, 30].

PACAP was discovered due to its ability to increase adenylate cyclase (AC) activity in rat pituitary cells, and was first isolated from the ovine hypothalamus in 1989 [31]. The gene of PACAP (ADCYAP1) is localized on the short arm of chromosome 18 [32]. The peptide exists in two biologically active amidated forms, containing 38 and 27 amino acids: PACAP-38 and PACAP-27 ***(Fig. 2)***. PACAP-38 is the predominant form, accounting for 90% of the total PACAP content in most mammalian tissues, but it is rapidly metabolized and its plasma elimination half-life is less than 5 min [33]. PACAP is widely distributed in the central nervous system [34-36], in peripheral organs [37], in the endocrine glands [38, 39], and in secretions from the exocrine glands [40], thereby functioning as a pleiotropic peptide [41, 42].

PACAP 27

His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Ala-Ala-Val-Leu-NH_2

PACAP 38

His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Ala-Ala-Val-Leu-**Gly-Lys-Arg-Tyr-Lys-Gln-Arg-Val-Lys-Asn-Lys**-NH_2

Figure 2. *Amino acid sequences of PACAP-27 and PACAP-38.*

It is a hypophysiotropic hormone [43], a neurotransmitter and a neuromodulator in the nervous system [44], and it exerts neuroprotective [45], antiapoptotic [46] and differentiation-inducing effects in the developing nervous system [47, 48]. Furthermore, it serves important regulatory and protective roles in the gastrointestinal [49], cardiovascular [50-52], reproductive [53, 54] and respiratory systems [55]. The effects of PACAP are mediated through three receptors: $VPAC_1$ (previously designated the VIP, VIP1 or PACAP type II receptor), $VPAC_2$ (known as the VIP2 or PACAP type III receptor) and PAC_1 (formerly known as the PACAP type I receptor); the latter has 1000-fold higher specific affinity for both forms of PACAP than for VIP [28, 56]. The binding of PACAP to its receptors induces two main signal transduction pathways. Through Gs- or Gq/11-protein activation, a number of kinases exert a variety of physiological and pathophysiological effects [28, 30].

PACAP – neuropeptides, nociception, trigeminal system, migraine

The role of PACAP in vasodilatation [57, 58] and nociceptive processes [59-65] has been confirmed in several studies. The presence of this peptide has been demonstrated in the trigeminal system [35, 66-68]. The co-localization of nociceptin and PACAP has been described, but their relationship is unknown. An investigation of human TRGs revealed that ~68% of the nociceptin-positive cells contained PACAP [69]. In another study, moderately dense CGRP and PACAP-containing fibres were observed adjacent to numerous SP-ir fibres, but VIP-ir fibres were not seen in the TNC or at the cervical$_1$-cervical$_2$ (C_1-C_2) levels of the spinal cord [35, 70]. The co-existence of PACAP and SP has also been reported [71, 72]. Moreover, PACAP co-exists with CGRP in sensory ganglia and nerve plexuses of inner organs [73].

The available human data point to the involvement of PACAP in the mechanisms of migraine. A clinical study has revealed that intravenously administered PACAP-38 induces headache in healthy volunteers and migraine-like attacks in patients with migraine without aura (6 h on average after the start of the infusion) [27], similarly to the effect of NTG in causing headache [74, 75]. Moreover, the decrease of the mean blood flow velocity in the middle cerebral artery and the increase of the diameter of the superficial temporal artery were also observed in the PACAP study of the migraineurs. This implies again that PACAP-38 has vasodilating effect and it has role in the migraine-related mechanisms and anatomical structures:

A broad range of data suggest that PACAP is an integrator of nociceptive and sensitization processes, besides being involved in neurogenic inflammation [59, 61, 65, 76]. This peptide is present in the primary sensory neurons of the TRG [68], the parasympathetic otic and the sphenopalatine ganglia [77, 78]. 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 [13, 35]. We earlier furnished evidence for this hypothesis with animal experimental results. PACAP-deficient mice displayed reduced light-aversive behaviour (photophobia), as well as decreased meningeal blood flow and c-fos expression in the TRG and TNC were detected relative to wild-type mice after NTG-induced TS activation [79].

Based on these results it is assumed that PACAP may be an important mediator, and therefore a diagnostic marker of TS activation. The receptors of PACAP have been implicated as potential therapeutic targets in migraine pathophysiology [28]. However, there are no direct experimental data to confirm this theory and no clinical data are available on endogenous alterations in PACAP levels in relation to migraine.

IV. AIMS

The general aim of our study was to determine whether there are any alterations in the concentration of PACAP in blood and nerve tissues in the case of TS activation and migraine disorder. Preclinical investigations were therefore conducted by the stimulation of the TS in animals in order to generate migraine-like conditions, while the specificity and relevance of PACAP in migraine were confirmed in our human clinical study.

1) PRECLINICAL ANIMAL EXPERIMENTS

Our goal was to investigate the potential peripheral and central effects of PACAP in two types of rat experiments, which are models of peripheral and central sensitization by different pathways:

a) NTG-induced chemical stimulation

b) Electrical stimulation of the TRG

PACAP-38-LI and PACAP-27-LI were measured following the development of the models in a time-dependent manner, in the venous blood plasma (the cranial vena cava) and the Gasserian ganglion (TRG), indicating peripheral alterations.

In order to evaluate the central changes in both forms of the peptide, the immunoreactivities were determined in the area of second-order sensory neurons (TNC), the lower spinal cord (C_3-C_4) by radioimmunoassay (RIA), and the cerebrospinal fluid (CSF) from the suboccipital cistern by mass spectrometry (MS).

2) CLINICAL HUMAN INVESTIGATIONS

It is possible to analyse the mechanisms of pain in animal models, but there is no real clinically relevant system with which to mimic the human specificity of headache diseases appropriately. Human investigations are therefore particularly important to identify the key mediators responsible for the development and progression of migraine.

Based on the literature, we hypothesized that the plasma concentration of PACAP-38 increases during migraine attacks. Our aims were therefore to reveal the potential relationship between the PACAP-38 level of the human plasma and the presence of migraine headache.

RIA measurements were carried out on peripheral blood plasma samples in order to determine the alterations in PACAP-38-LI during the ictal and interictal periods in migraine patients in comparison with healthy control subjects.

In addition, the clinical features of the disease, the plasma CGRP-like immunoreactivity (CGRP-LI) and the PACAP-38-LI were compared to explore possible correlations.

V. MATERIALS AND METHODS

1) PRECLINICAL ANIMAL EXPERIMENTS

Animals

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 in the NTG-induced chemical TS activation model, 20 in the electrical TRG-stimulation model, and 11 as intact animals in the control group ***(Table 1)***. The animals were bred and maintained under laboratory conditions on a 12-h dark 12-h light cycle at 22-24 °C and ~60% relative humidity in the Laboratory Animal House of the Department of Neurology in Szeged. Standard rat chow and tap water were available *ad libitum*.

Groups	**Subgroups**			**Total number of animals**
Control				n=11
NTG-model	90 min (n=14)	180 min (n=14)		n=28
ES-TRG-model	ES-TRG	90 min (n=5)	180 min (n=5)	n=20
	Sham ES-TRG	90 min(n=5)	180 min (n=5)	

Table 1. *Groups of animals in both models.*

Ethics

All experimental procedures performed in this study complied fully with the guidelines of Act 1998/XXVIII of the Hungarian Parliament on Animal Protection and the Decree on Scientific Procedures in Animal Experiments (243/1988), and with the recommendations of the International Association for the Study of Pain [80] and the European Communities Council (86/609/ECC). The studies were in harmony with the Ethical Codex of Animal Experiments and were approved by the Ethics Committee of the Faculty of Medicine, University of Szeged.

Models

Chemical stimulation of the TS

A commonly applied and well-established animal model of TS activation is the systemic administration of NTG. Extensive literature is available on this field regarding the mechanisms, the good reproducibility and the human relevance [81-85].

The NTG has proved to be useful as an antianginal drug; however, after discovery its headache producing side effect was immediately noted. The sublingually applied NTG induces a sudden, mild intensity 1-hour lasting headache after 20-30 min, in healthy subjects [86], but it occurs more often in migraineurs [87]. This first phase usually relieves spontaneously. After a delay of approximately 4 hours the NO-induced mechanisms trigger typical moderate or severe throbbing attack without aura only in migraine patients, which requires medication. Rarely, the migraine-like headache may also occur in healthy subjects, which can be explained by an anamnestic data predisposing the development of migraine [75].

The effect of NTG is based on the release of nitrogen oxide (NO), which produces a rapid vasodilatation. The effects of NO can be attributed to the soluble guanylate cyclase that enhances the conversion of guanosine triphosphate (GTP) to the second messenger cyclic guanosine-monophosphate (cGMP). The initial steps of signalling pathway are followed by activation of protein kinase-G (PKG), which can phosphorylate specific proteins in the vascular smooth muscles. As a result the RhoA monomer G-protein is inhibited, while the myosin light-chain protein is activated by the myosin phosphatase. The dural arteries dilate, which stimulate the pain-sensitive fibres around the blood vessels [88].
NO is an endogenous transmitter, formed during the conversion of L-arginine to citrullin on the action of nitric oxide synthase (NOS). The NOS molecule is one of the markers of trigeminal activation, since NO itself is a very unstable gaseous substance, difficult to detect. The constitutive, Ca^{2+}-dependent neuronal NOS isoform (nNOS) is the most important enzyme from the aspect of sensory information in trigeminal pain processing. It is abundant in the superficial layers of the dorsal horn of the spinal cord [89-91]. The inhibition of NOS was showed to be able to ameliorate the symptoms of spontaneous migraine attacks [92].

Experiments

Three groups were involved in the NTG-induced chemical stimulation (CS) studies. One group of 11 animals remained intact. In two other groups, 14 animals per group received a single i.p. injection of NTG (prepared from Nitrolingual Pumpspray, Pohl-Boskamp GmbH, Germany) in a dose of 10 mg/kg (0.13 ml/100 g of a 7.68 mg/ml solution) to induce CS of the TS ***(Fig. 3)***.

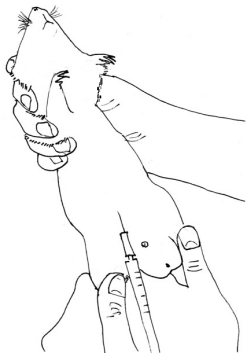

Figure 3. *NTG-injection in rat.*

In rats, NTG in the dose mentioned above, as a massive stimulus for the TS, can trigger physiological (arterial diameter, pulsation and blood flow [93]) and molecular (c-fos, CGRP, SP, nNOS and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [84, 94-98]) responses that resemble a common manifestation of activated TS.

In case of the intact group the CSF, blood sampling and tissue preparation followed immediately the anaesthesia, but in the two other groups it took place only 90 min or 180 min after NTG administration. The animals were anaesthetized with i.p. chloral hydrate solution (in a dose of 0.4 g/kg), which provided stable, deep anaesthesia. Before cupping, CSF (~150 µl per animal) was taken from the suboccipital cistern, while 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). Following cupping different nerve structures (the TNC, spinal cord (SC) and TRG) were excised from the animals at the 90 or 180 min time points. In preliminary experiments, sampling was also carried out after 15 and 30 min (data are shown in the case of plasma), but in view of the absence of changes in PACAP-38-LI at these times, this was not done later. Blood samples were kept at 4 °C until the plasma was separated by centrifugation (5,000 rpm for 10 min at 4 °C). Samples were stored at -80 °C until the measurement of PACAP-38- and PACAP-27-LI by RIA and determination of these peptides by MS ***(Fig. 4.)***.

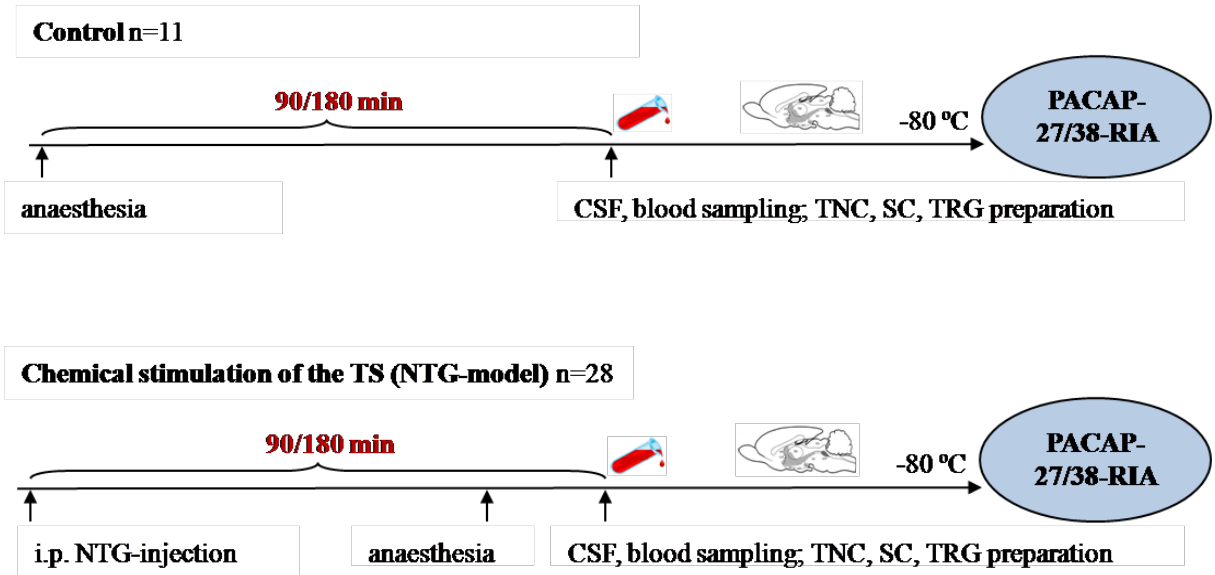

Figure 4. *Scheme of the NTG-model in rat.*

Electrical stimulation of the TS

Another possibility to develop an activated state of the TS is the electrical stimulation (ES) of the TRG. This is a well-described, widely used and generally certified method of TS activation with a broad range of stimulation parameters [99-105]. ES of the superior sagittal sinus can evoke a similar effect. Besides direct stimulation of the peripheral trigeminal afferents, ES can cause mast cell degranulation in the dura mater and the tongue. Pronounced neurogenic inflammation (vasodilatation and plasma protein extravasation) therefore develops on the brain surface. These responses can be explained by the release of inflammatory mediators, e.g. various vasoactive neuropeptides, which can trigger general neuronal activity in the area of the trigeminal complex or cause changes in blood flow [106, 107] and even induce structural alterations in the nerve terminals [100, 108-110].

Experiments

Five animal groups were created for these examinations: 11 rats served as non-stimulated intact animals; two groups of 5 rats each were followed up after sham stimulation until 90 and 180 min, respectively; and two groups of 5 rats each were investigated at 90 and 180 min after ES of the TRG. In earlier experiments, sampling was carried out after 30 min too (data are shown in the case of plasma), but as there was no change in PACAP-38-LI at this time, this was not done later. First, the rats were deeply anaesthetized with i.p. chloral hydrate solution (in a dose of 0.4 g/kg) and maintained throughout the experiment. The animals were placed in a stereotaxic setup where the head was fixed. After removal of the scalp, the localization of the TRG from the bregma was measured with micromanipulators according to the Watson-Paxinos Rat Brain Atlas (anteroposterior: 3.2 mm; mediolateral: 2.9 mm). The skull was drilled at the assigned point and the stimulating macroelectrode was passed into the brain to reach the TRG ***(Fig. 5)***. The TRG was stimulated according to the following parameters: duration of stimulation: 30 min; stimulation rate: 10 Hz; duration of impulse: 5 ms; current: 1 mA; stimulation mode: continuous.

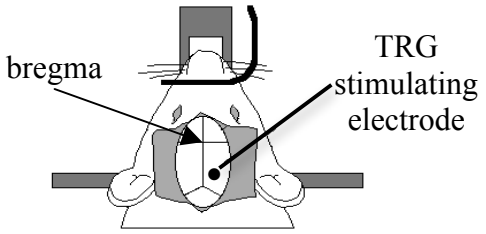

Figure 5. *Scheme of the ES-TRG in rat.*

This ES method can induce massive neuropeptide release from the pseudounipolar TRG neurons [111-113]. This neuropeptide depletion can be attributed to the more rapid firing of cells caused by the relatively high current and frequency and the long duration of the stimulation [114]. In cases of sham stimulation, the electrode was positioned in the same way

at the same location, but no current was applied. CSF, blood samples and neural tissues were collected, stored and analysed as described above ***(Fig. 6.)***.

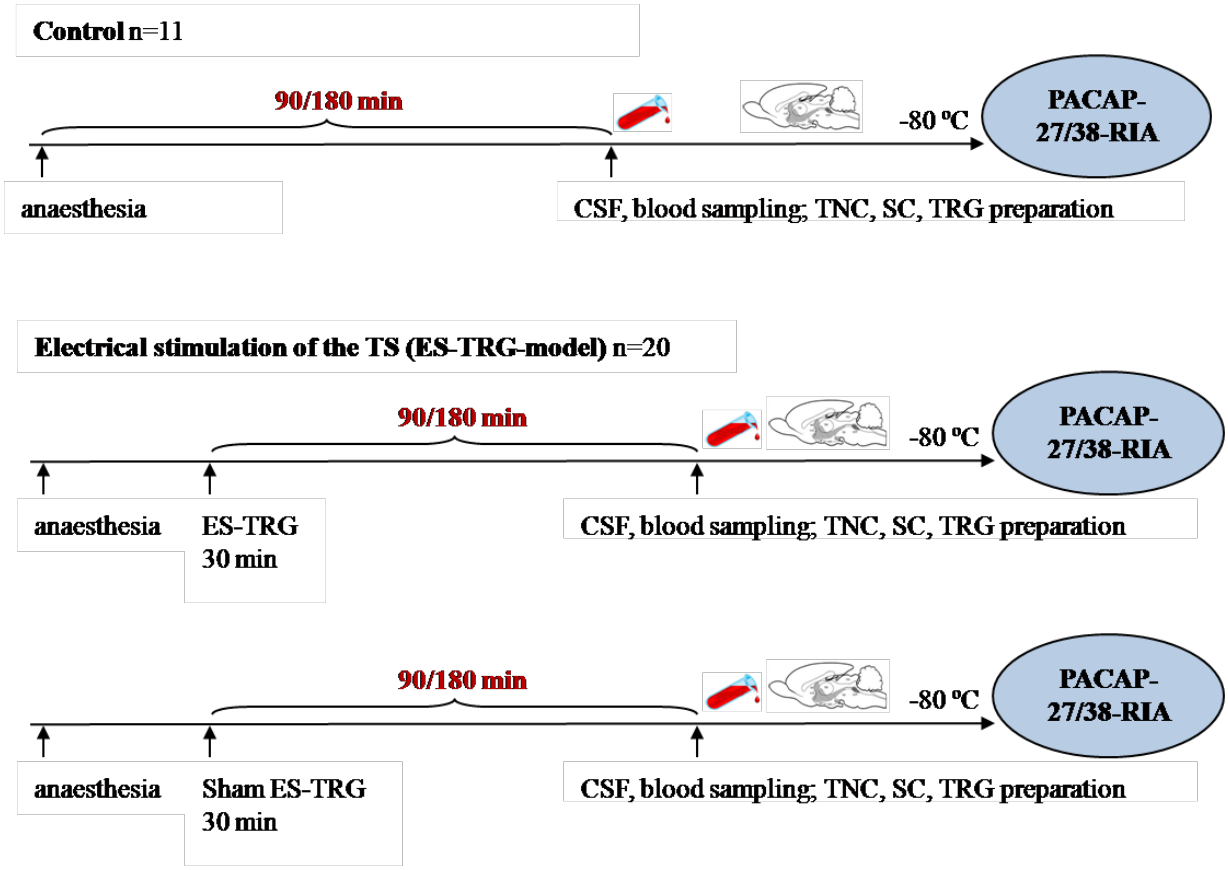

Figure 6. *Scheme of the ES-TRG model.*

RIA determination of plasma, CSF and tissue PACAP-38-LI and PACAP-27-LI

Plasma and CSF concentrations of PACAP-38 and PACAP-27 were determined with specific and sensitive RIA techniques developed earlier [115]. The "88111-3" PACAP-38 and the "88123-3" PACAP-27 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 this antibody were confirmed by cross-reactivity studies: no cross-reactivity was found with PACAP-27 in the PACAP-38 assay, with PACAP-38 in the PACAP-27 assay, or with other neuropeptides in either case. Following centrifugation of the blood samples (2000 rpm at 4 °C for 10 min) the peptide was extracted from the plasma into 3 volumes of absolute alcohol. After precipitation and a second centrifugation (2000 rpm at 4 °C for 10 min), the samples were dried under a nitrogen flow and resuspended in 300 µl assay buffer before RIA determination so as to achieve a 10 times

higher concentration for the RIA procedure [61, 115]. Brain segments (TNC, TRG and C_3-C_4 regions of SC) were frozen and stored at -80 °C until further processing. The samples were weighed and homogenized in 1 ml ice-cold bidistilled water with a manual potter homogenizer. The homogenates were centrifuged at 10000 rpm for 10 min and then at 12000 rpm for another 10 min, and 70 µl samples of the supernatants were used for RIA measurements.

The tracers were mono-^{125}I-labeled peptides prepared in our laboratory. Synthetic peptides were used as RIA standards in concentrations ranging from 0 to 1000 fmol/ml. The assay was prepared in 1 ml 0.05 M (pH 7.4) phosphate buffer containing 0.1 M sodium chloride, 0.25% (w/v) BSA and 0.05% (w/v) sodium azide. The antiserum (100 µl, 1:10000 dilutions), the RIA tracer (100 µl, 5000 cpm/tube) and the standard or unknown samples (100 µl) were measured into polypropylene tubes with the assay buffer. After incubation for 48-72 h at 4 °C, the antibody-bound peptide was separated from the free peptide by addition of 100 µl separating solution (10 g charcoal, 1 g dextran and 0.5 g commercial fat-free milk powder in 100 ml distilled water). Following centrifugation (3000 rpm at 4 °C for 15 min), the contents of the tubes were gently decanted and the radioactivity of the precipitates was measured in a gamma counter (Gamma, type: NZ310). The PACAP-38 and PACAP-27 concentrations of the unknown samples were read from calibration curves.

Examination of PACAP-38 and PACAP-27 in the rat plasma and CSF by MS

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. The aqueous solutions of the PACAP-38 and the PACAP-27 standards and the examined samples were loaded onto the target plate (MTP 384 massive target T, BrukerDaltonics, Bremen, Germany) by mixing 1 µL of each solution with the same volume of a saturated matrix solution, prepared freshly every day by dissolving α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile/0.1% trifluoroacetic acid (TFA) (1/2, v/v) [59]. The CSF samples were desalted and cleaned with 0.1% TFA solution with the use of $ZipTip_{18}$ pipette tips (Millipore Kft., Hungary). The purified proteins and peptides were eluted directly onto the MALDI target plate with 3 µl of acetonitrile/0.1% TFA (50/50, v/v) solution by mixing 1 µl of the saturated matrix solution described above. The ions were accelerated under delayed extraction conditions (200 ns) in positive ion mode with an acceleration voltage of 20.00 kV; each

spectrum was detected in linear mode. The instrument uses a 337 nm pulsed nitrogen laser, model MNL-205MC (LTB Lasertechnik Berlin GmbH., Berlin, Germany). External calibration was performed in each case with #206195 Peptide Calibration Standards (BrukerDaltonics, Bremen, Germany). Protein masses were acquired in the range of 1000 to 8000 m/z. Each spectrum was produced by accumulating data from 300 consecutive laser shots. The BrukerFlexControl 2.4 software was used for control of the instrument and the BrukerFlexAnalysis 2.4 software for spectrum evaluation.

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

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 [20]. The study groups were age-matched. The demographic and clinical characteristics of the patient and control populations are summarized in ***Table 2***.

A detailed questionnaire was used to compile a homogeneous group of migraineurs as concerns the features of their migraine disease: the duration of the migraine, the attack frequency, allodynia [116], the severity of pain during attacks as measured on a visual-analogue scale (VAS). The relation of the migraine attacks to the menstrual cycle [117] and to the presence of other non-migraine, chronic pain disorder (lumbago, low-back pain, knee- and hip-joint arthrosis) was also assessed. Depression was not clinically diagnosed in any of the cases. Healthy volunteers serving as controls were screened for non-reported/non-treated headaches. Subjects (both patients and controls) who displayed any significant and serious non-migraine chronic disorders were excluded from the study.

Healthy control subjects n=40	**Mean age** (years)	**Gender**
	36.60 ± 11.84	♀ n=26
		♂ n=14

Migraine patients n=87	**Mean age** (years)	**Gender**	**Type of migraine**	**Mean duration of disease** (years)	**Mean attack frequency/ year**	**Allodynia** n=23	**Mean VAS-score**
	37.91 ± 10.17	♀ n=79 ♂ n= 8	**MA** n=18 **MO** n=69	12.99 ± 9.61	36.80±28.44	0. level n=64 1. level n= 9 2. level n= 9 3. level n= 5	7.94± 1.75

Table 2. *Mean data on healthy control volunteers and 87 migraineurs (age, gender, type of migraine (with or without aura), disease duration (years), attack frequency, allodynia and VAS-score) are shown. MA: migraine with aura; MO: migraine without aura. Allodynia: 0: absence of allodynia; 1: mild allodynia; 2: moderate allodynia; 3: serious allodynia.*

Study design and procedures

The study was approved by the Ethics Committee of the Faculty of Medicine, University of Szeged (87/2009). All study participants gave their written informed consent, in accordance with the Declaration of Helsinki. There were no restrictions as regards food and drink intake. Blood samples were drawn from migraineurs during a migraine 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 of 21 migraineurs could be collected in both periods. Data of the 21 migraineurs are shown in ***Table 3***. 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.

Table 3. *Demographic data on 21 migraineurs, whose samples from both the ictal and the interictal periods were analysed. The age, gender, type of migraine (with or without aura), disease duration (years), attack frequency, allodynia, VAS-score, PACAP-38-LI in plasma samples (fmol/ml) originating from interictal and ictal phases, the time of the previous attack before interictal blood sampling (days) and the duration of the present headache (hours) are shown for each patient. MA: migraine with aura; MO: migraine without aura. Allodynia: 0: absence of allodynia; 1: mild allodynia; 2: moderate allodynia; 3: serious allodynia*

Patient	Age	Gender	Type of migraine	Duration of disease (years)	Attack frequency /year	Allodynia	VAS-score	Interictal PACAP-38-LI (fmol/ml)	Previous attack (days ago)	Ictal PACAP-38-LI (fmol/ml)	Duration of headache before sampling (hours)
1	24	♀	MO	3	17	0	10	**24.30**	9	**26.50**	4
2	39	♀	MO	26	30	0	9	**20.00**	2	**29.16**	96
3	46	♀	MO	18	24	0	9	**19.50**	7	**23.90**	4
4	44	♀	MO	30	47	3	7	**18.40**	9	**20.90**	12
5	45	♀	MO	2	12	0	9	**26.94**	6	**20.40**	5
6	27	♀	MO	14	30	2	8	**20.50**	6	**17.00**	24
7	35	♀	MO	20	12	0	8	**27.00**	6	**31.20**	7
8	39	♀	MO	30	109	2	9	**21.20**	10	**25.90**	7
9	31	♂	MA	10	52	0	7	**24.11**	7	**33.21**	8
10	21	♀	MO	5	24	0	7	**20.41**	7	**25.18**	3
11	38	♀	MO	2	30	0	7	**22.34**	5	**31.81**	24
12	46	♀	MO	10	36	0	8	**26.36**	7	**28.05**	10
13	17	♂	MO	10	12	0	8	**23.51**	10	**35.28**	2
14	30	♀	MO	10	12	1	5	**26.43**	7	**23.30**	24
15	39	♂	MO	10	66	0	8	**27.15**	1	**35.28**	2
16	37	♀	MO	9	30	0	8	**25.23**	7	**33.70**	48
17	50	♀	MO	14	12	0	8	**21.59**	21	**26.92**	5
18	52	♀	MO	14	26	3	8	**22.08**	5	**30.71**	60
19	35	♀	MO	25	52	0	10	**25.05**	1	**31.69**	8
20	58	♀	MO	15	21	0	8	**27.09**	7	**29.18**	14
21	35	♀	MA	6	20	2	10	**29.33**	20	**29.67**	20
Mean	**37.52**			**13.48**	**32.10**		**8.14**	**23.74**	**7.62**	**28.04**	**18.43**
SD	**10.34**			**8.61**	**23.23**		**1.20**	**3.09**	**4.95**	**5.00**	**23.33**

RIA measurements and data acquisition

Plasma concentrations of PACAP-38 were determined with specific and sensitive RIA techniques developed earlier [115]. 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, and no cross-reactivity was found with PACAP-27 or with other related neuropeptides in either case. Following centrifugation of the plasma samples (2000 rpm at 4 °C for 10 min) the peptide was extracted from the plasma into 3 volumes of absolute alcohol. After precipitation and a second centrifugation (2000 rpm at 4 °C for 10 min), the samples were dried under a nitrogen flow and resuspended in 300 µl of assay buffer before RIA determination, in order to achieve a 10-times higher concentration for the RIA procedure [61, 115]. The tracers were mono-^{125}I-labelled peptides prepared in our laboratory. Synthetic peptides were used as RIA standards in concentrations ranging from 0 to 1000 fmol/ml. The assay was prepared in 1 ml 0.05 M (pH 7.4) phosphate buffer containing 0.1 M sodium chloride, 0.25% (w/v) BSA and 0.05% (w/v) sodium azide. The antiserum (100 µl, 1:10000 dilution), the RIA tracer (100 µl, 5000 cpm/tube) and the standard or unknown samples (100 µl) were measured into polypropylene tubes with the assay buffer. After incubation for 48–72 h at 4 °C, the antibody-bound peptide was separated from the free peptide by the addition of 100 µl of separating solution (10 g charcoal, 1 g dextran and 0.5 g commercial fat-free milk powder in 100 ml of distilled water). Following centrifugation (3000 rpm at 4 °C for 15 min), the contents of the tubes were gently decanted and the radioactivity of the precipitates was measured in a gamma counter (Gamma, type NZ310). The PACAP-38 concentrations of the unknown samples were read from calibration curves.

Plasma concentrations of CGRP were determined with specific and sensitive RIA techniques developed earlier [118].

Statistical analysis

Data expressed as mean±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 $p<0.05$.

VI. RESULTS

1) PRECLINICAL ANIMAL EXPERIMENTS

Changes in PACAP-38-LI in rat plasma and different brain regions in response to CS of the TS

The level of PACAP-38-LI in the systemic circulation of intact, untreated rats, 18.5±3.6 fmol/ml, was not significantly changed within the 180-min examination period by CS of the TS with 10 mg/kg i.p. NTG ***(Fig.7)***. PACAP-27-LI was not measurable in the rat plasma; it was below the detection limit of the assay even when the total peptide content was extracted from a volume of 4 ml.

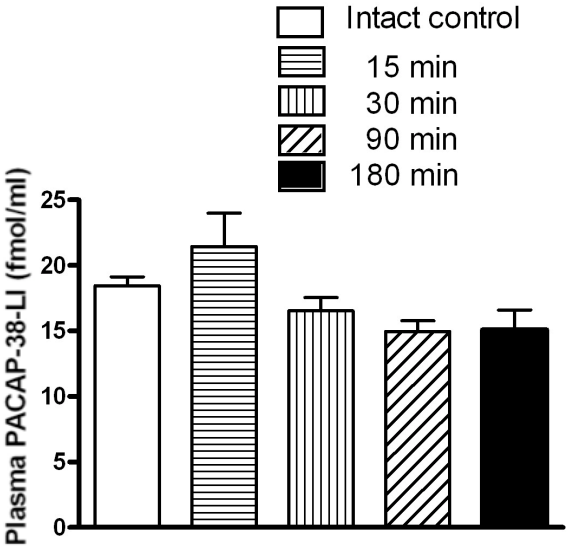

Figure 7. *PACAP-38-like immunoreactivity (PACAP-38-LI) determined by RIA in rat plasma 15, 30, 90 and 180 min after i.p. injection of 10 mg/kg nitroglycerin. Plasma samples of untreated intact rats served as control. Each column denotes the mean+S.E.M. of the results on n=11-28 animals. Significant differences were not observed with one-way ANOVA followed by Tukey's post-hoc test.*

RIA could reliably measure both PACAP-38-LI and PACAP-27-LI in the homogenates of different rat brain regions related to the trigeminal system. Their concentrations were ~5-6 fmol/mg and ~0.4-0.6 fmol/mg wet tissue respectively, in each of the TNC, the C_3-C_4 segments of the SC and the TRG. NTG injection evoked significant increases in PACAP-38-LI at both 90 min and 180 min in the TNC, but not in the other two examined areas. The concentrations of PACAP-27-LI were about 10 times lower than those of PACAP-38-LI in

both the TNC and the C_3-C_4 regions of the SC. The level of the shorter form was approximately half that of the longer one in the TRG. The NTG-induced alterations in PACAP-27-LI in each region were identical to the changes in PACAP-38-LI ***(Fig. 8)***.

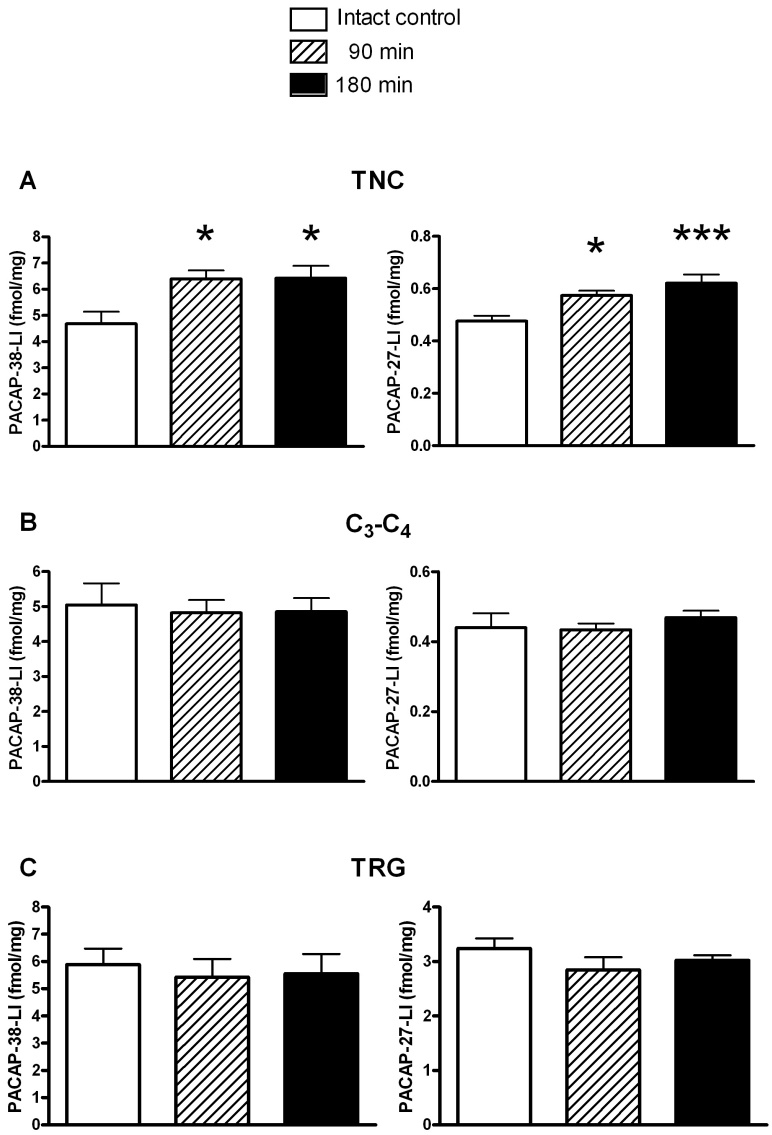

Figure 8. *PACAP-38-like and PACAP-27-like immunoreactivities (PACAP-38-LI and PACAP-27-LI) determined by RIA in homogenates of* ***(A)*** *the trigeminal nucleus caudalis (TNC),* ***(B)*** *the C_3-C_4 spinal cord segments, and* ***(C)*** *the trigeminal ganglia (TRG) of the rat 90 and 180 min after 10 mg/kg i.p. nitroglycerin injection. The respective brain segments of untreated intact rats served as control. Each column denotes the mean+S.E.M. of the results on n=11-28 animals; *p<0.05, ***p<0.001 vs. intact control (one-way ANOVA followed by Tukey's post-hoc test).*

Changes in PACAP-38-LI in rat plasma and different brain regions in response to ES of the TS

In contrast with what was observed on CS of the TS with NTG, ES of the right TRG led to a significant, ~30% elevation of the plasma PACAP-38-LI 90 min later. This elevation subsequently declined somewhat, but still remained significant at 180 min. Sham stimulation (electrode insertion without ES) did not influence the PACAP-38-LI in the systemic circulation ***(Fig. 9)***.

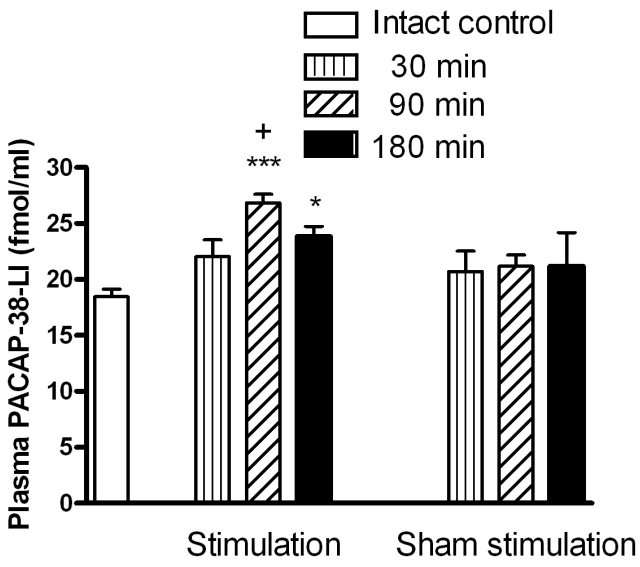

Figure 9. *PACAP-38-like immunoreactivity (PACAP-38-LI) determined by RIA in the rat plasma 30, 90 and 180 min after electrical stimulation of the TRG (10 Hz, 1 mA, 30 min). The plasma samples of untreated intact rats and sham-stimulated rats served as controls. Each column denotes the mean+S.E.M. of the results on n=11-20; *p<0.05, ***p<0.001 vs. intact control; $^{+}p<0.05$ vs. sham-stimulated control at the respective time (one-way ANOVA followed by Tukey's post-hoc test).*

Similarly to the effect of the NTG injection, electrical TRG stimulation gave rise to significant increases in both PACAP-38-LI and PACAP-27-LI levels in the TNC after 180 min, whereas no change was observed in the C_3-C_4 and the TRG regions. No change was detected in the sham-stimulated group ***(Fig. 10)***.

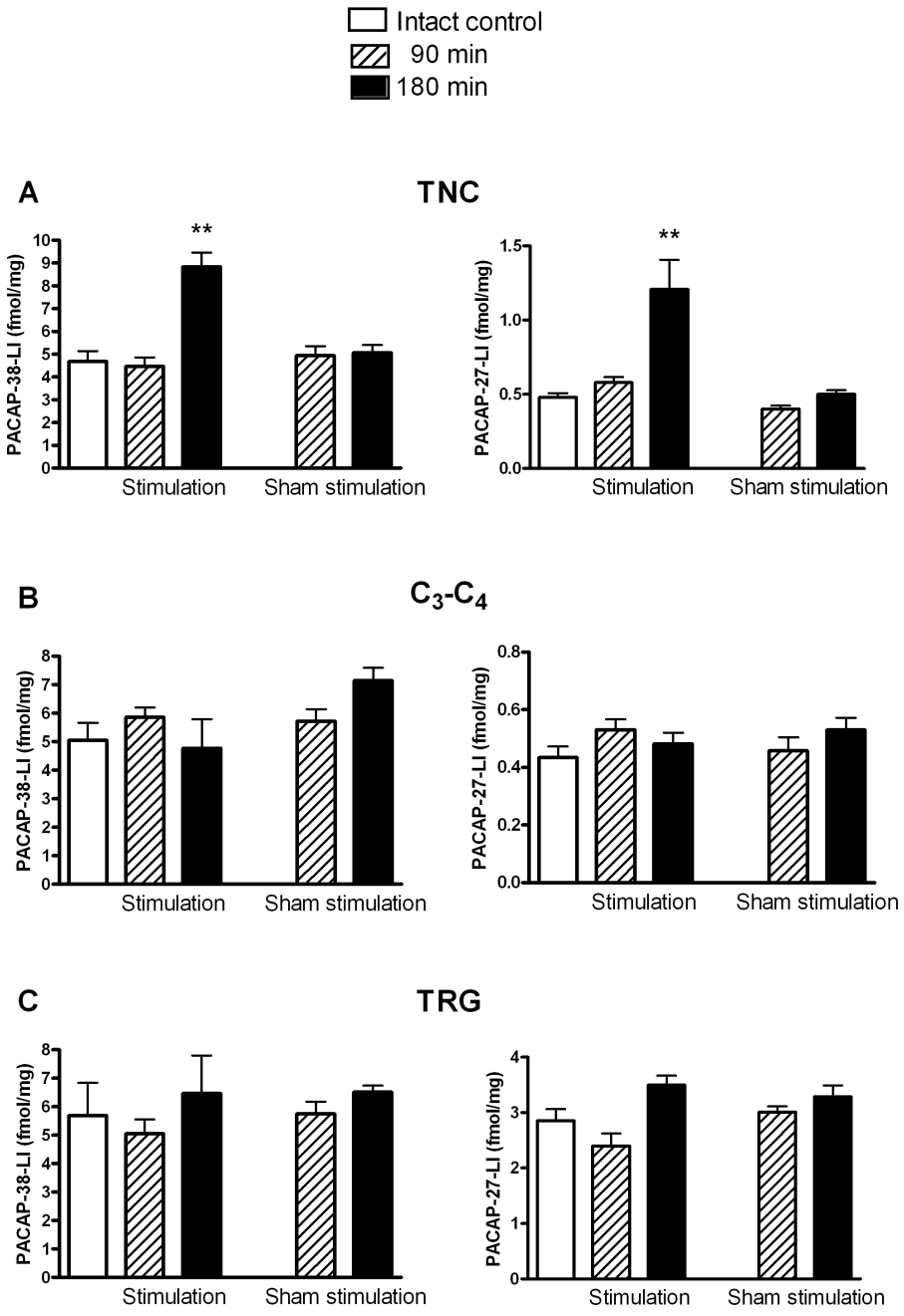

Figure 10. *PACAP-38-like and PACAP-27-like immunoreactivities (PACAP-38-LI and PACAP-27-LI) determined by RIA in homogenates of* ***(A)*** *the trigeminal nucleus caudalis (TNC),* ***(B)*** *the C_3-C_4 spinal cord segments, and* ***(C)*** *the trigeminal ganglia (TRG) of the rat 90 and 180 min after electrical stimulation of the TRG (10 Hz, 1 mA, 30 min). The respective brain segments of untreated intact rats and sham-stimulated rats served as controls. Each column denotes the mean+S.E.M. of the results on n=11-20 animals; **p<0.01 vs. intact control (one-way ANOVA followed by Tukey's post-hoc test).*

Identification of PACAP-38 and PACAP-27 in rat plasma and CSF

PACAP-38 was clearly identified by MS at m/z 4535.3 (PACAP-38 H^+ adduct) in the intact rat plasma samples, but PACAP-27 was not detectable ***(Fig. 11C)*** relative to the standard spectra ***(Fig. 11A,B)***. However, neither form could be found in the CSF samples obtained from any group ***(Fig. 11D)***. RIA measurements confirmed the lack of PACAP-38-LI and PACAP-27-LI in the CSF (data not shown).

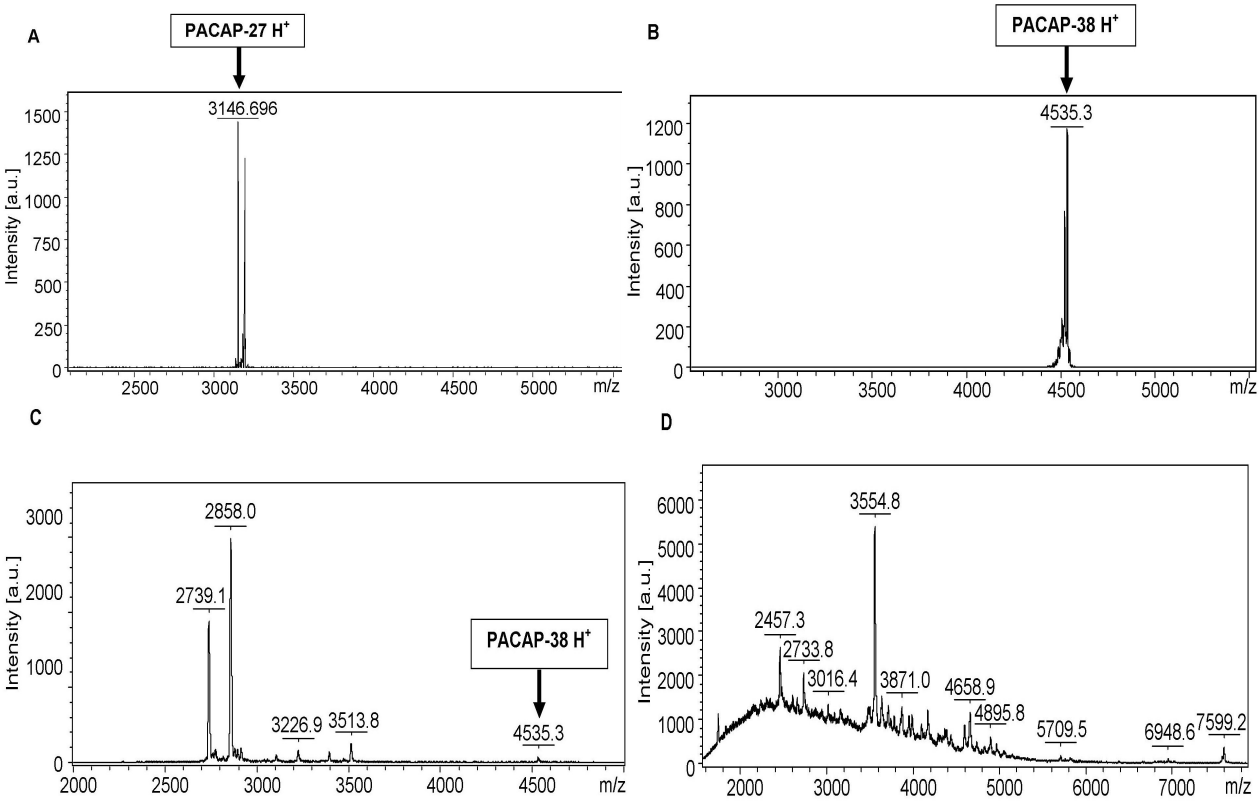

Figure 11. *Identification of PACAP-38 in* ***(B)*** *the standard and* ***(C)*** *intact rat plasma samples by MS at m/z 4535.3 Da, representing the average mass of the protonated quasimolecular ion of PACAP-38. Neither the other biologically active form, PACAP-27 (3147.6 Da), nor its* $[M+Na]^+$ *could be detected as compared to the* ***(A)*** *PACAP-27 standard. Panel* ***D*** *is a MALDI TOF spectrum of the intact rat CSF sample in positive ion mode using linear detection, where the characteristic peaks of PACAP-38 (4535.3 Da) and/or PACAP-27 (3147.6 Da) were not observed.*

2) CLINICAL HUMAN INVESTIGATIONS

Differences in plasma PACAP-38-LI between migraineurs and healthy controls

As concerns the total of 87 migraine patients (n=87), a significantly lower PACAP-38-LI was determined in the interictal plasma of the migraineurs (n=80; 24.60±3.59 fmol/ml) than in that of the healthy volunteers (n=40; 26.54±4.43 fmol/ml; Student's unpaired t-test, $p<0.011$, $t=2.578$) ***(Fig. 12/A)***. However, the plasma samples from the patients during their migraine attacks (n=28) exhibited a significantly higher PACAP-38 concentration (27.39±4.67 fmol/ml) as compared with the interictal samples (n=59; 24.91±3.73 fmol/ml; Student's unpaired t-test, $p<0.009$, $t=-2.676$) (the interictal data of those 21 migraineurs whose plasma samples were collected from both periods were excluded from this analysis to avoid the statistical problems caused by the paired samples) ***(Fig. 12/B)***. No difference was found when the ictal samples were compared with those of the controls (Student's t-test for unpaired comparisons, $p<0.447$, $t=-0.765$) ***(Fig. 12/C)***.

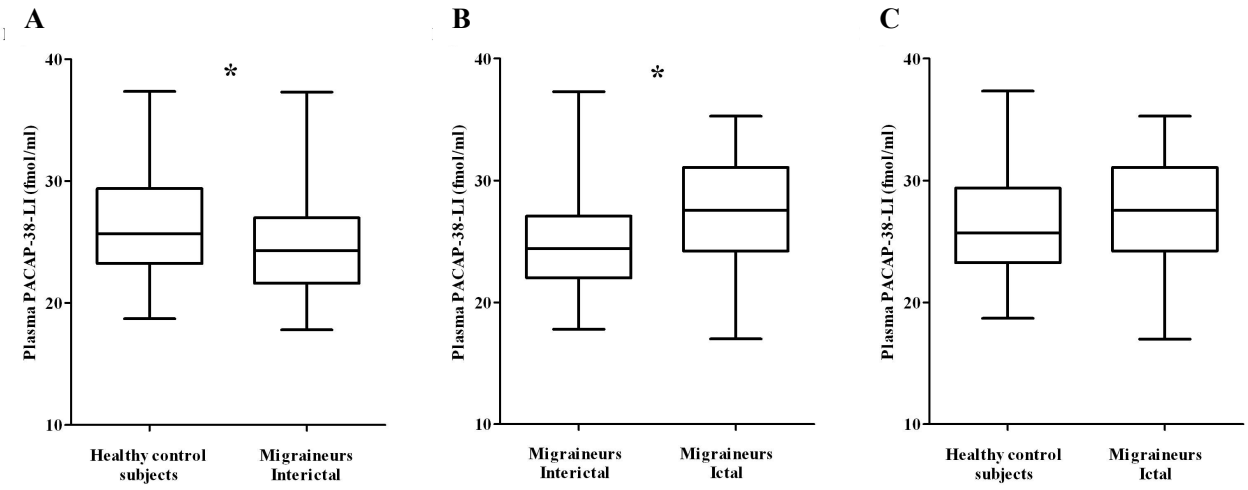

Figure 12. *PACAP-38-LI (fmol/ml) was determined by RIA in the plasma of the migraineur groups in comparison with those of healthy volunteers. Boxes indicate PACAP-38-LI (median±SD, minimum and maximum values) of healthy control subjects (n=40), and of migraineurs (n=87) during the interictal (n=80) and ictal periods (n=28). Significant PACAP-38-LI decrease was observed in the interictal group vs. the control with Student's unpaired t-test, $p<0.011$* ***(A)****. Leaving out the interictal data of the paired samples, the interictal (n=59) vs. the ictal (n=28) group comparison showed significantly higher PACAP-38-LI during migraine attacks with Student's unpaired t-test, $p<0.009$* ***(B)****. There were no significant PACAP-38-LI differences between the control and the ictal group with Student's t-test for unpaired comparisons, $p<0.447$* ***(C)****.*

Association between plasma PACAP-38-LI and duration of migraine

A mild negative correlation was found between the duration of the migraine and the interictal PACAP-38-LI (n=87; linear regression, $p<0.044$, $R=-0.231$) ***(Fig. 13)***. Plasma PACAP-38-LI did not correlate with the age, attack frequency, allodynia, and the VAS-score (ANOVA, linear regression, $p>0.05$) and differences were not found regarding the gender, hormonal changes and pain (Student's unpaired t-test, $p>0.05$).

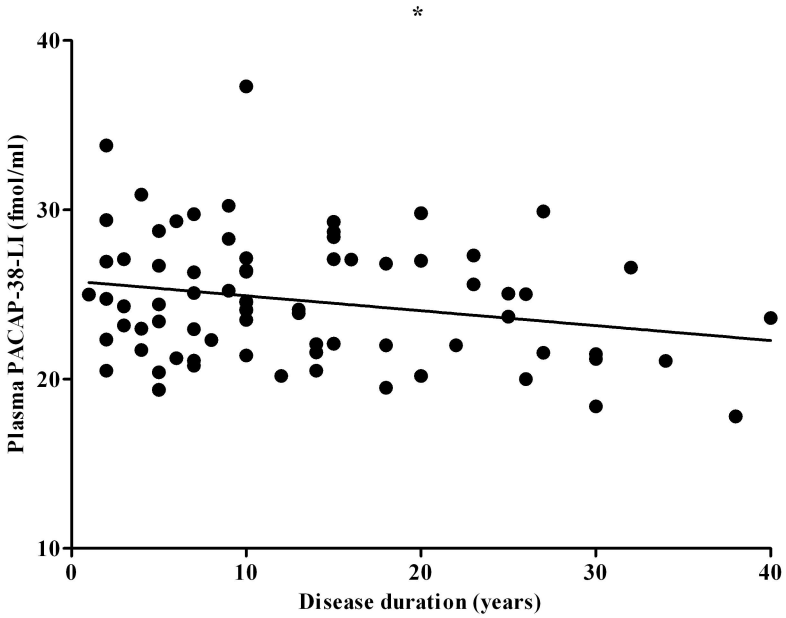

Figure 13. *Interictal plasma PACAP-38-LI (fmol/ml) depending on the duration of the migraine disorder in the group of migraineurs (n=87). A negative correlation was observed with linear regression on the graph, p<0.044.*

Changes in plasma PACAP-38-LI and CGRP-LI in 21 migraineurs

To gain insight into the changes of neuropeptide levels, we measured the plasma concentrations of PACAP-38 and CGRP plasma concentration in the same subject during a headache attack and interictally. The plasma PACAP-38-LI was significantly higher in the ictal period (28.04±5.00 fmol/ml) than in the interictal period (23.74±3.09 fmol/ml) (n=21; Student's paired t-test, $p<0.001$, $t=-4.134$) ***(Fig. 14/A)***.

The CGRP-LI was determined simultaneously with the PACAP-38-LI in both phases in 18 migraineurs. Significantly higher CGRP levels were observed in the plasma samples during the ictal period (53.74±31.52 fmol/ml) as compared to the interictal period (39.74±27.49 fmol/ml) (n=18; Wilcoxon-test, $p<0.035$) ***(Fig. 14/B)***.

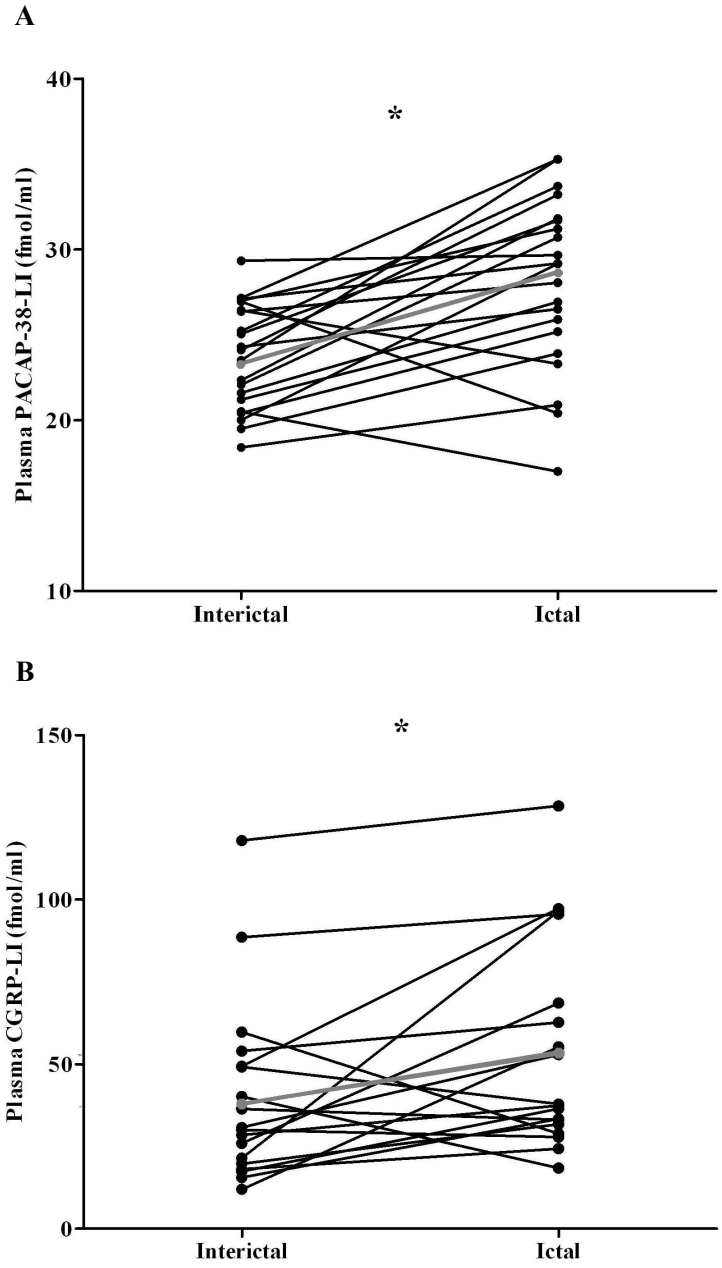

Figure 14.

A: *PACAP-38-LI (fmol/ml) was determined by RIA on plasma samples from migraineurs in both interictal and ictal periods. Plots of individual data for each patient (n=21). A significant difference was observed between the levels in the two phases with Student's paired t-test, $p<0.001$.*

B: *CGRP-LI (fmol/ml) was determined by RIA on plasma samples from migraineurs in both interictal and ictal periods. Plots of individual data for each patient (n=18). A significant difference was observed between the levels in the two phases with Wilcoxon-test, $p<0.035$.*

The thick grey lines represent the mean values.

Associations of changes in plasma PACAP-38-LI with the menstruation cycle sensitivity and chronic pain conditions in 21 migraineurs

Changes in the plasma PACAP-38-LI proved to be influenced by 2 parameters: There was a significant PACAP-38-LI elevation in the ictal phase (31.01±3.32) compared to the interictal phase (24.18±2.52) in patients whose migraine headache was not sensitive to the menstruation cycle (Group 1: n=11; Student's paired t-test, $p<0.00002$, t=-7.250). Meanwhile, there was no such significant increase during the ictal phase (24.78±4.56) compared to the interictal phase (23.26±3.70) in patients whose migraine headache was sensitive to the menstruation cycle (Group 2: n=10; Student's paired t-test, $p<0.344$, t=-0.998) ***(Fig. 15)***.

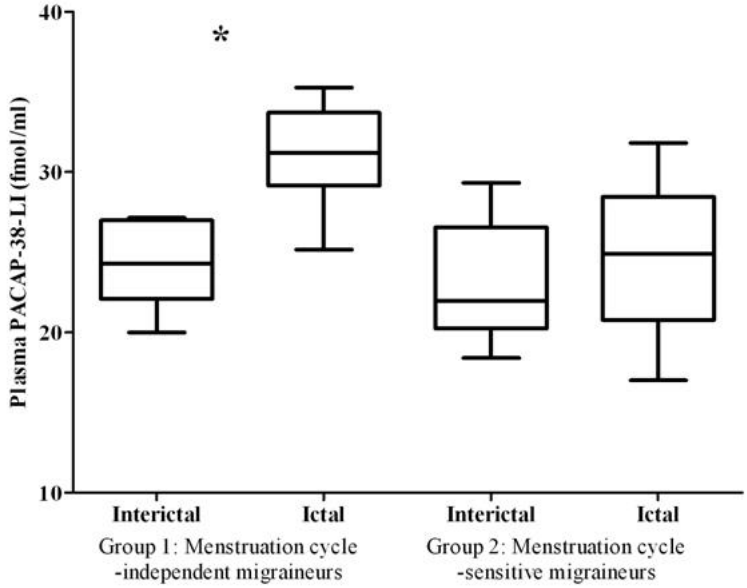

Figure 15. *Plasma PACAP-38-LI (fmol/ml) in the interictal and ictal periods of two subpopulations of 21 migraineurs, characterized on the basis of the menstruation cycle dependence. Group 1: menstruation cycle-independent migraineurs (n=11); group 2: migraine patients, whose headache was sensitive to their menstruation cycle (n=10). Each box represents the median±SD, minimum and maximum values of the results. A significant difference in PACAP-38-LI was observed between the interictal and ictal phases in group 1 with Student's paired t-test, p<0.00002.*

Similar PACAP-38-LI increase was detected in the ictal phase (30.01±3.69) vs. the interictal phase (24.15±2.47) of patients, who did not have chronic pain-related conditions (Group 1: n=15; Student's paired t-test, $p<0.00005$, t=-5.716). However, there was no difference in the ictal phase (23.13±4.64) compared to the interictal phase (22.71±4.40) in patients, who had

other non-migraine, chronic pain disorders (Group 2: n=6; Student's paired t-test, p<0.833, t=-0.222) ***(Fig. 16)***.

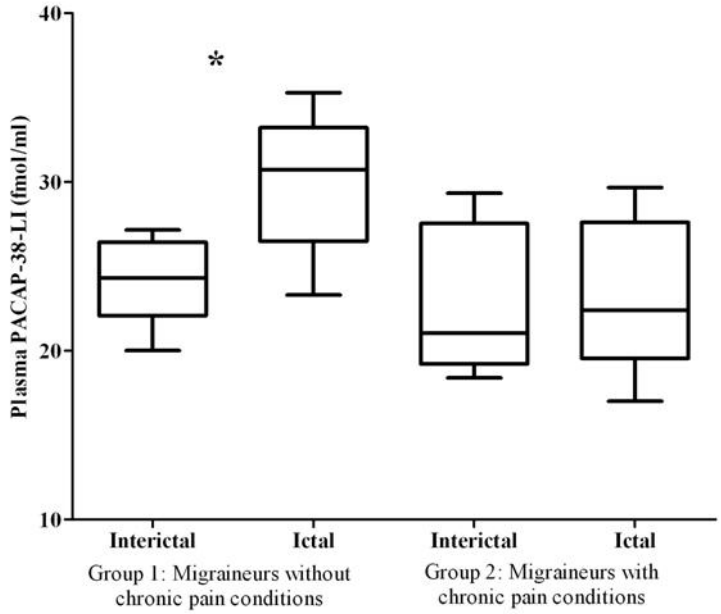

Figure 16. *Plasma PACAP-38-LI (fmol/ml) was characterized in the interictal and ictal periods of two subpopulations of 21 migraineurs, separated on the basis of chronic pain conditions. Group 1: patients without chronic low back pain (n=15); group 2: patients with simultaneous low back pain (n=6). Each box represents the median±SD, minimum and maximum values of the results. A significant difference in PACAP-38-LI was observed between the interictal and ictal phases in group 1 with Student's paired t-test, p<0.00005.*

To reveal the relationship between ictal-interictal PACAP-38 levels and menstruation cycle and chronic pain, repeated measure ANOVA was used. Multivariate test showed significant main effect of PACAP-38 measurements in different phases of the disease ($F(1,19)=22.579$, $p<0.0001$) and main effect of headache related to the menstruation cycle ($F(1,19)=22.257$, $p<0.0001$). The interaction of the two factors was also significant ($F(1,19)=9.096$, $p<0.007$). Similar ANOVA was carried out for identifying if ictal/interictal PACAP-38 level changes are influenced by concomitant chronic pain conditions. While significant main effects were identified by analysis (ictal/interictal: $F(1,19)=11.392$, $p<0.003$, chronic pain condition: $F(1,19)=0.553$, $p<0.446$) the interaction was not significant in this case ($p>0.05$).

No correlations were found between the levels of peptide and age, gender, the attack frequency, allodynia or the VAS-score.

VII. DISCUSSION

According to our results the alteration of PACAP-38-concentration in the peripheral blood plasma is associated with the migraine disease, which was also evidenced in animal models of the activated TS. The role of certain neuropeptides has being investigated for a long time in the development of migraine, but the function of PACAP in headache disorders may be a new perspective for the research.

1) PRECLINICAL ANIMAL EXPERIMENTS

Numerous animal experiments [59, 60, 62, 65] and some clinical studies [27, 28] have pointed out the key role of PACAP in nociceptive signalling mechanisms. Although its involvement in migraine has recently been indicated by human data [27, 223], and its immunohistochemical localization has been described in the TS [35], our work provides the first experimental evidence that its concentration is specifically altered in the TNC and the plasma in response to stimulation of the TS in rat models.

Our results support the neuropeptide theory of the development of activated TS. Both peripheral and central sensitizations were accompanied by PACAP-level 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.

Blood plasma

Chemical stimulation of the TS

It is well known that NO has vasodilating effect hence the NTG administration often causes immediate dull headache in sensitive subjects. The delay in the action of NO triggers a typical attack in migraineurs, which is possibly related to the activation of trigeminal Aδ and C fibres leading to central sensitization at the level of TNC [89].

In our experiment, the CS of the TS was might not strong enough to generate pronounced alterations in plasma PACAP-38-level. The higher PACAP-38-LI 15 min after NTG injection as compared with the control groups is explained by the acute and short-term effect of NO. In another study, we showed that the systemic blood pressure decreases in the first 10 min following NTG injection, after that it remains unchanged [79]. These phenomena are manifested in alterations in vessel wall tension, which can be one of the triggers of the pseudounipolar neurons of TRGs, since the intracranial vasculature is mainly innervated by

trigeminal nerves [119]. The initial vessel wall tension changes, as an exciter, stimulate the peripheral trigeminal nerve terminals, and therefore a small amount of PACAP-38 can be released into the systemic circulation. Later, when the blood pressure starts to become normal, the vessel wall tension will not change as much as earlier. It does not function as a trigger, so it cannot evoke PACAP-38 release.

The other explanation of the decreasing PACAP-38-LI 30, 90 and 180 min after NTG injection is the short (less than 5 min) plasma elimination half-life of PACAP-38 in the blood.

Central sensitization is generally confirmed in the NTG model, as observed in our experiments by the elevated PACAP-38- and PACAP-27-LI in the TNC. NTG evokes migraine attacks [75, 120] and develops sensitization [121] in human studies. Similarly, in animal experiments, NTG activates the second-order neurons and selectively elevates the levels of nNOS- [89], CaMKII- [122] and c-fos [123] -immunoreactivity, which are involved in nociception and central sensitization.

Electrical stimulation of the TS

The ES of the TRG caused significant elevations of PACAP-38-LI in the blood plasma and both peptide forms in the TNC, which may be a result of PACAP release from both the peripheral [69, 124] and presumably the central terminals of the primary sensory neurons. This is in line with previous findings that capsaicin can induce PACAP release from peripheral sensory nerves [125]. The fact that the highest PACAP level in the circulation was measured 90 min after the ES is probably due to its release from the peripheral terminals of the activated neurons that is peaking at this time. The PACAP elevation was still significant at 180 min, but it was tending to decrease due to the peptide depletion.

Trigeminal nucleus caudalis

Chemical stimulation of the TS

The NTG-induced PACAP increase in the region of the TNC is likely to have an important role in the trigeminal activation. The fact that systemic administration of PACAP can evoke sensitization and migraine-like attacks in migraineurs [27] is in line with the results of our animal experiments. Moreover, PACAP release in the dorsal horn has been reported in other experimental conditions of peripheral stimulation [126-130]. The potential relationship between PACAP and pain is enhanced by the co-localization and functional link of the NOS and PACAP systems, which has been described in a variety of studies [63, 76, 131-134].

Electrical stimulation of the TS

The PACAP-38-LI increase in the plasma preceded the changes seen in the TNC after ES, which suggests a rapid peripheral release, followed by activation and sensitization of the second-order trigeminal neurons. The ES-induced TS activation is followed by enhanced PACAP release from the central terminals of the TRG neurons in the TNC. A similar change was reported in the cyclophosphamide-induced chronic cystitis model, where the expression of PACAP-27/38 increased significantly in the spinal segments and dorsal root ganglia (DRG) involved in micturition reflexes [135]. This PACAP release is also in accordance with the elevated PACAP-level noted after capsaicin administration into the subarachnoid space [125].

Briefly, the up-regulation of this peptide may indicate a general trigeminal activation. The most noteworthy result was the elevation of both PACAP-38- and PACAP-27-LI in the TNC, which occurred selectively after both CS and ES of the TS. We have recently reported that i.p. injected PACAP-38 evokes a marked photophobia, meningeal vasodilatation and an increased number of c-fos-positive neurons in the TNC of wild-type, but not PACAP-deficient mice [79]. These data are in complete agreement with our present conclusion that PACAP released in the TNC is responsible for central sensitization of the TS. Sensitization associated molecular changes restricted to the "trigeminal area" were observed in previous experiments: c-fos, CGRP [98, 136], nNOS-immunoreactivity in the NTG model [89], and c-fos [137], nNOS [111], CGRP alterations [110] following the ES of the TRG.

Trigeminal ganglion

Our experiments did not reveal significant PACAP changes of any kind in the TRG in either model. In a previous study PACAP-immunoreactivity was showed in a small number of nerve cell bodies of the human TRG, but expression changes of this peptide in the ganglia were not fully evaluated. Increased PACAP-levels in the sensory neurons of the rat DRG have been described several days after irreversible peripheral nerve damage (transection) [65] and nerve compression [138]. Moreover, the role of PACAP both in nociception and regeneration was emphasized in a sciatic nerve transection rat model, where the PACAP-immunoreactivity has changed in the DRG and different laminae of spinal cord compared to the control and transected side [139]. The lack of alteration in PACAP expression in the TRG in our experiments might be related to the fact that we applied acute stimulations. The short stimulation period and latency was not sufficient to cause substantial expression changes in

the ganglion. The ES method of TRG differs from the peripheral nerve damage applied in the studies mentioned above, but it is massive enough to cause damage and depletion of PACAP-containing cells from the TRG. Further experiments are needed to emerge the real PACAP changes in the TRG and to get on to unexceptional results.

Cerebrospinal fluid

An earlier study revealed decreased somatostatin- and beta-endorphin-like immunoreactivities of the plasma or CSF obtained by suboccipital puncture, while the neuropeptide Y-like immunoreactivity did not change during the attack period in patients suffering from common migraine [140]. The present MS results confirmed the RIA detection of the presence of PACAP-38 in the rat plasma. In contrast, the presence of PACAP in the CSF was not detected with either the highly sensitive and specific RIA technique or MS, which suggests that PACAP was unable to cross the blood-CSF barrier. It is unknown that whether the barriers stay intact or damaged in our models. PACAP-27 was not detectable in either the plasma or the CSF, which might be explained by the generally lower concentration (10-100 times lower) of PACAP-27 in the mammalian tissues examined so far [30].

There is a few literature about the presence of PACAP in the CSF, however, one paper reported that PACAP is demonstrable in an artificial CSF perfusate in a specific rat experimental setup, where capsaicin was added to the perfusate [125]. In addition, a human study suggests that PACAP-38 could be detected in the CSF of healthy control subjects, moreover, its concentration in the CSF significantly increases in multiple sclerosis patients [141]. Nevertheless, under these conditions, disruption of the blood-CSF barrier cannot be excluded.

Functions of PACAP in other pain conditions

A number of investigations have demonstrated various actions of PACAP in nociceptive processes: intrathecally administered PACAP dose-dependently decreased the flinching of the hindpaw in the formalin test [65, 142]. In contrast, PACAP induced hyperalgesia after administration into the mouse spinal cord [60]. There is evidence that PACAP and its receptors can modulate the activity of spinal dorsal horn neurons in rat following a chronic constriction injury [126].

The neuroregulatory functions of PAC_1 receptor were demonstrated in a study, where the chronic nociceptive response of PAC_1 receptor knock-out mice was markedly reduced in formalin, thermal, laser and mechanical stimulation-initiated models of inflammation [143].

The inflammatory/neuropathic pain due to the effect of carrageenan and spinal nerve transection can be suppressed by the absence of the PACAP gene. Intrathecal administration of N-methyl-D-aspartic acid (NMDA) did not cause mechanical allodynia in PACAP knock-out mice, but it was evoked by the application of PACAP and NMDA together. It suggests that PACAP can be the promoter of excitotoxic and nociceptive processes [76]. Nevertheless, the typical sensitization phenomena of migraine can be evoked by the systemic application of PACAP-38 in humans [27].

Further examinations are needed to clarify these controversial data of PACAP related to its pronociceptive or antinociceptive effects and peripheral or central features.

2) CLINICAL HUMAN INVESTIGATIONS

Our work provided the first evidence that the plasma concentration of PACAP-38 is significantly lower in the interictal period of migraineurs as compared with that in healthy volunteers, however, the amount of peptide increases in the blood during migraine headaches. These results suggest that PACAP-38 might be an important mediator of the pathophysiology of migraine. Moreover, the different peptide levels regarding the two phases of disease indicate that the PACAP-38 is involved in the development of attacks.

Possible mechanisms behind reduced PACAP-38-LI during interictal phase of migraine patients

Since correlation was found between the interictal lower plasma concentration of PACAP-38 and the disease duration hereby we think that the amount of PACAP-38 constantly decreases in the neuronal elements. Therefore diminishing peptide release and reduced plasma PACAP-38 level can be detected during the attack-free period in the long term. The correct mechanisms are unknown, but we suggest that at the beginning of the ictal period a specific, but unknown trigger (stress, hormonal changes, foods, sleep deprivation, even more the lower plasma concentration of peptide, etc.) might induce PACAP-38 release from the sensory nerve terminals, which subsequently increases the plasma PACAP-38 level. However, the PACAP-38-LI elevation in the ictal period is not a definitive increase rather than return to the baseline, since this peptide concentration is just a little bit higher than the plasma PACAP-38 level of healthy controls. We think that the presence of headache might serve to adjust the balance in the plasma PACAP level. It needs long term follow-up and multiple sampling investigations in order to determine the correct plasma PACAP-38 alterations and the functions of this peptide in migraineurs.

The reduced peptide concentration might be explained indirectly in terms of brain energy deficit (an impairment of the cerebral and striated muscle energy metabolism [144, 145], elevated lactate levels [146, 147], abnormalities of mitochondrial compartments [148] and imbalanced Mg^{2+} concentration in the neurons [149, 150]). It may additionally be

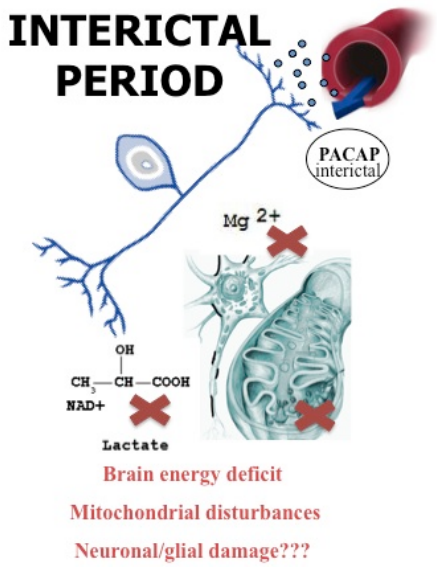

Figure 17. *Possible theories of interictally decreased PACAP-38-LI.*

hypothesized that the low interictal peptide concentration might be associated with degenerative changes affecting the PACAP-releasing circuitries (cortical atrophy [151], iron deposition in the periaqueductal grey matter [152] and increased levels of markers indicative of neuronal and glial damage [153] ***(Fig. 17)***).

Possible mechanisms related to the elevated PACAP-38-LI during migraine attacks

Among proposed mechanisms, the activation of vascular and neuronal elements of TS via several regulatory peptides seems to be essential in the formation of migraine. Dilation of meningeal vessels and sensitization of trigeminal neurons have been implicated as different components underlying this disease. There are some anatomical and physiological factors, which might be involved in the migraine-like headache induced by PACAP-38.

Vascular effects of PACAP-38 related to migraine

Some studies have approached the effects of PACAP-38 in headache from the aspect of vessels:

- In 1995 Zagami and co-workers have published [154] that stimulation of the superior sagittal sinus causes extracranial release of PACAP.

- An MRI angiographic study has revealed that PACAP-38 infusion-induced headache is related to significant dilatation of middle meningeal arteries (MMAs), in contrast with middle cerebral arteries [155]. A PAC1 receptor antagonist can influence the vasodilating action of PACAP-38 on pressurized MMAs. Multiple variants of the PAC_1 receptor have been found besides the $VPAC_2$ receptors in the rat MMAs [156]. However, there are some controversial results regarding the relationship of PACAP-38 and these arteries [57, 156-158].

- There is evidence that the intracarotid infusion of PACAP-38 produces significant dilatation of the dural arteries in rat, which administration route has proved to be more effective than the intravenous [58].

- Recently, the PACAP and VIP receptors were characterized by means of pharmacological modulators in human meningeal and coronary arteries. These peptides were found to have lower potency and efficacy in meningeal vasculature than in coronary arteries. This study concluded that processes of PACAP-38-induced

migraine-like headache might not involve meningeal vasodilatation rather than sensitization of peripheral and central sensory trigeminal fibres [57].

- The vasodilating properties of PACAP in a small extent or indirectly can contribute to the development of headache, e. g. PACAP induces vascular effects mediated via activation of perivascular nerves [159]. The vascular effects cannot be excluded, but based on the literature it seems that these may be less relevant in migraine [57].

Mast cell effects of PACAP-38

PACAP-38 is more potent to sensitize trigeminal sensory fibres directly and also through mast cell degranulation [27, 160, 161]. It was recently reported that PACAP-38 is associated with mast cells, MMA dilation and migraine [162], but different PACAP fragments were also emphasized many times in several aspects: these peptides dose-dependently induce histamine [163] and serotonin release on rat peritoneal mast cells. The mechanism probably involves the direct activation of G_i-type proteins [164] or another PACAP receptor-independent direct activation of one or more G proteins, which may then activate the phopholipase-C (PLC) - dependent signal-transduction pathway [165]. It was also found that PACAP is capable of releasing histamine from skin mast cells [166], moreover, the mast cell degranulation contributes to the development of PACAP-induced dermal oedema in mice [167].
The reported effects of PACAP infusion regarding neurogenic inflammation and mast cell degranulation in relation to trigeminal activation are very poor and controversial [160]. Effects of different truncated PACAP and VIP fragments were tested on rat peritoneal and dural mast cells, which has concluded that PLC-mediated mast cell degranulation is implicated in PACAP-induced migraine [161].

The pleiotropic effects of PACAP might be attributable to the three kinds of receptors and the two main known signalling pathways. According to the literature the hypothetic mechanisms of vasodilation and mast cell degranulation caused by PACAP are the followings:

1. In the vascular smooth muscle cells through the Gs-protein activation, PACAP stimulates the activity of AC, leading to increased cyclic adenosine monophosphate (cAMP) level. The cAMP can activate the protein kinase A (PKA), which activates the ATP-sensitive K^+-channels by phosphorylation. Subsequently the K^+ ions effuse to the extracellular space and the cell becomes hyperpolarised. The Ca^{2+} influx will give up through the L-type voltage-gated Ca^{2+}-channels, which results decreased

intracellular Ca^{2+}concentration and vascular relaxation. Furthermore, the cAMP can inhibit the myosin light-chain kinase (MLCK) and induces vasodilation [168, 169] ***(Fig. 18)***.

2. In the mast cells via the Gq/11-protein-coupled process, it activates the β-subunit of PLC, which generates diacyl-glycerol (DAG) and inositol-triphosphate (IP_3) from the phosphoinositol-diphosphate (PIP_2) in the plasma membrane. The IP_3 can mobilise the Ca^{2+} storages in the endoplasmatic reticulum, the DAG anchors the protein kinase C (PKC) to the cell membrane. The Ca^{2+} release can activate the PKC, which contributes to the secretion of histamine and other inflammatory mediators to the extracellular space inducing neurogenic inflammation [170-172] ***(Fig. 18)***.

   Indirectly the increased Ca^{2+}-level can promotes the synthesis of NO, since the NOS I and NOS II are Ca^{2+}-dependent enzymes, so the NO also causes vasodilation on another pathway [28, 173].

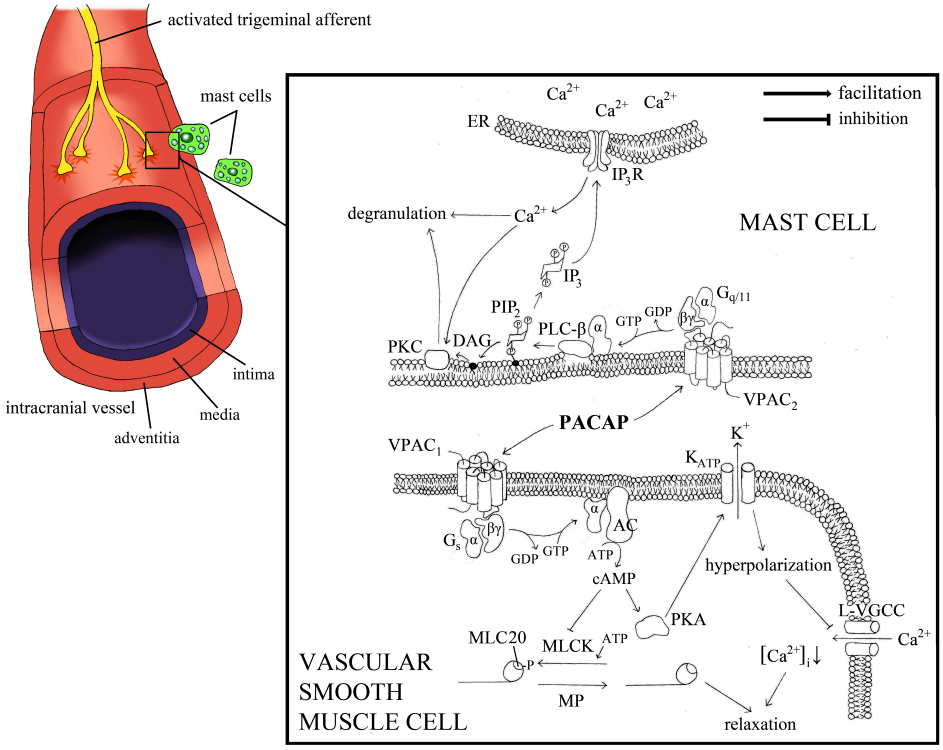

Figure 18. *Different signalling pathways of PACAP in the smooth muscle cells and mast cells*

AC: adenylate cyclase; ATP: adenosine-triphosphate; Ca^{2+}: calcium-ion; cAMP: cyclic adenosine monophosphate; DAG: diacyl-glycerol; ER: endoplasmatic reticulum; GDP: guanosine-diphosphate; Gq/11: heterotrimer G-protein subfamily; Gs: facilitating G-protein; GTP: guanosine-triphosphate; IP_3: inositol triphosphate; IP_3R: inositol triphosphate receptor; K_{ATP}: ATP-sensitive K^+-channel;L-VGCC: L-type voltage-gated Ca^{2+}-channel; MLC20: myosin 20 kDa light chain; MLCK: myosin light chain kinase; MP: myosin phosphatase; P: phosphate group; PIP_2: phosphoinositol-diphosphate; PKA: protein kinase A; PKC: protein kinase C; PLC-β: phospholipase C-β; $VPAC_{1,2}$: PACAP receptor **Graph: Mészáros Á.**

Neuronal effects of PACAP-38 related to migraine

PACAP-38 is a sensory [68], sympathetic [77] and parasympathetic [78] neuropeptide [174], which is released from the nerve endings [13, 35, 68, 175] at the dural or other cranial compartments. It can modulate both vessels and nerve fibres through its receptors leading to elevated intracellular cAMP level [176]. There are several evidences that the increased cAMP causes sensitization and activates the trigeminal neurons [177] and meningeal nociceptors [178, 179], therefore induces delayed headache [180].

The headache-inducing action of PACAP-38 was first described in 2009. Schytz and co-workers have demonstrated that PACAP-38 has a simple headache-evoking effect in healthy volunteers, but provokes severe migraine-like attacks in susceptible subjects. A decreased mean blood flow velocity in the middle cerebral artery and an increase in diameter of the superficial temporal artery were observed 20 min following the infusion [27].

Since it is unknown that which alteration (development of headache or elevation of PACAP-38-LI) occurs earlier, it is possible that PACAP-38 might cause a self-amplifying positive feedback, which can contribute to the maintenance and aggravation of headache. The causative role of PACAP-38 is equivocal, but its involvement is unquestioned.

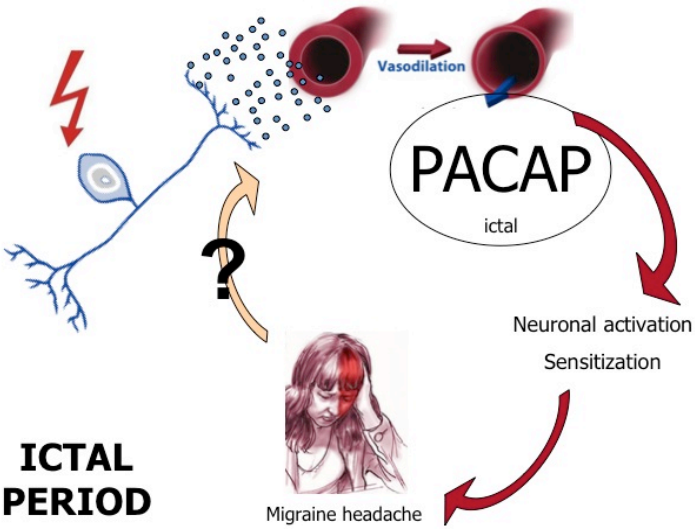

Figure 19. *Possible theories of ictally increased PACAP-38-LI.*

Based on similar features and receptors of PACAP and VIP, as well as the complex mechanism of migraine, it is likely that migraine cannot be related only to PACAP-38. This peptide can contribute to the evolution of migraine attacks in co-operation with other regulatory neuropeptides, molecules and enzymes, like VIP, CGRP, 5-HT, SP and NO. When the headache starts in response to specific triggers (environmental factors, TS activation (vasodilation, neuropeptide release, plasma protein extravasation), CSD, energy deficit), the presence and aggravation of pain may lead to the release of PACAP-38 from the nerve terminals, as a self-triggering process. This may be an explanation of the finding that the increase of plasma PACAP-38-LI in the ictal phases of migraineurs is moderate, though significant compared to the interictal period. Hereby the

phenomenon of throbbing headache can be related to the increased PACAP-38-LI during the ictal phase of migraineurs found in our study. Subsequently, it would be plausible that the activation of second order trigeminal neurons by PACAP-38 can result in direct central sensitization [27] ***(Fig. 19)***.

According to the wide distribution and pleiotropic effects of PACAP it is also certain that actually there are unknown receptors and signalling mechanisms of PACAP, which may have important functions in neurological diseases. Hereby our presumption is that the vascular and neuronal events caused by PACAP are not separable in the case of migraine. During attacks the PACAP-38 can release from both the central and peripheral terminals of the pseudounipolar neurons of TRG [66]. The peptide binds to its receptors and enters the systemic circulation. It seems, that activation of PAC_1 and $VPAC_2$ receptors can elicit vasodilation in the meningeal vessels, unlike the VPAC1 receptors are important in the vascular responses of cerebellar arteries [156]. The PAC_1 receptors are also emphatic in the activation of second-order sensory neurons [79], which appears consistent with previous interpretations implicating PAC_1 receptors in migraine [27, 181]. This is confirmed by the results that intradermal injection of PACAP-38 or VIP can elicit mild short-lasting cutaneous pain, increased skin blood flow, flare and wheal in healthy volunteers, which support that primarily the VPAC and not the PAC_1 receptors mediate these alterations. It is evidenced that PACAP induces neurogenic inflammation, mast cell degranulation, neuronal activation and sensitization [27, 160]. Participation of PACAP in processes of migraine can be confirmed by administration of serotonin 5-HT1B and 5-HT1D receptor agonist sumatriptan, which attenuates the PACAP-induced MMA vasodilation and headache pain [27, 155].

Summarized, PACAP can be involved in many aspects to promote the development and aggravation of severe headache attacks.

Possible function of blood-brain barrier (BBB) in PACAP-related migraine disease

In accordance with the molecular weights of PACAP-27 (3 kDa) and -38 (4.5 kDa) ***(Fig. 11/A, B)***, the PACAPs can penetrate the blood-brain barrier (BBB). PACAP is able to pass through the intact BBB [182, 183], the blood–spinal-[184, 185] and the blood–testis barrier [186]. However, the efficiency of PACAP transport across the BBB is dependent on the saturable peptide transport system [186-188]. Moreover, these mechanisms require specific transporters in specific brain areas [189, 190]. The hypothalamus and the hippocampus have the fastest uptake [189]. The accumulation of circulating peptide is limited in the brain by the brain-to-blood efflux transporters [186, 191].

Peptide carriers located at the BBB are often regulated and affected by diseases. Slower transport of PACAP can be observed into the whole brain, the olfactory bulb, the hypothalamus and the hippocampus in aged Alzheimer's disease mice model [189]. The BBB transport of PACAP can alter following spinal cord injury, stroke, and cardiac arrest [184, 185, 187].

There are assumptions that the integrity of BBB is disrupted in migraineurs [192, 193]. Transient brain dysfunction, vasogenic cerebral oedema and damaged BBB can develop in lipopolysaccharide-induced brain injury in rats in response to the over-expression of matrix metalloproteinase 9 [194]. This enzyme can degrade the basal membrane, resulting in structural impairment of the BBB and altered plasma levels of certain molecules, which have been observed in patients with migraine [195-198]. The enhanced BBB permeability in migraine may facilitate PACAP to penetrate into the brain parenchymal elements and exert its central effects. From the opposite aspect, the PACAP released in the brain [30] can also penetrate through the BBB, and hence may be detected in the plasma.

Correlation of PACAP-38-LI with the menstruation cycle and other chronic pain conditions in migraineurs

Menstruation cycle

Migraine attacks are often closely related to female hormonal changes [117]. Hence, it is interesting that PACAP-38-LI increases significantly during the ictal period only in those women whose headache is not related to the menstrual cycle. Although, the influence of menstruation cycle in the PACAP-38-LI changes cannot be excluded, but there is evidence that the plasma concentration of PACAP-38 is relatively stable and independent of the gender, age, food intake or female hormonal cycles in healthy subjects [40].

Chronic pain and stress

It is well established that PACAP-38 plays an important role in a variety of other pain conditions [62, 199, 200]. Contrarily in our study there was no increased PACAP-38 LI in the ictal phase in patients with other chronic pain conditions. Moreover, we did not find any statistical proof of the influence of chronic pain and the alterations of PACAP-38 level.

The role of stress might arise in the release of PACAP. This peptide may be involved in the hypothalamic-pituitary-adrenal (HPA) axis activation. Stress may be an inducing factor of

PACAP-38-release, because it was observed that the effects of different types of stressors were markedly ameliorated in PACAP-deficient mice [201].
In our animal experiments the rats were anaesthetized in the ES-TRG model, so the effect of stress can be excluded, however, in the NTG-induced model the rats were awake.
The influence of stress may also be excluded in the human study, because significant alterations were found only in the group of migraineurs without any other chronic pain syndrome, which condition can be perceived as a kind of stress factor.

Possible relations between the PACAP-38 and CGRP in migraineurs

CGRP is a 37-amino-acid neuropeptide, identified in 1982 [202]. It has diverse biological functions in the peripheral and central nervous system [203, 204]. More than 20 years ago, the presence of CGRP-immunoreactivity in the rat TRG [205] and the release of CGRP was demonstrated in the extracerebral circulation of humans and cats in response to TS activation [108, 206]. CGRP-containing neurons have also been detected in the human TRG [68, 207] and elevated plasma CGRP levels have been described in migraine [16, 17] and other types of primary headache [208]. CGRP caused MMA dilation was detected in normal volunteers and it was reversed by sumatriptan administration [209]. Moreover, an intravenous infusion of human alpha-CGRP causes migraine headache [180, 210] and there is a significant positive correlation between plasma levels of CGRP and headache severity scores in NTG-induced migraine attacks [87]. However, there are inconsistent results, which found increased plasma levels of CGRP in migraine during attack-free periods [17]. A controversial study questions the importance of CGRP in migraine [211]. Additionally, a study reported no changes in plasma CGRP-level during migraine attacks compared to the interictal period [18].
Despite these contradictions, the importance of CGRP in migraine became firmly established [212]. In fact, intravenous and oral CGRP-receptor antagonists [213, 214] might also be effective in the treatment of migraine [215]. However, their side-effect profile, with special emphasis on hepatotoxicity, currently makes this drug development direction problematic [216-218]. Co-expression patterns were described between the CGRP and other peptides, molecules [13, 219]. Our results draw attention to a possible influence between the PACAP and CGRP systems in migraine pathogenesis [125, 220, 221]. In addition there are evidence that PACAP shares expression and regulation simultaneous with CGRP [27, 155, 222].

VIII. CONCLUSIONS

In the animal experiments, both PACAP-38-LI and PACAP-27-LI were specifically elevated in the TNC in response to both the chemical and electrical stimulation of the TS. Furthermore, a marked elevation of PACAP-38 was detected in the systemic circulation only in the ES-TRG-model. The results indicate that this peptide is closely connected with the NO system, and PACAP might therefore play a pivotal role in nociception and the TS.

From the human study, it was concluded that PACAP-38 might be implicated in the development of migraine headache. There are associations between the migraine periods (ictal and interictal) and alterations in plasma PACAP-38 levels. The quantitative changes of this peptide are related to the disease duration, the menstruation cycle and the presence of other pain-related disorders. The causative role of PACAP-38 in migraine headache demands further studies [223].

These data facilitate the understanding of the mechanisms of TS activation, and answer certain questions relating to clinically relevant sensitization processes. Our results indicate the need of investigations of the role of plasma PACAP-38 as a putative biomarker in migraine, which might provide new perspectives as concerns the identification of a new target in the therapy of migraine.

The relevance and potential future applications of PACAP

PACAP seems to be an interesting and promising target understanding the actions of TS. Although the poor metabolic stability, the wide distribution and diverse effects of PACAP limit its application as a potent therapy for diseases, even so there have been attempts to utilize the functions of PACAP.

A novel fusion molecule, PACAP-TAT, which contains the 11-amino acid human immunodeficiency virus protein TAT, endows PACAP with an enhanced ability to penetrate the BBB, than that of PACAP alone. The i.p. injection of PACAP-TAT induces a stronger inhibitory effect on food intake, which indicates the localization of protein in the brain [224].

It was recently demonstrated that therapeutic amounts of PACAP could be delivered to the brain by the intranasal administration route [190]. The occipital cortex and striatum were the highest uptake regions. The cyclodextrin-containing injections may therefore be useful in the therapeutic targeting of peptides to specific brain regions, because the various cyclodextrins can produce unique PACAP distribution patterns. Besides pharmacological interventions, the determination of PACAP plasma levels and their alterations may serve as biomarkers.

IX. 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).

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XII. SUMMARY

BACKGROUND

Migraine is a common, paroxysmal primary headache disorder. Characteristically, this is a highly complex, restrictive and extremely costly disease, which has high socio-economic and personal impacts on the quality of life. Although there have been extensive researches in the field of migraine, the exact details of the pathomechanism remain unknown at present. Epidemiological studies have revealed that the prevalence of migraine in the adult population in the developed countries is approximately 12%. Migraine may develop at any age and gender, but it is relatively common in young adult women. The 2004 guidelines of the Headache Classification Committee of the International Headache Society (IHS) state that there are two main forms of this disease: migraine with or without aura. Both types can be characterized by interictal (attack-free) and ictal phases. The period of the migraine attack can be divided into 4 sections: Prodromal phase; Aura phase (in the case of migraine with aura); Headache phase with the accompanying symptoms; Resolution or postdrome period. In clinical practice, various drug treatments are available for the therapy of migraine. However, the effectiveness of these pharmaceuticals is not sufficient in all patients and the migraine-specific drugs usually have undesired side-effects. Hence, the investigation of new molecules, targets and markers involved in the pathomechanism of migraine is indispensible, with the development of effective drugs and successful therapy.

Several hypotheses have been proposed to explain the mechanisms of headache diseases. The activation of the trigeminovascular system (TS) and the releases of different neuropeptides have been confirmed in the processes of migraine. Recent studies have potentially implicated the vasoactive, pituitary adenylate cyclase-activating polypeptide (PACAP) in the pathophysiology of migraine. 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 function in the human organism. The role of PACAP in vasodilatation, nociceptive and sensitization processes as well as in the mechanisms of neurogenic inflammation has been confirmed in numerous studies. PACAP is present in the primary sensory neurons of the TRG, the parasympathetic otic and the sphenopalatine ganglia. Moreover, the available human data point to the involvement of PACAP in the mechanisms of migraine. Schytz and his co-workers demonstrated 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.

These results suggest that PACAP may be an important mediator and diagnostic marker of the TS activation. The receptors of PACAP have been implicated as potential therapeutic targets in migraine pathophysiology, but there are no direct experimental data to confirm this theory. Additionally, no clinical data are available on endogenous alterations in PACAP levels in relation to migraine. We therefore set out to examine the relationship between migraine and the level of expression of PACAP in animal models and also in human conditions involving migraine.

AIMS

The aim of our study was to determine whether there are any alterations in the concentration of PACAP in blood and nerve tissues in the case of TS activation and migraine disorder. Preclinical investigations were therefore conducted by the stimulation of the TS in animals in order to generate migraine-like conditions, while the specificity and relevance of PACAP in migraine were confirmed in our human clinical study.

MATERIALS AND METHODS

Preclinical animal experiments

Fifty-nine young adult Sprague-Dawley rats of either sex (8-12 weeks old, 250-350 g body weight) were used in these studies in two different rat models of the activated TS: one induced by systemic nitroglycerine (NTG) injection and the other by electrical stimulation (ES) of the trigeminal ganglion (TRG). These are commonly applied and well-established animal models of TS activation. Extensive literature is available on this field regarding the mechanisms, the good reproducibility and the human relevance. In case of the control group the cerebrospinal fluid (CSF), the blood samples and the different trigeminal nerve structures were taken after the anaesthesia, but in the TS-activated groups the samples were excised only 90 min or 180 min after the stimulations. PACAP-27- and PACAP-38-like immunoreactivities (PACAP-LI) were determined in the plasma and specific trigeminal components (trigeminal nucleus caudalis (TNC), cervical regions (C_3-C_4) of spinal cord (SC), TRG) by means of radioimmunoassay (RIA) and in the CSF by means of mass spectrometry.

Clinical human investigations

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. 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. Blood samples were drawn from healthy control subjects and migraineurs during a migraine attack and/or in an attack-free period. There were no restrictions as regards food and drink intake. Affected patients were asked not to start their usual attack treatment until blood samples had been taken. From among the 87 patients, blood samples of 21 migraineurs could be collected in both periods. Plasma samples were stored at −80 °C until the PACAP-38- and calcitonin gene-related peptide (CGRP)-LI were measured by RIA. Additionally, we have analysed the correlations between the concentrations of peptides (PACAP-38, CGRP) and the features of migraine disease.

In both study procedures the plasma PACAP-27/38-LI were determined with specific and sensitive RIA techniques developed earlier. The PACAP-38 ("88111-3") and PACAP-27 ("88123-3") 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. No cross-reactivity was found with each other or with other related neuropeptides in either case. Plasma CGRP-LI was measured with specific and sensitive RIA techniques by earlier published method.

RESULTS

Preclinical animal experiments

In the animal experiments, the concentrations of PACAP-27 and -38 were significantly elevated in the TNC 90 and 180 min after the NTG-injection ($p<0.05$ and $p<0.001$) and 180 min after the ES of the TRG ($p<0.01$). The plasma PACAP-38-LI was increased 90 and 180 min following the ES ($p<0.05$ and $p<0.001$), but not in the NTG-induced model. PACAP-27 was not present in the plasma, and the tissue concentrations of this peptide were lower than those of PACAP-38. The alterations in both peptides in the tissue homogenates in response to both TS stimulations were identical. Significant changes were not observed in the upper regions of the cervical SC or in the TRG in either of the models. Neither PACAP form could be identified in the CSF.

Clinical human investigations

In the human study, significantly lower PACAP-38-LI was detected in the interictal plasma of the migraineurs as compared with the healthy control group ($p<0.05$). In contrast, elevated PACAP-38 and CGRP levels were measured in the ictal phase relative to the attack-free

period in the 21 migraineurs ($p_{PACAP-38}<0.001$; $p_{CGRP}<0.05$) and in the case of PACAP-38-LI in the overall population of migraineurs ($p<0.01$). A mild negative correlation was observed between the interictal PACAP-38-LI and the disease duration.

DISCUSSION

Preclinical animal experiments

Our results support the neuropeptide theory of the development of activated TS. Both peripheral and central sensitizations were accompanied by elevated PACAP levels, 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 up-regulation of this peptide may indicate a general trigeminal activation. The most noteworthy result was the elevation of both PACAP-38- and PACAP-27-LI in the TNC, which occurred selectively after both CS and ES of the trigeminal system. These selective and significant peptide concentration changes in the brainstem nuclei suggest that the trigeminovascular trigger induces a marked release of PACAPs from the central terminals of the primary sensory neurons. It seems that ES of the TRG generates a massive TS activation. In response, PACAP can release from the peripheral branches, hence it enters the circulatory system and presents in elevated concentration in the blood.

We have recently reported that i.p. injected PACAP-38 evokes a marked photophobia, meningeal vasodilatation and an increased number of c-fos-positive activated neurons in the TNC of wild-type, but not PACAP-deficient mice. These data are in complete agreement with our present conclusion that PACAP, releasing in the TNC is responsible for the central sensitization.

Clinical human investigations

We presume that similar mechanisms occur in migraineurs. In consequence of an unknown trigger, the systemic level of PACAP-38 increases. The PACAP, similar to the CGRP 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.

Among proposed mechanisms, the activation of vascular and neuronal elements of TS via several regulatory peptides seems to be essential in the formation of migraine. Dilation of

meningeal vessels and sensitization of trigeminal neurons have been implicated as different components underlying this disease. There are anatomical and physiological factors, which might be involved in the PACAP-38-induced migraine-like headache: PACAP-38 is a sensory, sympathetic and parasympathetic neuropeptide, which is released from the nerve endings at the dural or other cranial compartments. It can modulate both the vessels and the trigeminal nerve fibres (directly or through mast cell degranulation) through its receptors leading to elevated intracellular cyclic adenosine monophosphate (cAMP) levels. There are several evidences that the increased cAMP causes sensitization and activates the trigeminal neurons and meningeal nociceptors, therefore induces delayed headache.

There are assumptions that the integrity of blood-brain barrier (BBB) is disrupted in migraineurs. The matrix metalloproteinase 9 can degrade the basal membrane, resulting in structural impairment of the BBB, and altered plasma levels of certain molecules that have been observed in patients with migraine. The enhanced BBB permeability in migraine may facilitate 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.

Although there are contradictions in the literature of CGRP, the importance of this peptide in migraine became firmly established. Our results draw attention to a possible influence between the PACAP and CGRP systems in migraine pathogenesis. In addition there are evidences that PACAP shares its expression and regulation simultaneously with CGRP. The correlations between the PACAP-38-LI and the menstruation cycle/other chronic pain conditions can confirm that the alterations of plasma PACAP-38 concentrations may be migraine specific features.

CONCLUSION

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 TS activation, and indicate the need for further investigations. The plasma PACAP-38 alterations might be a putative biomarker of migraine and provide new perspectives in the therapy of migraine.

XIII. ÖSSZEFOGLALÓ

HÁTTÉR

A migrén egy gyakori, rohamokban jelentkező primer fejfájásbetegség, mely jelentősen rontja a betegek életminőségét. A genetikai, kísérletes és klinikai vizsgálatok ellenére, a migrén pontos pathomechanizmusa máig nem tisztázott. Epidemiológiai adatok szerint a felnőtt populáció kb. 12 %-át érinti a betegség, mely leggyakrabban a fiatal felnőtt nőknél (20-40 év) jelentkezik. A migrénnek 2 fő típusa ismert: 1. aurás; 2. aura nélküli. Mindkét forma jellemzője az interiktális (rohammentes) és iktális (roham alatti) fázisok váltakozása. A migrénes rohamperiódus további szakaszokra osztható: 1. bevezető fázis (prodróma); 2. esetleg jelentkezhet aura; 3. fejfájás és kísérőtünetei; 4. lábadozás, felépülés (reconvalescencia). A fejfájások gyógyszeres intervallum és rohamterápiája sok esetben nem hatékony és számos nem kívánt mellékhatással rendelkezik. Ezért szükség van olyan kutatásokra, melyek a migrén pathomechanizmusában szerepet játszó molekulák, targetek és markerek azonosítását célozzák. Azok funkcióinak pontos felderítése segítséget nyújthat új, hatékony gyógyszerek és terápiás megoldások kifejlesztésében.

A fejfájások kórfolyamata számos elmélettel magyarázható, azonban a trigeminális rendszer, az általa beidegzett érrendszer (trigeminovascularis rendszer, TR), valamint bizonyos neuropeptidek és neuroaktív anyagok szerepét már többször bizonyították. Az utóbbi időkben néhány migrénkutatás a vazoaktív, hipofízis adenilát cikláz-aktiváló polipeptid (PACAP) jelentőségére hívta fel a figyelmet. A PACAP a VIP/szekretin/glukagon szupercsalád tagja és „brain-gut peptidként” az emberi szervezetben széles körben előfordul, számos funkcióval rendelkezik. A PACAP-nak szerepe van a vazodilatációban, fontos integrátor a nociceptív és szenzitizációs mechanizmusokban, valamint a neurogén gyulladás folyamataiban. Jelen van többek között a trigeminális ganglion (TRG) neuronjaiban és perivascularis idegvégződéseiben, a paraszimpatikus ganglion oticumban és sphenopalatinumban. Egy 2009-es klinikai vizsgált megerősítette a PACAP szerepét migrénes folyamatokban: Schytz és munkatársai kimutatták, hogy a 38 aminosavas peptid, a PACAP-38, intravénás infúzióban migrénszerű rohamokat és jelentős érválaszokat provokál migrénes betegekben, míg egészséges egyénekben fejfájást indukál. A PACAP tehát fontos mediátora és diagnosztikus markere lehet a TR aktivációjának, azonban az endogén PACAP-szint változásokról még keveset tudunk. Ezért vizsgálatainkban arra kerestük a választ, hogy hogyan szabályozza a PACAP a migrénes fejfájások kialakulását.

CÉLOK

Célul tűztük ki a PACAP koncentráció-változásának vizsgálatát a vérben és a trigeminális rendszerrel kapcsolt agyi régiókban az aktivált TR állatmodelljeiben, valamint klinikánkon gondozott migrénes betegek vérmintáiban, a migrén periódusainak, jellemzőinek összefüggésében.

ANYAGOK ÉS MÓDSZEREK

Preklinikai állatkísérletek

Vizsgálatainkban 59 fiatal felnőtt Sprague-Dawley patkányt (hím/nőstény; életkor: 8-12 hét; testsúly: 250-350 g) használtunk. Egyrészt kémiai úton szisztémás nitroglicerin injekcióval, másrészt az egyik oldali TRG elektromos ingerlésével 2 különböző mechanizmusú aktivált TR modellt alakítottunk ki, melyek jól kidolgozott, reprodukálható és humán szempontból is releváns rendszerek. A stimulált csoportokban a modellek kialakítását követő 90. és 180. percben, míg a kontroll csoport esetében az altatást követően, liquor és a vérmintát gyűjtöttünk, majd eltávolítottuk a különböző trigeminális idegi struktúrákat. Ezt követően a mintákat -80°C-on tároltuk, majd radioimmunoassay (RIA) módszerrel meghatároztuk a PACAP-27- és PACAP-38-immunoreaktivitást (IR) a vérplazmában és a trigeminális komponensekben (agytörzsi trigeminal nucleus caudalis = TNC, C_3-C_4 nyaki gerincvelő, TRG), illetve tömegspektrometria segítségével a liquorban.

Klinikai humán vizsgálatok

87 aurás és aura nélküli migrénes beteget, illetve 40 egészséges kontroll alanyt vontunk be tanulmányukba. A betegeket a Nemzetközi Fejfájás Társaság 2004-es kritériumrendszere alapján válogattuk. A csoportokat korban egyeztettük. Részletes migrén, allodynia és depresszió kérdőív alapján igyekeztünk homogenizálni a migrénes betegek csoportját. A kontroll alanyoktól egyszer, míg migrénes betegektől a rohammentes és/vagy roham alatti időszakban is gyűjtöttünk vérmintát. A pácienseket megkértük, hogy fejfájások idején ne alkalmazzanak gyógyszeres terápiát a vérvétel előtt. 21 migrénes beteg esetében mind az interiktális, mind az iktális periódusban történt vérvétel. A plazma mintákat −80 °C-on tároltuk a PACAP-38- és kalcitonin gén-rokon peptid (CGRP)-IR, RIA módszerrel történő meghatározásáig. Vizsgáltuk a betegség jellemzőinek és a plazma PACAP-38, CGRP mennyiségi változásainak összefüggéseit.

A PACAP-27/38 méréseket egy korábban a PTE ÁOK Anatómiai és Farmakológiai Intézeteiben kifejlesztett, specifikus és szenzitív RIA technikával határoztuk meg. A nyúlban

termeltetett antiszérumok (PACAP-38, „88111-3”; PACAP-27, „88123-3”) nagy specificitását és C-terminális érzékenységét keresztreakciós mérésekkel igazolták. Az ismeretlen minták peptid koncentrációit kalibrációs görbe alapján határoztuk meg. A CGRP-IR meghatározását korábban publikált metodika alapján végeztük.

EREDMÉNYEK

Preklinikai állatkísérletek

Az állatkísérletekben a TNC területén szignifikánsan emelkedett PACAP-27 és -38 koncentrációt mértünk a kémiai stimulációt követő 90. és 180. percben ($p<0.05$ és $p<0.001$), valamint a TRG elektromos ingerlése után 180 perccel ($p<0.01$). Jelentős PACAP-38-IR növekedést detektáltunk az elektromos stimulációt követő 90. és 180. percben ($p<0.05$ és $p<0.001$), ellenben a NTG-modellben ilyen nagymértékű változást nem találtunk. A vérplazmában a PACAP-27-et nem tudtuk kimutatni, és szöveti koncentrációja is alacsonyabb volt, mint a PACAP-38 esetében. A szöveti homogenizátumokban a peptidek hasonló arányban változtak mindkét modellben. Sem a kémiai, sem az elektromos modellben nem találtunk szignifikáns peptid-szint változást a TRG-ben és a C_3-C_4-es nyaki gerincvelői régió területén. A liquorban nem tudtuk kimutatni a PACAP egyik formáját sem.

Klinikai humán vizsgálatok

A humán vizsgálatokban a migrénes betegek rohammentes periódusában szignifikánsan alacsonyabb plazma PACAP-38-IR-t mértünk a kontroll alanyokhoz képest ($p<0.05$). Ezzel ellentétben szignifikánsan emelkedett PACAP-38 és CGRP szinteket találtunk az iktális fázisban az interiktálishoz viszonyítva a 21 „önkontrollos” beteg esetében ($pPACAP-38<0.001$; $pCGRP<0.05$), valamint a vizsgált teljes migrénes populációban ($p<0.01$). Enyhe negatív korrelációt találtunk az interiktális PACAP-38 plazma szintje és a betegség fennállásának időtartama között.

MEGBESZÉLÉS

Preklinikai állatkísérletek

Eredményeink alátámasztják az aktivált TR kialakulásában szerepet játszó neuropeptid-felszabadulás elméletet. A neuro-vaszkuláris rendszer komplexitására utal, hogy modelljeinkben a perifériás és a centrális szenzitzáció mechanizmusait PACAP-szint változások kísérik. A modellek között megfigyelhető kismértékű eltérések a különböző aktivációs mechanizmusoknak tulajdoníthatók. A legszembetűnőbb eredmények a TNC

PACAP-27 és PACAP-38-IR emelkedése, melyek szelektíven jelentkeztek mind a kémiai, mind az elektromos ingerlés következtében. Mindez arra utal, hogy a TR aktiválódása – mely úgy tűnik, hogy a TRG elektromos ingerlése következtében még jelentősebb –, nagymértékű PACAP felszabadulást indukál az elsődleges érző neuronok perifériás és centrális terminálisaiból. Feltehetően ezek után belép a keringési rendszerbe, ahol emelkedett koncentrációban lesz jelen. Korábbi vizsgálatainkból kiderül, hogy vad típusú egerekben az ip. alkalmazott PACAP-38 fotofóbiát, meningeális vazodilatációt vált ki és megemeli a TNC-ben a c-fos pozitív, aktivált neuronok számát, ellentétben a PACAP-deficiens állatokkal szemben. Ezen eredmények összefüggésbe hozhatók jelen következtetéseinkkel, miszerint a TNC-ben felszabaduló PACAP felelős a centrális szenzitizációért, a TR aktiválódásáért.

Klinikai humán vizsgálatok

Feltételezzük, hogy hasonló folyamatok történnek a migrénes betegekben, bár az ok-okozati összefüggések még nem tisztázottak. Egy ismeretlen trigger következtében a PACAP-38 szisztémás szintje megemelkedik, és a CGRP-hez hasonlóan kifejti vazodilatatív és szenzitizáló hatását. A mechanizmusok hozzájárulhatnak a fejfájás kialakulásához és súlyosbodásához. Az interiktálisan csökkent PACAP-38-szint és a betegség időtartamával mutatott összefüggés (kismértékű negatív korreláció) alapján felmerül, hogy a rohamok idején felszabaduló nagyobb mennyiségű peptid fokozatosan kiüríti a PACAP-tartalmú rostokat.

Úgy tűnik, a TR neuronális és vaszkuláris elemeinek aktivációja nélkülözhetetlen a migrén kialakulásához. A meningeális erek dilatációja, a trigeminális neuronok szenzitizációja illetve hízósejt degranuláció is befolyásolhatja a PACAP-38 indukálta migrén-szerű fejfájást. A dura környezetében az idegvégződésekből felszabaduló PACAP-38 képes modulálni az erek és az idegrostok receptorain keresztül az intracelluláris cAMP szintet. Az emelkedett cAMP szint a trigeminális neuronok szenzitizációját, a meningeális nociceptorok aktivációját okozhatja, fejfájást indukálhat.

Irodalmi adatok szerint migrénesekben csökken a vér-agy gát (BBB) integritása. Megfigyelték, hogy migrénesekben a megváltozott neuropeptid szintek, laktát és magnézium koncentrációk, valamint mitokondriális deficitek mellett, a BBB strukturáltsága is romlik. Ebben a matrix metalloproteináz 9, mint a bazális membránt degradáló enzimnek jelentős szerepe van. Tehát feltételezzük, hogy a migrénesekben fellelhető fokozott BBB permeabilitás elősegítheti a PACAP átjutását az agy parenchimás szöveteibe és így az

kifejtheti centrális hatásait. Másrészről, az agyban felszabadult PACAP is penetrálhat a BBB-n és nagy mennyiségben átjuthat a plazmába.

Tanulmányunk újabb bizonyítékul szolgál arra, hogy megemelkedik a CGRP plazmaszintje migrénes rohamok idején. Eredményeink felhívják a figyelmet arra, hogy kapcsolat lehet a PACAP és CGRP rendszerek között a migrén pathogenezisében, melyet más vizsgálatok is igazoltak. A PACAP-38-IR és a menstruációs ciklus/ krónikus fájdalom között tapasztalt összefüggések azt jelzik, hogy a peptid plazmakoncentrációjának változásai migrén specifikus jellemzők lehetnek.

KÖVETKEZTETÉSEK

Eredményeink alapján arra következtetünk, hogy a PACAP egy speciális modulátora lehet a TR-nek. A peptidnek szerepe lehet a szenzitizáció folyamataiban, melyet a humán tanulmányban megfigyelt, migrénes fázisokhoz kötött plazma PACAP-38 koncentrációváltozások igazolhatnak. Vizsgálataink hozzájárulnak a betegség pathomechanizmusának részletesebb megismeréséhez, és perspektívákat kínálnak új targetek azonosítására a migrén terápiájában. További kutatások szükségesek a PACAP és a fájdalom vonatkozásában, melyek révén a PACAP egy új biomarkere lehet a fejfájásbetegségeknek.

XIV. APPENDIX

NYILATKOZAT

Kijelentjük, hogy Tuka Bernadett munkája meghatározó jelentőségű az alábbi, Doktori (PhD) Értekezése és Tézisei alapjául szolgáló közleményekben, melyeket mindeddig nem használtuk fel tudományos fokozat megszerzésére, mint ahogyan azt a jövőben sem fogjuk megtenni.

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