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THE ROLE OF SEROTONIN AND KYNURENINE METABOLISM IN MIGRAINE

PhD thesis

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Table of Contents

List of Abbreviations

5-HT - 5-hydroxytryptamine, serotonin

AAAD - aromatic amino acid decarboxylase

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

CORT - cortisol

DHEA-S - dehydroepiandrosterone sulphate

dlPFC - dorsolateral prefrontal cortex

dmPFC - dorsomedial prefrontal cortex

fMRI - functional magnetic resonance imaging

HPA – hypothalamic-pituitary-adrenal axis

ICHD - International Classification of Headache Disorders

IDO - indoleamine 2,3-dioxygenase

KAT - kynurenine aminotransferase

KMO - kynurenine 3-monooxygenase

KYN - kynurenine

KYNA - kynurenic acid

LAT1 - L-type amino acid transporter 1

LC-MS/MS - Liquid Chromatography Mass Spectrometry

LNAA - large neutral amino acids

mGLUR5 - metabotropic glutamate receptor 5

MNI - Montreal Neurological Institute

NMDA - N-methyl-D-aspartate

PAG - periaqueductal gray matter

PFC - prefrontal cortex

STAI - trait-anxiety scores

TDO - tryptophan-2,3-dioxygenase

TPH - tryptophan hydroxylase

TRP - tryptophan

ZUNG - depressive symptoms scores

1. Introduction

Migraine is a widespread neurological disorder with an estimated global prevalence of 14-15% (1). The first attack most often occurs in adolescence and has a peak in the 30s of patients, affecting more women (35%) than men (18%)(2). Migraine is the second burdensome disorder in the world, but first among young women (3). Its financial impact extends to both individuals and healthcare systems, encompassing direct medical costs, such as healthcare visits and medications, as well as indirect costs related to lost productivity and disability (4).

Migraine attack, typically lasting 4-72 hours, is characterized by a debilitating, moderate to severe, pulsating headache, which is usually unilateral. Migraine patients become hypersensitive to light (photophobia) and sound (phonophobia) during an attack, seeking a dark and quiet environment (5). Gastrointestinal symptoms, including nausea and vomiting, are also prevalent and can contribute to the overall discomfort. One third of migraine patients experience aura symptoms 5-60 minutes before the headache phase, mainly in form of visual disturbances, but it could cause sensory, auditory, language or motor problems as well (6).

The theories of migraine development are still debating (5). Over several decades it was considered as a vascular disorder based on the pulsating nature of the pain and the vasodilation in the vessels (7). Nowadays, migraine is considered a neurovascular disorder emphasizing the involvement of the brain (8). Regarding the neuronal mechanisms, both peripheral and central components are contributing to migraine attack development (5). Premonitory symptoms (such as fatigue, mood, and appetite changes) usually precede the headache phase with 24-48 hours and appear as alterations in hypothalamic and midbrain activation (9, 10). Migraine triggers initiate the activation of the trigeminovascular system, either inducing the firing of the first-order peripheral trigeminal neurons or indirectly modulating the nociceptive inputs to the second-order trigeminocervical complex leading to activation of ascending projections from the brainstem structures (8, 11). Migraine attacks are associated with the release of vasoactive peptides, such as calcitonin gene-related peptide (CGRP)(12, 13). The released neuropeptides induce neurogenic inflammation (14). These processes could lead to peripheral and central hypersensitization (15).

1.1. The migraine brain

The hypersensitivity of "migraine brain" is not limited to the headache phase, but persist between the attacks, in interictal period (16). Central sensitization contributes to the interictal hypersensitivity in migraine, where the central nervous system becomes more responsive to pain signal and sensory stimuli such as light, sound or smells (17-19). Cortical hyperexcitability in pain-free period, particularly in the visual network also plays a role in sensitivity to stimuli (20). In addition, altered pain processing pathways result in an increased sensitivity to various stimuli, making individuals more prone to experiencing pain or discomfort even in the absence of an active migraine attack (21, 22). The hypersensitivity to endogenous (e.g., hormone) and exogenous stressors (e.g., weather, light) could drive the migraine brain more sensitive, thus making it difficult to adapt to changes (23).

Recent studies hypothesized that migraine could be a disease of allostatic load (24). Allostasis is the process of maintaining homeostasis in stressful situations, while allostatic load is the "price" that the body must pay for adaptation (25). Migraine attacks are the consequences of inability to habituate to repeated stressors of a similar nature (both physiological or psychological) and an impaired ability to terminate the stress response in the usual manner (24). Specific stressors linked to migraine encompass both psychological or emotional factors and physiological elements (such as noise, certain foods, odors, and bright light)(26). Migraine patients often identify perceived stress as the most prevalent trigger for their attacks (27). Based on this concept, the hypersensitivity/hyperexcitability of the migraine brain is energy-consuming, thus the price to pay is a metabolic imbalance of the brain, that manifests in form of migraine attack (28). Therefore, episodic migraine attacks (less than 14 headache days/month (6)) are considered as allostatic response to maintain homeostasis and let the brain energy recover (29). This idea is supported by the fact that patients during a migraine attack are avoiding any energy consuming activities (6, 8).

However, chronic activation of these responses could lead to allostatic overload, when maladaptive responses in form of repeated attacks lead to a vicious circle (30). The chronification of migraine, an increase in headache frequency (>14 headache days/month), could originate from recurrent abnormal stressors and the overuse of specific medications (e.g., triptans, opioids), potentially intensified by genetic factors (6).

The process of migraine chronification indicates a progressive maladaptive response of the brain (23). One of the brainstem regions that could have potential role in migraine attack generation is the periaqueductal gray matter (PAG) (31).

1.2. The role of periaqueductal gray matter (PAG) in migraine

The PAG is located in the midbrain orchestrating several autonomic functions through its rich connection to brainstem and cortical structures (32). PAG plays a crucial role in the descending pain modulatory system, influencing the perception and processing of pain signals (33). The electrical stimulation of PAG induces analgesia (34) and inhibition of nociceptive trigeminovascular neurons (35). It shows increased activation during spontaneous and induced migraine attacks suggesting its involvement in migraine pathophysiology (8, 36, 37). Moreover, PAG exhibits heightened resting-state and pain-induced functional connectivity with regions involved in nociceptive and somatosensory processing pathways in migraine patients, and these connections are correlated with migraine frequency (38-40). Previous studies demonstrated a reduced functional connectivity of PAG with prefrontal cortical and limbic areas, responsible for top-down pain modulation, in migraine patients compared to non-migraineurs (41).

Beyond pain modulation, the PAG plays a role in coordinating passive and active defensive behaviors, commonly known as “fight or flight responses” (42-44). Based on a recent hypothesis, migraine attacks are a sort of inescapable pain situations, when “flight” autonomic-behavior response is activated to avoid threatening situations (29, 45). During migraine pain, visceral C fibers are activated, and by projecting to ventrolateral PAG, they promote sympathetic system inhibition, thus contribute to developing avoidance behavior (46). The “sickness behavior” of migraine patients is considered an evolutionary mechanism to counterbalance allostatic load (29). However, chronic exposure to stress induces a decreased glutamatergic transmission in the ventrolateral PAG leading to depression, another maladaptive behavior (47). Thus, the pathological expression of fight or flight responses could contribute to anxiety and depression, which are highly comorbid disorders with migraine (48, 49). More than 50% of migraine patients experience anxiety or mood disorders in their lifetime (50), and the disease burden of migraine patients with psychiatric comorbidity is elevated (51). In the background of comorbidity, shared common biological and genetic processes are assumed including the tryptophan (TRP) metabolic pathway and its impact on the network of PAG (52).

1.3. Tryptophan (TRP) pathways in migraine

TRP is an essential amino acid that serves as a precursor of various metabolic pathways involved in migraine pathophysiology, such as serotonin (5-HT) and kynurenines (53, 54). Only 5% of TRP is metabolised into 5-HT through 5-hydroxytryptophan and the majority is transformed via kynurenine pathway (**Figure 1.**) (55). The role of 5-HT in migraine has been investigated since the findings of Sicuteri, who discovered elevated levels of 5-HT metabolites in migraine patients' urine during attacks (56). 5-HT has vasoconstrictor and antinociceptive effects that led to the discovery of the first migraine specific attack reliever drugs, triptans. Triptans enhance the 5-HT signaling by activating 5-$HT_{1B,1D}$ and 5-HT_{1F} receptors in cranial blood vessels and nerve endings (57). They alleviate pain by constricting blood vessels and inhibiting the release of peptides, including CGRP, while other mechanisms of action remain to be fully understood (58). Hence 5-HT is not able to cross the blood–brain barrier, the 5-HT synthesis of the brain depends on the dietary intake and plasma TRP concentration (55). The transport of TRP to the brain relies on its competition with other large neutral amino acids (LNAAs) (such as leucine, isoleucine, valine, phenylalanine or tryrosine) for their common transporter L-type amino acid transporter 1 (LAT1) (59).

Although the TRP/LNAA ratio is commonly used to describe the proportion of TRP uptake to the brain, the plasma TRP/LNAA ratio in migraine remains unexplored. Studies examining the blood TRP concentration in migraine patients yield inconsistent results, reporting both higher (60-62) and lower (63, 64) TRP concentrations compared to healthy controls. Moreover, migraine patients appear to be more sensitive to alterations in plasma TRP concentration. More precisely, TRP depletion studies reported that migraine patients consuming TRP free, but LNAA containing drinks developed migraine-like headache, nausea, and photophobia (65). Additionally, relatively lower dietary intake of TRP is associated with an increased risk of migraine development in susceptible individuals (66). Moreover, fluctuation of plasma TRP concentration influences brain serotonin synthesis, thus contributing to development of depressive symptoms and anxiety in susceptible persons – as above mentioned, migraine patients are among them (67, 68).

The kynurenine pathway has gained more and more attention in migraine research over the past decades (54). TRP is metabolized into N-formyl-kynurenine by hepatic

tryptophan-2,3-dioxygenase (TDO) and peripheral or central indoleamine-2,3-dioxygenase (IDO). N-formyl-kynurenine undergoes degradation by formamidase to produce L-kynurenine (KYN). KYN can be further metabolized into several neuroactive components such as the neuroprotective kynurenic acid (KYNA) by kynurenine 3-monooxygenase (KMO) enzyme and the neurotoxic quinolinic acid (69). The kynurenine pathway interferes with glutamatergic neurotransmission, as KYNA could act on N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors exerting antinociceptive effect (70). Glutamate is the most important excitatory neurotransmitter in the brain, playing a role in pain perception and central sensitization. Thus, the kynurenine pathway could be relevant in interictal hyperexcitability of migraine brain and pain generation during attacks (71).

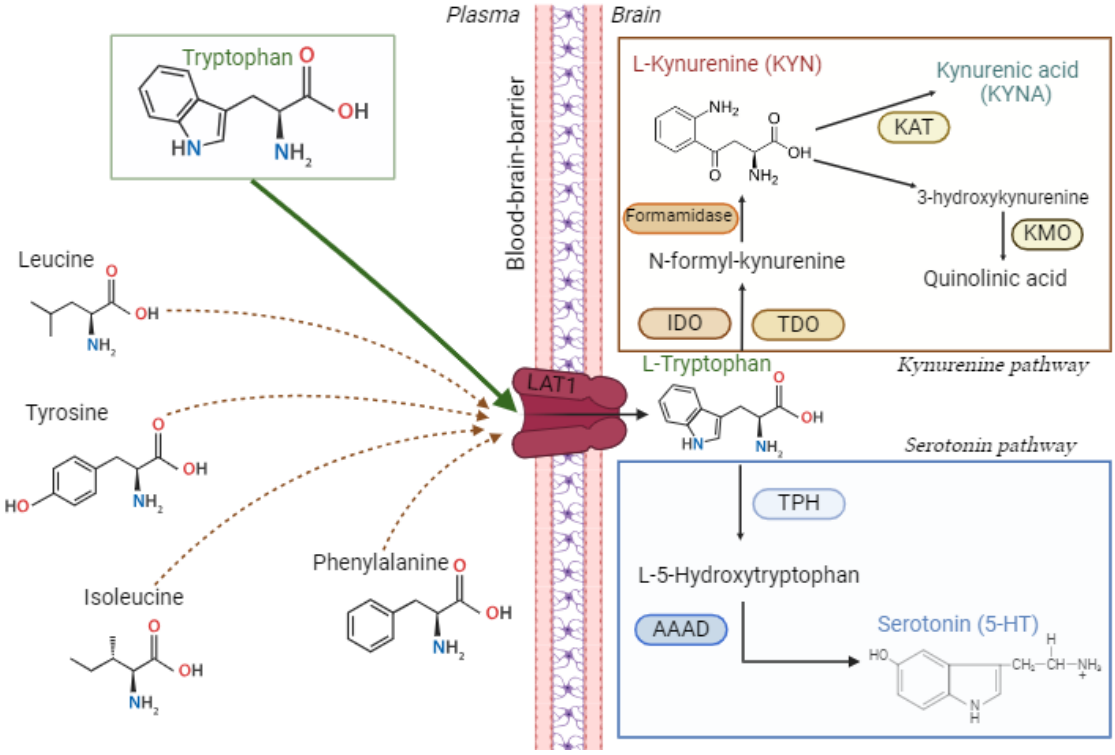

Figure 1. *The tryptophan metabolic pathways: serotonin and kynurenine pathways.*

Note: Tryptophan competes with large neutral amino acids for the transporter (LAT1) to enter to the brain. In the brain, the major metabolic route of tryptophan is the kynurenine pathway. Only a few percent are metabolized through serotonin pathway. AAAD: aromatic amino acid decarboxylase, IDO: indoleamine 2,3-dioxygenase, KAT: kynurenine aminotransferase, KMO: kynurenine 3-monooxygenase, LAT1: large neutral

amino acid transporter, TDO: tryptophan-2,3-dioxygenase, TPH: tryptophan hydroxylase. The figure was created in BioRender.com.

Furthermore, kynurenine pathway mediates the communication between the central nervous system and the immune system (72). During inflammation, cytokines promote the metabolism of TRP into kynurenine pathway by inducing the activity of IDO, which is usually described with the ratio of KYN/TRP (73). Kynurenine metabolites have regulatory effects on immune and stress response, for example KYNA could reduce cytokine expression, meanwhile KYN contributes to monocytes activation (74). The neurogenic inflammation in the central nervous system is a pathological feature of migraine attacks, as several inflammatory neuropeptides are released, among them CGRP, which is inducing vasodilation and trigeminal neuron sensitization (14, 75). The kynurenine pathway, exerting a neuroimmunoregulatory role, is a potential key player in the comorbidity of migraine and inflammation-induced depression (76).

1.4. Relationship between PAG and TRP pathways

The TRP metabolic pathways, both 5-HT and KYN pathways are demonstrated to influence the neuronal transmission of PAG interfering with pain and emotion processing (69, 77). More precisely, high concentration of 5-HT receptors (mainly 5-HT_{1A} and 5-HT_{2C}) situated in the ventrolateral PAG are involved in descending pain modulation, even proposed to interact with opioid mediated antinociception (78). In animal models, the gold standard acute migraine medication, sumatriptan could inhibit the synaptic transmission in the PAG acting on 5-$HT_{1B/1D}$ receptors (79).

Moreover, previous studies demonstrated that serotonergic neurons in dorsal and ventral PAG play a role in defensive behaviour such as freezing, alertness or escaping (80). Based on the Deakin/Graeff hypothesis, serotonergic neurons originated from the dorsal raphe projecting to PAG inhibit panic and escape-like physiological and behavioural responses via 5-HT_{1A} and 5-HT_{2A} receptors (81). Thus, the dysfunction of these projections contributes to anxiety and affective disorders (82, 83).

Focusing on the kynurenine pathway, microinjection of KYNA, an NMDA antagonist in the dorsal PAG showed anxiolytic effect and inhibited cell activity of PAG through blocking glutamatergic neurons (84). Thus, the imbalance of kynurenine

pathway, favouring to cytotoxic metabolites could lead to anxiety and depression (85, 86).

Therefore, it can be hypothesized that TRP metabolic pathways in association with PAG functional connectivity network may contribute to migraine pathophysiology and related vulnerability to anxiety and depressive symptoms.

2. Objectives

This work is aimed to investigate the potential role of tryptophan (TRP) pathway in the interictal hyperexcitability of migraine brain through altered brain connectivity and in development of maladaptive stress response.

Three hypotheses are proposed to be tested:

1) Migraine patients have altered TRP pathway metabolism between attacks, which shows association with migraine frequency.
2) The maladaptive stress response of migraine patients is associated with altered TRP pathway.
3) Migraine patients have hypersensitive emotional and pain regulation neuronal networks that show association with TRP pathway.

In order to address the above listed hypotheses, a neuroendocrine stress challenge using acute citalopram infusion and a resting-state functional magnetic resonance imaging (fMRI) study with PAG as seed region were designed with multiple blood sample collections.

1) We compared the baseline plasma TRP, KYN concentrations and TRP/LNAA, KYN/TRP ratios in two independent blood samples between episodic migraine patients without aura and non-headache, healthy controls.
2) We compared the alterations in plasma TRP, KYN, cortisol (CORT) concentrations and in TRP/LNAA, KYN/TRP, CORT/dehydroepiandrosterone sulfate (DHEA-S) ratios during placebo controlled, double-blind, acute citalopram challenge between episodic migraine patients without aura and non-headache, healthy controls.
3) We compared the resting-state neuronal network of PAG between episodic migraine patients without aura and non-headache, healthy controls, and investigated for the first time the association of plasma TRP concentration and PAG functional connectivity. Furthermore, we assessed the relationship between migraine parameters (migraine frequency, disease onset) and emotional symptoms (depressive symptoms, trait-anxiety) with PAG functional connectivity in episodic migraine patients without aura.

3. Methods

3.1. Participants

The initial study population consisted of 33 episodic migraine without aura patients and 48 healthy controls. All participants were screened by expert neurologists and clinical psychologists/psychiatrists. In addition to diagnostic interview, Mini-International Neuropsychiatric Interview was used to check their mental health status (87). Exclusion criteria were the following: having any past or current serious medical, neurological (except episodic migraine without aura) or psychiatric disorders, using any daily medication (except contraceptives), using preventive migraine medication (87, 88). Further inclusion criteria were the following: between 18-50 years of age, right handedness (89), free from headache and medication 48 h before and 24 h after the examination days, refraining from alcohol 24 h and caffeine 4 h before blood sampling.

Episodic migraine without aura was diagnosed by expert neurologists based on the International Classification of Headache Disorders-III criteria (ICHD)(6, 88). Clinical variables of migraine (age at migraine onset and frequency of attacks per month) were recorded for migraine patients.

The entire study was conducted in accordance with the Declaration of Helsinki and participants provided written informed consent. The study protocol was approved by the Scientific and Research Ethics Committee of the Medical Research Council in Hungary (23609-1/2011-EKU, 23421-1/2015-EKU) (87, 88).

3.2. Study design

Our study consisted of two separate experimental days at least 14 days apart (mean = 50.81 ± 48.34 days) (**Figure 2**.). On each experimental day, three blood samples were collected from the participants after an acclimatization period. The first blood samples preceded any intervention, thus indicate baseline plasma concentrations. After the first blood sampling, acute citalopram challenge took place where a 10-minute baseline saline infusion was followed by 7.5 mg citalopram infusion during 7.5 min (infusion rate: 6 mL/min, concentration: 10 mg/60 mL) in a randomized, double-blind, crossover design (87). The dose of the citalopram infusion was determined based on previous studies (90-92). The acute administration of citalopram increases the extracellular 5-HT level in the brain and induces an acute neuroendocrine stress by stimulating the hypothalamic-

pituitary-adrenal (HPA) axis (90, 93, 94). The placebo arm received normal saline infusion. During the infusion, participants were asked yes/no questions every 5 minutes to describe how they felt about the following statements: anxiety, nausea, drowsiness, dizziness, restlessness and discomfort. Finally, blood samples were collected 20 min and 60 min after the start of the infusions. On the second experimental day, participants completed psychological questionnaires to measure their current depressive symptoms (ZUNG) (95) and trait-anxiety (STAI) (96). Before second experimental day's citalopram challenge, participants underwent a 6-minute resting-state fMRI session. The experiments were scheduled between 4:00 pm and 8:00 pm to avoid the impact of diurnal changes in CORT and DHEA-S, which shows a slight decrease in the afternoon (97).

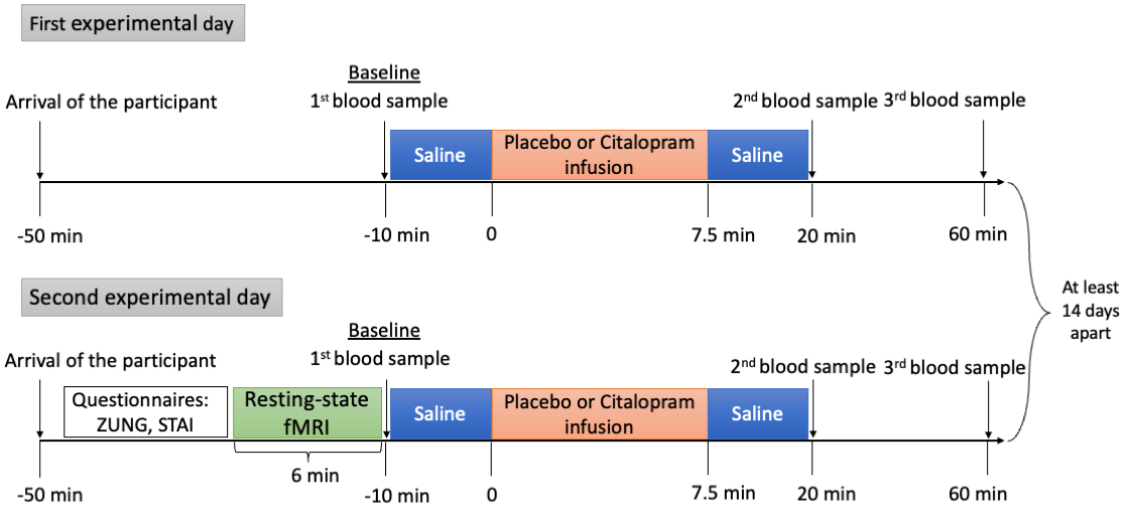

Figure 2. *Schematic representation of the study design.*

Note: The randomized, double-blind, crossover study consisted of two experimental days with three blood samplings. fMRI: functional magnetic resonance imaging, STAI: trait-anxiety score, ZUNG: depressive symptoms score.

3.3. Biological samples

Blood samples were centrifuged and kept frozen at -80°C until the assay (87, 88). Liquid Chromatography Mass Spectrometry (LC-MS/MS) method was used to determine plasma concentration of citalopram, TRP, KYN, and LNAA (valine, leucine, isoleucine, phenylalanine, and tyrosine) (98). Plasma CORT and DHEA-S concentrations were measured by competitive ELISA kits (87).

3.4. Imaging data acquisition

At the beginning of the imaging session, high resolution (1x1x1 mm^3) structural data were acquired using T1-weighted 3D turbo field echo sequence with a 3 T Philips Medical System, Achieva 3T scanner. Participants were instructed to close their eyes and remain awake during the resting-state fMRI measurement. The acquisition parameters of T2*-weighted echo-planar pulse-sequence were the following: repetition time = 2.500 ms, echo time = 30 ms, field of view = 240x240 mm^2, 3x3x3 mm^3 resolution (88).

3.5. Statistical analysis

3.5.1. Statistical analysis of baseline data related to Hypothesis 1

For statistical analysis of the plasma concentrations and baseline characteristics, SPSS (SPSS Statistics for Windows, version 27.0) was used. Based on Fernstrom et al., the sum of LNAA affecting TRP uptake to the brain was calculated as the sum of tyrosine, phenylalanine, leucine, isoleucine and valine concentrations (59). The ratio of TRP/LNAA, KYN/TRP and CORT/DHEA-S was calculated. CORT/DHEA-S ratio is a sensitive marker of stress response (99). Mann-Whitney test was applied to determine any differences between migraine patients and controls in age and baseline plasma concentrations. Significance threshold was $p < 0.05$ (88).

3.5.2. Statistical analysis of citalopram challenge data related to Hypothesis 2

The final sample size for acute citalopram challenge analysis consisted of 17 episodic migraine without aura women and 17 healthy women. Those subjects had to be excluded from the analysis who had some problems during at least one blood sampling. Among the 17-17 participants, 10 migraine patients and 9 healthy controls received citalopram intervention during the first experimental day, the others were allocated to placebo. On the second experimental day, the 10 migraine patients and 9 healthy controls were allocated to cross-over placebo. Males were also excluded from this part of the study because of the low number of males and potential gender specific effect (100).

The plasma concentrations did not show normal distribution, thus Friedman test was used to determine the effect of the citalopram infusion on plasma concentrations in the whole population, and in separately analysing the control and migraine groups (87). Post-hoc Wilcoxon signed-rank tests was used with a Bonferroni correction. Spearman correlations were applied to determine the relationship between baseline and citalopram-

induced plasma concentrations with the age at migraine onset and migraine frequency (87).

GraphPad Prism version 8.0.1 for Windows software was used for data visualization of plasma concentrations.

3.5.3. Statistical analysis of fMRI data related to Hypothesis 3

The final sample size for PAG functional connectivity analysis was 54 participants. In case-control arrangement: 27 episodic migraine without aura patients (21 females, 6 males; mean age: 25.9 ± 4.6 years) and 27 sex-matched, healthy control subjects (21 females, 6 males; mean age: 25.6 ± 4.0 years).

In order to characterize the mean TRP intake, the mean of the baseline TRP concentrations was calculated. As there was no significant difference between the two baseline blood samples' TRP concentration, (88). Comparing migraine patients to healthy controls in trait-anxiety and depressive symptoms scores, independent t-test was applied. Significance threshold was $p < 0.05$ (88).

The state-of-the-art preprocessing steps were the following: 1) intensity non-uniformity correction and intensity normalization on the T1-weighted images; 2) Segmentation of T1-weighter images 3) Spatial normalisation into 2 mm MNI152 space 4) Deleting the first four frames of functional time-series 5) Primary motion correction 6) Co-registration of functional images with structural scans and transformation 7) Spatial filtering with 6 mm Gaussian-kernel 8) Signal correction with ICA-AROMA 9) Temporal band-pass filtering (88). After preprocessing, seed-to-voxel functional connectivity analysis was carried out, where time-series of a chosen region is correlated with all other voxels across the brain. Left and right PAG as seed regions were defined based on previous literature (MNI peak coordinates left PAG: -2; -28; -6; right PAG: 2; -28; -6; with 3 mm radius) (38). The seed-based connectivity map definition steps are detailed in Gecse, K. et al., 2022 (88).

Statistical Parametric Mapping (SPM12) software package implemented in Matlab 2016a was used to analyse within- and between-group comparisons. "First, PAG intrinsic functional connectivity was determined in whole group analysis using one sample t-test. Next, two sample t-test was conducted to compare PAG connectivity between migraine and control groups. Correlation between plasma TRP and PAG connectivity in the whole population was determined by including the mean TRP

concentration of the first blood samplings as a regressor. TRP correlation with PAG connectivity based on diagnosis was performed by using F-contrast" (88).

Then, the correlation between mean plasma TRP concentration and PAG intrinsic functional connectivity was investigated in the migraine and control group separately. Also, the correlation between migraine-related emotional features, STAI and ZUNG scores and migraine indicators (attack frequency and age of onset) with PAG connectivity were investigated. Next, TRP concentration was used as covariate to determine whether it alters the association between PAG connectivity and migraine indicators, and STAI or ZUNG (88).

All analyses were corrected for motion parameters, age and sex. To better model TRP influx to the brain, all TRP related analyses were corrected for LNAA levels. "An initial threshold of $p < 0.001$ uncorrected for multiple comparison and at least twenty contiguous voxels were used in the analyses. All reported results survived family-wise error correction at a cluster-level threshold of $p_{FWE} < 0.05$" (88).

As the first part of the thesis involved only female participants, in order to investigate the possible confounding effect of sex on fMRI data, were repeated migraine and control group comparison and TRP effect analysis using female participants only.

For data visualization, significant clusters were added as overlay on MNI 152 brain template in MRIcroGL.

4. Results

4.1. Descriptive statistics

The original study population consisted of 33 migraine patients (27 females and 6 males), and 48 healthy controls (29 females and 19 males). Some healthy controls have not completed the ZUNG (n = 3) and STAI (n = 5) questionnaires. There were no differences between migraine and control groups in age, depressive symptoms, and trait-anxiety scores (**Table 1.**). The mean frequency of monthly migraine attacks was 3.30 ± 2.82 and the mean age at migraine onset was 15.27 ± 6.47 years.

4.2. Baseline TRP pathway

Both examination days started with blood sample collection to determine the baseline concentration of TRP, KYN, CORT and the ratio of TRP/LNAA, KYN/TRP and CORT/DHEA-S (**Table 1.**). The TRP concentration (1.: $U = 488$, $p = 0.016$, 2.: $U = 363$, $p = 0.006$) and TRP/LNAA ratio (1.: $U = 458$, $p = 0.007$, 2.: $U = 334$, $p = 0.002$) were significantly higher in migraine group compared to controls in both blood samples. The KYN concentration was significantly higher ($U = 399$, $p = 0.029$) in migraine patients compared to controls in the second experimental day. However, there was no difference in KYN concentration between the two groups in the first experimental day ($U = 668$, $p = 0.591$). There were no further differences in baseline plasma concentrations between migraine and control groups.

Table 1. *Descriptive statistics and baseline plasma concentrations of all participants*

	Migraine	N	Control	N	Test statistic (U)	p-value
Age (years)	26.48 ± 4.77	33	25.69 ± 4.05	48	730.5	0.553
ZUNG score	33.86 ± 5.72	33	32.63 ± 6.40	45	610	0.179
STAI score	37.75 ± 8.64	33	35.47 ± 9.31	43	653	0.365
Migraine frequency (attack per month)	3.30 ± 2.82	33	NA			
Age at migraine onset	15.27 ± 6.47	33	NA			
1. experimental day - Baseline blood sample						
TRP [µg/mL]	9.26 ± 3.00	32	7.81 ± 3.08	45	488	**0.016**
TRP/LNAA	0.09 ± 0.03	32	0.07 ± 0.02	45	458	**0.007**
KYN [µg/mL]	0.56 ± 0.21	32	0.52 ± 0.17	45	668	0.591
KYN/TRP	0.06 ± 0.02	32	0.07 ± 0.02	45	901	0.061
CORT [ng/mL]	81.48 ± 59.61	31	77.23 ± 63.18	42	616	0.696
CORT/DHEA-S [µg/mL]	57.99 ± 53.25	31	43.98 ± 36.51	42	577	0.409
2. experimental day - Baseline blood sample						
TRP [µg/mL]	9.16 ± 2.67	27	7.46 ± 2.10	44	363	**0.006**
TRP/LNAA	0.10 ± 0.04	27	0.07 ± 0.02	44	334	**0.002**
KYN [µg/mL]	0.60 ± 0.19	27	0.56 ± 0.30	43	399	**0.029**
KYN/TRP	0.07 ± 0.02	27	0.07 ± 0.02	43	695	0.167
CORT [ng/mL]	71.41 ± 52.26	28	54.18 ± 45.99	38	425	0.165
CORT/DHEA-S [µg/mL]	49.62 ± 46.72	28	32.23 ± 24.49	38	494	0.509

Note: Values demonstrate mean ± SD. Significance threshold was $p < 0.05$. CORT: cortisol, DHEA-S: dehydroepiandrosterone sulfate, KYN: L-kynurenine, LNAA: large neutral amino acids, N: number of analysable samples, STAI: trait-anxiety score, TRP: Tryptophan, ZUNG: depressive symptoms score.

4.3. Acute citalopram challenge

4.3.1. Effect of acute citalopram

In the acute citalopram challenge, data of 17 female migraine patients (mean age: 25.53 ± 4.65 years) and 17 healthy control females (mean age: 25.71 ± 4.16 years) were analysed. The mean BMI of the participants was: 20.97 ±3. 29kg/m^2. Plasma citalopram concentration significantly increased in migraine patients (20 min: 21.13 ± 6.67 [µg/mL]; 60 min: 11.50 ± 3.97 [µg/mL]) and healthy controls (20 min: 17.91 ± 11.83 [µg/mL]; 60

min: 13.15 ± 7.22 [μg/mL]). There were no significant differences in plasma citalopram concentration between the two groups (20 min: $H(1) = 2.75$, $p = 0.097$; 60 min: $H(1) = 0.15$, $p = 0.904$)(87).

Acute citalopram induced subjective experiences of the participants were the following: 9.1% anxiety, 63.6% drowsiness, 24.2% dizziness, 21.2% nausea, 30.3% restlessness, 48.5% discomfort. There were no significant differences in reported subjective experiences between the migraine and the control groups (anxiety ($p = 0.601$), nausea ($p = 1.000$), drowsiness ($p = 0.721$), dizziness ($p = 1.000$), restlessness ($p = 1.000$), discomfort ($p = 0.494$))(87).

Significant increases in TRP ($\chi2(2) = 6.35$, $p = 0.042$), KYN ($\chi2(2) = 11.53$, $p = 0.003$) and TRP/LNAA ($\chi2(2) = 6.59$, $p = 0.037$) concentrations were observed as main effect of acute citalopram challenge in the study population. In case of placebo, the circadian decrease in CORT concentration ($\chi2(2) = 31.83$, $p < 0.001$) and CORT/DHEA-S ($\chi2(2) = 26.65$, $p < 0.001$) were observed, that cannot be detected after citalopram infusion (87).

4.3.2. Diagnosis dependent effect of acute citalopram challenge

Acute citalopram challenge showed different effects on TRP pathway in migraine patients than in healthy controls (**Figure 3.**). Citalopram infusion induced significant increases in TRP ($\chi2(2) = 14.94$, $p < 0.001$), KYN ($\chi2(2) = 8.94$, $p = 0.011$) concentrations and TRP/LNAA ($\chi2(2) = 13.18$, $p < 0.001$) ratio in healthy control group. However, these citalopram-induced changes were not observed in migraine patients. Only KYN/TRP ratio ($\chi2(2) = 7.41$, $p = 0.025$) showed significant decrease in migraine group (87).

Citalopram as a neuroendocrine challenge induced the increase in CORT and CORT/DHEA-S concentration, thus the circadian decrease of CORT and CORT/DHEA-S after citalopram infusion were not observed in migraine and control groups. In case of placebo, CORT (migraine: $\chi2(2) = 23.48$, $p < 0.001$; control: $\chi2(2) = 9.88$, $p = 0.007$) and CORT/DHEA-S (migraine: $\chi2(2) = 20.24$, $p < 0.001$; control: $\chi2(2) = 7.88$, $p = 0.019$) showed time-dependent concentration decrease in both groups (87).

Post-hoc analysis revealed that 20 minutes after citalopram infusion TRP concentration ($Z = -1.12$, $p = 0.003$) and TRP/LNAA ($Z = -1.18$, $p = 0.002$) were significantly elevated compared to baseline concentration in healthy controls. Significant reduction in TRP concentration ($Z = 1.18$, $p = 0.002$) and TRP/LNAA ratio ($Z = 0.94$, p

= 0.018) were observed between the 60 minutes sample compared to 20 minutes. However, there were no significant differences in TRP concentration (Z = 0.06, p = 1.000) and TRP/LNAA ratio (Z = -0.24, p = 1.000) between baseline and 60 minutes after citalopram infusion. The same pattern described the citalopram-induced changes in KYN concentration. Namely, 20 minutes after citalopram infusion KYN concentration was significantly elevated compared to baseline concentration (Z = -0.82, p = 0.049) in healthy controls. Significant reduction in KYN concentration was observed between the 60 minutes sample compared to 20 minutes (Z = 0.94, p = 0.018). However, there was no significant difference between baseline KYN concentration and 60 minutes after citalopram infusion (Z = 0.12, p = 1.000) (87).

In migraine group, post-hoc analysis showed significant reduction in KYN/TRP ratio between 20 minutes and 60 minutes after citalopram (Z = 0.88, p = 0.003). There were no significant changes in KYN/TRP ratio between baseline and 20 minutes (Z = -0.71, p = 0.119) or 60 minutes after citalopram (Z = 0.18, p = 1.000) (87).

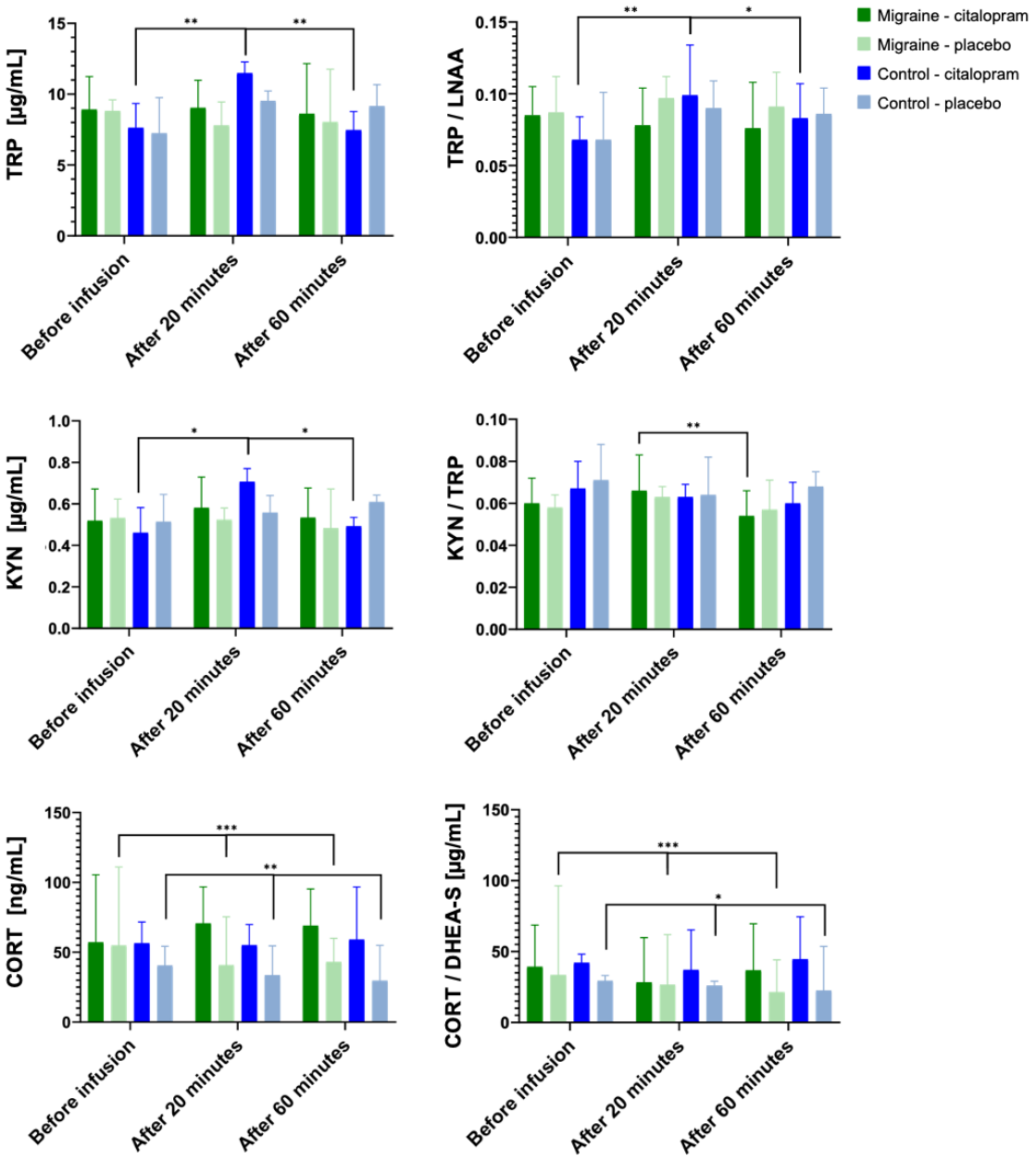

Figure 3. *The effect of citalopram challenge on plasma TRP, KYN, CORT concentrations and TRP/LNAA, KYN/TRP and CORT/DHEA-S ratios.*

Note: CORT: cortisol, DHEA-S: dehydroepiandrosterone sulfate, KYN: L-kynurenine, LNAA: large neutral amino acids, TRP: Tryptophan *p < 0.05, **p < 0.01, ***p < 0.001 (87).

4.3.3. Associations with clinical variables of migraine

Significant negative correlation was found between baseline KYN/TRP ratio and monthly migraine frequency (before citalopram: r_s= -0.451, p = 0.046, before placebo: r_s= -0.525, p = 0.018) (**Figure 4.**).

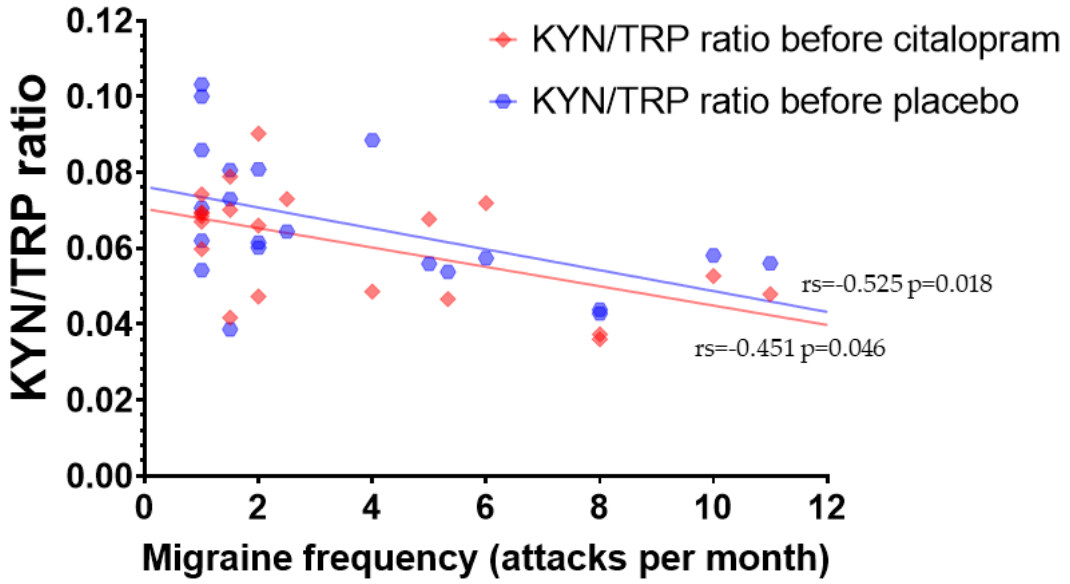

Figure 4. *Negative correlation between monthly migraine frequency and kynurenine (KYN) / tryptophan (TRP) ratio* before citalopram challenge and placebo (87).

In addition, citalopram-induced KYN concentration elevation 20 minutes after infusion positively correlated with migraine frequency (r_s = 0.766, p < 0.001) (**Figure 5**.). There were no significant associations between age at migraine onset and plasma biomarkers.

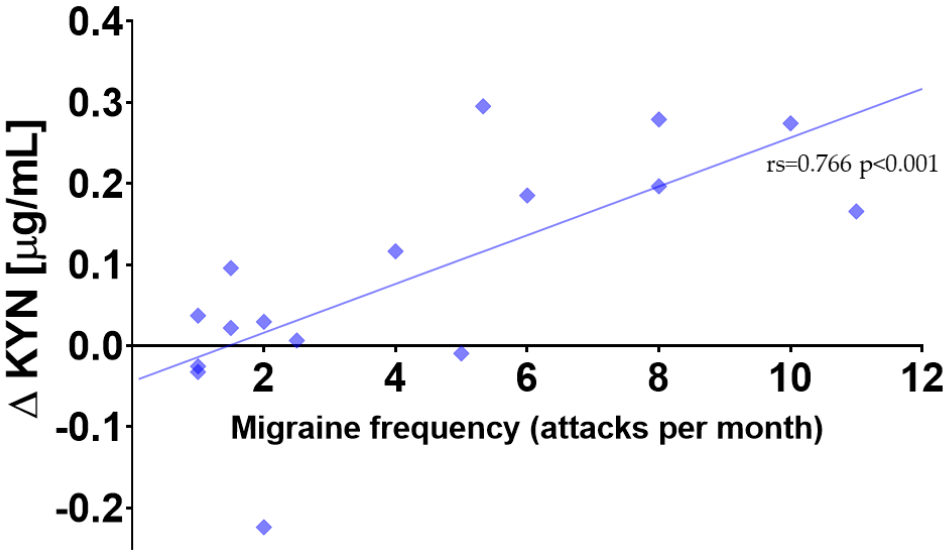

Figure 5. *Correlation between monthly migraine frequency and acute citalopram-induced kynurenine (KYN) concentration changes* (87).

Note: Δ = The difference between 20 minutes after citalopram infusion and baseline.

4.4. Resting-state functional connectivity of PAG

4.4.1. Intrinsic connectivity of PAG

Left and right side of PAG showed significant positive intrinsic functional connectivity with nearby brainstem structures, thalamus (**Table 2**.). Right PAG showed additional significant connectivity with cerebellum. There was no negative functional connectivity that survived the cluster-level $p_{FWE} < 0.05$ significance threshold.

Table 2. *Intrinsic functional connectivity of left and right side of PAG* (88)

Seed region	Region	Cluster size (voxel)	Peak T-value	MNI coordinates (x y z)		
Left PAG	R Thalamus	324	4.37	10	-28	-2
	R Precuneus	139	4.85	4	-70	54
Right PAG	R Thalamus	514	19.87	12	-22	10
	L Cerebellum Crus 1	1576	5.42	-40	-52	-34
	L Cerebellum VI		4.62	-12	-68	-26
	L Cerebellum Crus 2		3.86	-38	-70	-44
	L Vermis VI		3.73	-4	-58	-22
	R Cerebellum VI	428	4.41	26	-62	-30
	R Cerebellum Crus 1		3.60	32	-56	-36
	R Cerebellum VIII		3.49	18	-64	-38

Note: Results are corrected for age and sex. All the listed clusters survived the cluster-level $p_{FWE} < 0.05$ significance threshold. R: right, L: left, MNI: Montreal Neurological Institute

4.4.2. Differences between migraine and control groups in PAG connectivity

Left PAG showed increased functional connectivity with left postcentral gyrus (Peak-T value = 4.09 at cluster-level $p_{FWE} < 0.05$, cluster size: 285 voxels) in migraine group compared to control group. In migraine patients, right PAG had increased functional connectivity with left orbital part of superior frontal gyrus (Peak-T value = 6.65 at cluster-level $p_{FWE} < 0.05$, cluster size: 137 voxels) compared to controls. There was no increased functional connectivity of PAG in control group compared to migraine group.

Analysing only female participants, left PAG showed increased functional connectivity with left postcentral gyrus (Peak-T = 4.69 at cluster-level pFWE < 0.05, cluster size: 231 voxels) in migraine group compared to control group. Right PAG showed increased functional connectivity with right superior temporal gyrus (Peak-T value = 5.10 at cluster-level pFWE < 0.05, cluster size: 388 voxels) and right middle cingulate cortex (Peak-T value = 3.97 at cluster-level pFWE < 0.05, cluster size: 236 voxels) in migraine patients compared to controls.

4.4.3. Effect of TRP on PAG connectivity

The mean plasma TRP concentration did not show main effect on PAG intrinsic functional connectivity in the whole study population.

However, TRP showed diagnosis dependent effect on PAG intrinsic functional connectivity. More precisely, when comparing migraine group to control group, a significant difference was observed between the plasma TRP concentration effect on left PAG intrinsic functional connectivity with middle and superior occipital gyri and fusiform gyrus and left cerebellum (**Table 3**). TRP also showed different effects on right PAG intrinsic functional connectivity with left fusiform gyrus, left superior and middle occipital gyrus in migraine patients compared to controls.

Table 3. *Brain clusters with significantly different association between plasma TRP and intrinsic PAG functional connectivity in migraine patients compared to controls* (88).

Region	Cluster size (voxel)	Peak F-value	MNI coordinates (x y z)		
Left PAG					
R Superior Occipital gyrus	1763	26.26	26	-94	20
R Middle Occipital gyrus		20.75	32	-80	6
L Superior Occipital gyrus	1267	23.17	-14	-96	24
L Middle Occipital gyrus		22.19	-26	-86	16
L Fusiform gyrus	1244	25.18	-44	-70	-18
L Cerebellum IV-V		22.40	-6	-64	-6
R Fusiform gyrus	176	19.69	30	-46	-16
Right PAG					
L Fusiform gyrus	698	19.77	-36	-80	-14
L Superior Occipital gyrus	287	25.10	-20	-96	18
L Middle Occipital gyrus		18.22	-26	-88	16

Note: Results are corrected for age, sex and LNAA concentration. Significance threshold was cluster-level $p_{FWE} < 0.05$ including at least 20 contiguous voxels. R: right, L: left, MNI: Montreal Neurological Institute.

Analysing only female participants, the detected results did not change significantly. Namely migraine patients showed significantly different effect of plasma TRP on left PAG functional connectivity with middle and superior occipital gyri (Peak-F = 32.57 at cluster-level pFWE < 0.05, cluster size: 3671 voxels), left (Peak-F = 24.63 at cluster-level pFWE < 0.05, cluster size: 1450 voxels) and right (Peak-F = 21.78 at cluster-level pFWE < 0.05, cluster size: 180 voxels) fusiform gyri and right PAG functional connectivity with left fusiform gyrus (Peak-F = 28.24 at cluster-level pFWE < 0.05, cluster size: 1115 voxels), left middle occipital gyrus (Peak-F = 25.09 at cluster-level pFWE < 0.05, cluster size: 753 voxels), and right lingual gyrus (Peak-F = 19.79 at cluster-level pFWE < 0.05, cluster size: 251 voxels) compared to controls.

In post-hoc analysis of migraine group (**Figure 6**.), plasma TRP concentration showed positive correlation with intrinsic connectivity between PAG and superior and superior medial part of frontal gyrus. In addition, negative correlation was found between plasma TRP concentration and functional connectivity of left PAG with left and right fusiform gyrus, right cerebellum, both side of middle part of occipital gyrus and left superior occipital gyrus. Further significant negative correlation was found in case of right PAG connectivity with left fusiform gyrus.

In female migraine patients the significant negative correlation between plasma TRP and PAG functional connectivity showed the same pattern. Namely, plasma TRP concentration showed negative correlation with connectivity between left PAG and left fusiform gyrus, right cerebellum, right middle occipital gyrus. Also, negative correlation was found between plasma TRP and right PAG connectivity with left fusiform and right precentral gyrus.

During post-hoc analysis, there was no significant association between plasma TRP and functional connectivity of PAG in control group.

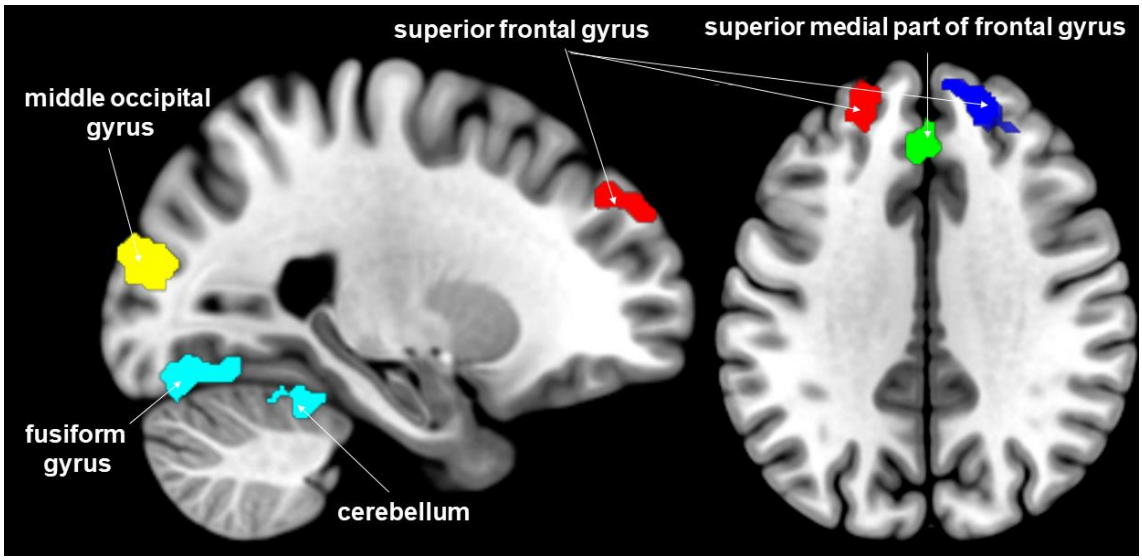

Figure 6. *Brain clusters where intrinsic functional connectivity with PAG were significantly associated with plasma TRP concentration in migraine patients* (88). *Note:* Secondary cluster-level threshold $p_{FWE} < 0.05$. Results are corrected for age, sex and LNAA concentration. LNAA: large neutral amino acids, PAG: periaqueductal gray matter, TRP: Tryptophan.

4.4.4. Associations with clinical variables of migraine, depressive symptoms, and trait-anxiety

Clinical variables of migraine (monthly migraine frequency and the age of migraine onset) significantly correlated with functional connectivity of PAG, but plasma TRP concentration did not affect these associations (**Table 4**.).

In migraine patients, depressive symptoms score positively correlated with functional connectivity between PAG and middle frontal gyrus (Peak-T value = 6.65 at cluster-level $p_{FWE} < 0.05$). Additional negative correlation between functional connectivity of right PAG and right fusiform gyrus (Peak-T value = -4.89 at cluster-level $p_{FWE} < 0.05$) and parahippocampal gyrus (Peak-T value = -4.14 at cluster-level $p_{FWE} < 0.05$) was revealed. To evaluate the association of TRP with above mentioned correlations, the analysis was corrected for plasma TRP concentration (and corrected for LNAA level). After that, there was no significant correlation between depressive symptoms and functional connectivity of PAG with middle frontal gyrus, fusiform gyrus or parahippocampal gyrus. However, depressive symptoms negatively correlated with functional connectivity between left PAG and left precuneus (Peak-T value = -4.44 at cluster-level $p_{FWE} < 0.05$), and right middle cingulum (Peak-T value = -4.08 at cluster-level $p_{FWE} < 0.05$) even after correction for plasma TRP concentration (**Table 4**).

Significant positive correlation was found between trait–anxiety with connectivity of left PAG and left middle frontal gyrus (Peak-T value = 5.33 at cluster-level $p_{FWE} < 0.05$). Additional positive correlation was revealed between trait-anxiety with connectivity of right PAG and left middle (Peak-T value = 5.73 at cluster-level $p_{FWE} < 0.05$) and superior medial frontal gyrus (Peak-T value = 5.09 at cluster-level $p_{FWE} < 0.05$). After correcting for plasma TRP concentration (and corrected for LNAA), trait-anxiety level did not show any significant correlation with functional connectivity of PAG (**Table 4**).

In healthy control group, trait-anxiety and depressive symptoms scores showed no correlation with functional connectivity of PAG.

Table 4. *Alterations of PAG connectivity in association with migraine frequency, migraine onset, trait-anxiety level and depressive symptoms in migraine patients with and without correction for plasma TRP concentration* (88).

Seed	Region	Cluster-size	Peak T-value	MNI-coordinates (x y z)		
Without correction for TRP concentration						
Attack frequency						
L PAG	R Triangular part of inferior frontal gyrus	181	5.68	50	40	10
	R Middle frontal gyrus	141	5.04	24	48	4
R PAG	R Triangular part of inferior frontal gyrus	272	5.4	50	28	26
	R Middle frontal gyrus	161	5.21	24	50	4
Age of onset						
L PAG	R Precuneus	197	5.23	14	-44	4
	R Lingual gyrus		4.31	6	-56	4
	R Cuneus		4.18	6	-64	20
Trait-anxiety						
L PAG	L Middle frontal gyrus	166	5.33	-30	56	28
R PAG	L Middle frontal gyrus	190	5.73	-26	54	30
	L Superior medial frontal gyrus	123	5.09	-4	36	32
Depressive symptoms						
L PAG	L Middle frontal gyrus	283	6.65	-26	54	28
	R Fusiform gyrus	158	-4.89	26	-40	-18
	R Parahippocampal gyrus		-4.14	30	-30	-16
R PAG	L Middle frontal gyrus	309	6.70	-26	54	30
After correction for TRP concentration						
Attack frequency						
L PAG	R Triangular part of inferior frontal gyrus	232	7.12	50	40	10
	R Middle frontal gyrus		3.98	54	34	22
R PAG	R Triangular part of inferior frontal gyrus	271	5.47	50	28	26
	R Superior frontal gyrus	126	4.07	32	58	14
Age of onset						
L PAG	R Lingual gyrus	289	5.45	14	46	4
	R Cuneus		4.94	6	66	20
Trait-anxiety						
No significant result						
Depressive symptoms						
L PAG	R Middle cingulate gyrus	206	-4.08	4	-40	42
	L Precuneus		-4.44	-10	56	26

Note: Significance threshold was cluster-level pFWE < 0.05 including at least 20 contiguous voxels. Results are corrected for age, sex and LNAA concentration. R: right, L: left, LNAA: large neutral amino acids, MNI: Montreal Neurological Institute, TRP: Tryptophan

5. Discussion

5.1. Hypothesis 1: Migraine patients have altered TRP pathway metabolism between attacks, which shows association with migraine frequency.

In our study, elevated TRP and TRP/LNAA ratio were detected in interictal, episodic migraine without aura patients compared to healthy controls during two independent timepoints. These results align with previous studies reporting elevated TRP concentration in episodic (60) and chronic migraine patients (61). Opposing results in some studies (63, 64) could be attributed to differences in study populations (e.g., usage of prophylactic drugs) and the timing of blood sampling (63). The TRP/LNAA ratio's reliability as a marker of TRP brain uptake was highlighted (59), however never previously investigated in migraine. The replicated, increased TRP/LNAA ratio potentially reflects decreased breakdown of TRP due to impaired TDO and IDO enzyme activity in migraine (54). The decreased expression of KYN pathway enzymes was already demonstrated in nitroglycerin-based animal migraine model (101).

Furthermore, in our study the reduced KYN/TRP ratio exhibited a negative correlation with the increasing number of migraine attacks per month in both blood samplings. While traditionally, the KYN/TRP ratio was used to indicate IDO activity, the contemporary perspective favours its utility in illustrating the shift of the TRP pathway from 5-HT to KYN (73). Consequently, our findings provide additional evidence supporting a less active KYN pathway in migraine.

5.2. Hypothesis 2: Migraine patients have maladaptive stress response affecting altered TRP pathway.

To investigate the maladaptive stress response of migraine patients, we used an acute citalopram challenge that serves as a neuroendocrine probe assessing serotonergic responsivity and induces acute neuroendocrine stress (93). After the citalopram infusion, an immediate elevation of TRP, KYN, and TRP/LNAA ratio were observed in healthy controls reflecting adaptive stress-responsivity. However, migraine patients did not exhibit this elevation, supporting the hypothesis of maladaptive stress response in migraine (102).

During acute stress situation, various mechanisms could increase plasma TRP and KYN level (103). Meanwhile HPA-axis activation is the best-known pathway to increase plasma amino acid levels in stress (104), our study demonstrated that plasma TRP

elevation preceded a significant increase in CORT response after citalopram infusion. Based on another feasible explanation, sympathetic nervous system activation may contribute to plasma TRP concentration elevation (105). The elevated brain influx of TRP is a short-term citalopram effect indicated by the increased TRP/LNAA ratio (106). Only a few percentages of plasma TRP are not bound to circulating albumin, thus free to enter the central nervous system. However, the activation of sympathetic nervous system during physical exercise or neuroendocrine stress leads to the increase of non-esterified fatty acids level that could displace TRP from protein binding, resulting increased TRP brain uptake (104). The increased TRP concentration in the brain promotes 5-HT synthesis and stress-coping (107).

The concurrent increase in TRP and KYN, without altering the KYN/TRP ratio, suggests an acute elevation in KYN pathway activity. The citalopram challenge as an acute stress may induce IDO and TDO enzymes contributing to KYN shunt activation (108, 109). In healthy controls, as indicated by our results and previous animal studies the balance between TRP and KYN metabolites may contribute to stress resilience (110). These alterations appear temporary, as the increase in TRP and KYN concentrations was followed by a rapid decrease within 60 min, possibly caused by the activation of degradation enzymes.

In contrast, migraine patients did not show an increase in TRP and TRP/LNAA plasma concentrations during the citalopram challenge. This suggests that the already elevated TRP concentration in migraineurs, possibly associated with sustaining the interictal phase or heightened stress sensitivity (88), could not be further increased by acute sympathetic nervous system activation. The induction of IDO and TDO enzymes might also be less effective in migraine, considering the reduced expression of KYN pathway enzymes (111), thus the citalopram challenge resulted in a modest increase in KYN plasma level. The diminished response for the citalopram induced acute neuroendocrine stress strengthened the notion of maladaptive stress response in migraine where downregulated TRP-KYN pathway may play a role (71, 102).

Notably, KYN concentration did not significantly increase in migraine patients after acute citalopram infusion, but the extent of the elevation positively correlated with migraine frequency. Although not measured in our study, previous research suggests lower KAT expression in migraine patients, potentially leading to reduced KYN

conversion to KYNA (111). KYNA, known for its antinociceptive effect, could contribute to analgesia in migraine by blocking CGRP release and reducing serotonergic neuron activation (112, 113). The diminished KYNA production may heighten NMDA receptor hyperactivity in migraine, contributing to migraine brain hyperexcitability (70, 114). Based on our results, we suggest that despite increased plasma TRP levels interictally, insufficiently elevated plasma KYN during acute stress and a downregulated KYN metabolic pathway may contribute to higher migraine attack frequency.

5.3. Hypothesis 3: Migraine patients have hypersensitive emotional and pain regulation neuronal networks that shows association with TRP pathway.

5.3.1. Hypersensitive PAG network in migraine

First, comparing migraine patients to healthy controls, our results, in line with previous studies, revealed a stronger connection between PAG and postcentral and superior frontal gyri indicating a sensory hypersensitivity of the migraine brain even in attack-free period (38, 39, 41). The cluster of the postcentral gyrus that showed significant connection to PAG matches the head and neck area of sensory homunculus.

5.3.2.TRP concentration dependent PAG functional connectivity in migraine

Then, we investigated the effect of plasma TRP on functional connectivity of PAG and a significant association was revealed between plasma TRP concentration and functional connectivity of PAG with regions involved in defence cascade only in migraine patients, but not in healthy controls (115). Namely, elevated plasma TRP concentration correlated with reduced functional connectivity of PAG with fusiform gyrus, middle occipital gyrus, and cerebellum; simultaneously an increased connectivity of PAG with the superior and superior medial parts of the frontal gyrus, specifically the dorsolateral prefrontal cortex (dlPFC) and dorsomedial prefrontal cortex (dmPFC).

The fusiform gyrus as part of the defence cascade is responsible to identify potential threat by evaluating the environment (encompassing movements, vocalizations, and facial expressions)(116). Threatening situation triggers the activation of PAG that is under top-down control of various regions such as the fusiform gyrus (117). In a safe situation, the fusiform gyrus exerts inhibitory control on defensive responses. Regarding migraine, a recent study noted increased PAG connectivity with the fusiform gyrus during painful stimuli, suggesting heightened activity of the fear-cascade under painful conditions in migraineurs (39). Our findings reveal that the functional connectivity

between PAG and fusiform gyrus diminishes with elevated plasma TRP concentration in migraine patients. These results might suggest that in the absence of pain there is a reduction in the perception of threat due to a less active fear-cascade in migraine patients with elevated plasma TRP concentration.

Since the dlPFC and dmPFC are involved in integrating the selection and maintenance of appropriate emotional behaviour (118) and in the regulation of pain perception (119). The positive correlation between elevated plasma TRP concentration and stronger functional connectivity of PAG with dlPFC and dmPFC suggests an impaired top-down pain and fear response that is depending on TRP pathway in migraine patients. Notably, the connection between PAG and dmPFC is important in the process of contextual fear discrimination, which employs past experiences to assess perceived threats in the current situation (120). Consequently, this connection is pivotal in determining suitable defensive or exploratory behaviours. While previous studies independently demonstrated positive functional connectivity of PAG with dmPFC and dlPFC in healthy controls (40) and migraine patients (41), they showed a weaker connectivity between PAG and dmPFC/dlPFC in migraine patients compared to controls (38, 39, 121), thus this connection may contribute to impaired top-down control of pain and fear responses in migraine. Considering our results, the elevation in plasma TRP concentration may strengthen the connectivity between PAG and dmPFC/dlPFC, thereby optimizing cortical control over the fear-cascade in migraine patients.

Another pivotal brain structure of the defence cascade is the cerebellum, that also showed TRP dependent functional connectivity with PAG in migraine patients (122). Cerebellum is a key region in developing fear-evoked freezing behaviour (115) and has been more frequently associated with migraine pathophysiology as it shows high expression of migraine-related genes (122, 123). In addition to its crucial role in motor function, the cerebellum is involved in cognition, pain processing and encoding aversive stimuli such as unpleasant images (124). Previous studies have reported reciprocal connection between PAG and cerebellum, which circuit is important in fear response modulation (125, 126). Moreover, migraine patients exhibited decreased functional connectivity between PAG and the cerebellum during heat pain stimuli compared to healthy controls (39, 124). However, in migraineurs, PAG—cerebellum IV-VI connectivity showed a negative correlation, meanwhile PAG—cerebellum crus I and IX

displayed a positive correlation with migraine attack frequency during painful stimuli, showing that the subregions of the cerebellum are functionally different (39). Our study revealed an association between decreasing functional connectivity of PAG with cerebellum IV-VI and increased TRP concentration in migraine patients, suggesting a potential decrease in fear-cascade activation in migraineurs with increased plasma TRP concentration. However, cerebellar lobules IV-V are mainly involved in motor functions, but cerebellar lobule IV has a complex role in both motor and cognitive tasks (127).

Another region that showed TRP concentration dependent association with PAG connectivity in migraine patients is the middle occipital gyrus. This region is involved in multisensory processing, thus spatial localization of visual, tactile, and auditory stimuli occurs there (128). Previous studies reported negative functional connectivity between PAG and middle occipital gyrus in healthy individuals (33, 40) and increased middle occipital gyrus activity during mind-wandering away from pain (129). In addition, during viewing threatening images, it exhibited increased activation concurrently with defence cascade activation compared to less arousing stimuli (130). Previously, Solstrand et al. reported decreased pain-induced functional connectivity between PAG and middle occipital gyrus in migraineurs compared to controls, that showed negative correlation with migraine attack frequency (39). Despite that the exact role of PAG-middle occipital gyrus functional connectivity in the defence cascade is not fully understood, based on our results and previous findings, we could hypothesize that the decreased functional connectivity of PAG-middle occipital gyrus in association with increased plasma TRP concentration may be involved in decreased pain-related fear response in migraine patients.

5.3.3. TRP concentration modulates the correlation between PAG connectivity with trait-anxiety and depressive symptoms in migraine

Although a positive association was demonstrated between migraine indicators, namely migraine attack frequency and age at migraine onset, with functional connectivity of PAG and frontal gyrus, this association was not correlated with plasma TRP concentration. The functional connectivity between PAG and triangular part of the inferior frontal gyrus and middle frontal gyrus was previously implicated in migraine pathology (38, 39, 121). Our findings align with prior observations that dietary TRP manipulation to boost serotonin

synthesis in the brain is not sufficient to decrease migraine attack frequency in most patients (131, 132).

Conversely, the positive correlation between functional connectivity of PAG-dlPFC/dmPFC with trait-anxiety and current depressive symptoms showed association with plasma TRP (LNAA corrected) concentration. Previous animal study demonstrated the analgesic and anxiolytic effect of the excitatory descending pathway from dmPFC to PAG (133). Moreover, PAG plays a role in cognitive processes, it orchestrates negative emotions and pain-related autonomic and behavioural responses through its afferent and efferent projections with the prefrontal cortex (134). As mentioned above, the connectivity between PAG and dmPFC is pivotal in contextual fear discrimination, thus the impaired defensive behaviour could lead to anxiety disorders (115, 120). Previously, the hypoactivity of dlPFC was showed in depressed patients, that normalized after treatment (135). Interestingly, TRP depletion has no mood-lowering effect in never-depressed healthy individuals, however, predisposing factors such as a high familial risk for depression or remitted depression make people susceptible to depressive symptoms in case of decreased TRP concentration (67). Similarly to previous observations, our study showed no significant association between TRP concentration and depressive symptoms or trait-anxiety level, nor PAG functional connectivity in healthy controls. Nonetheless, TRP and its metabolites could play modulating role in the functional coupling of PAG with dmPFC/dlPFC in migraine patients. The increased TRP concentration may be beneficial in migraine patients, by decreasing pain-induced negative affect and improving emotion processing through enhancing PAG-PFC connectivity (120). It is essential to emphasize that our patients were free from any psychiatric disorders, and the depressive symptom and trait-anxiety scores were in normal range, without any significant difference compared to healthy controls.

Finally, additional negative correlation was found between depressive symptoms and PAG connectivity with fusiform gyrus and right parahippocampal gyrus after correcting for plasma TRP concentration. Both regions are involved in fear processing circuits that show higher activation in depressed patients with migraine compared to non-depressed migraine patients (136). Thus, this result aligns with our suggestion that TRP pathways might interfere with fear processing by influencing PAG functional connectivity, potentially decreasing the activity of the fear-cascade in migraine patients (117).

6. Limitations

One of the main limitations of our study is the relatively low sample size. However, our population was screened by expert neurologists, so migraine patients consisted of a homogenous episodic migraine without aura population, free from any comorbid disorders or medication intake. In addition, the randomized, double-blind crossover design of the citalopram challenge is a strength of our study. Another methodological limitation is that plasma total TRP concentration was detected and free to bound TRP ratio was not investigated. At the same time, we emphasized that the TRP/LNAA ratio is a better marker for TRP brain influx, thus we focused on controlling for LNAA concentration in all analysis. Unfortunately, the expression and activity of KYN metabolising enzymes and metabolites were not assessed in this study. Finally, the determination of bilateral PAG as seed region in fMRI analysis was based on literature data. The intrinsic functional connectivity of the seed was in line with previous results and in our complex preprocessing pipeline we tried to manage all fMRI related challenges.

7. Synthesis of our hypotheses

We provided further evidence for the hypothesis of migraine as allostatic load disorder. In addition, our study provided specific mechanisms that could possibly be involved in interictal maladaptive stress response, thus be useful to identify novel drug targets influencing the kynurenine pathway (**Figure 7**.)(24, 28). First, we identified an interictal hyperexcitability of pain pathways in migraine patients compared to healthy controls by demonstrating increased PAG connectivity within pain-matrix of migraine patients. Second, we proved that the downregulated kynurenine pathway plays a role in maladaptive stress response of migraine patients by identifying that migraine patients failed to activate TRP pathway during stress challenge, and thus this mechanism is possibly involved in recurrent migraine attacks. Third, the TRP pathway alterations are associated with the dampened connectivity of fear-cascade regions that are responsible to the control of the “fight or flight” response. The “fight or flight” response is suggested to contribute to the behavioural meaning of pain in migraine thus our results further support the involvement of TRP pathway in interictal maladaptive stress response of migraineurs. (29). Finally, trait-anxiety and depressive symptoms are contributory risk factors and

emotional symptoms of migraine attacks that showed association with TRP modulated PAG-PFC connectivity suggesting a TRP dependent alteration in top-down control of pain and emotions in interictal period of migraine.

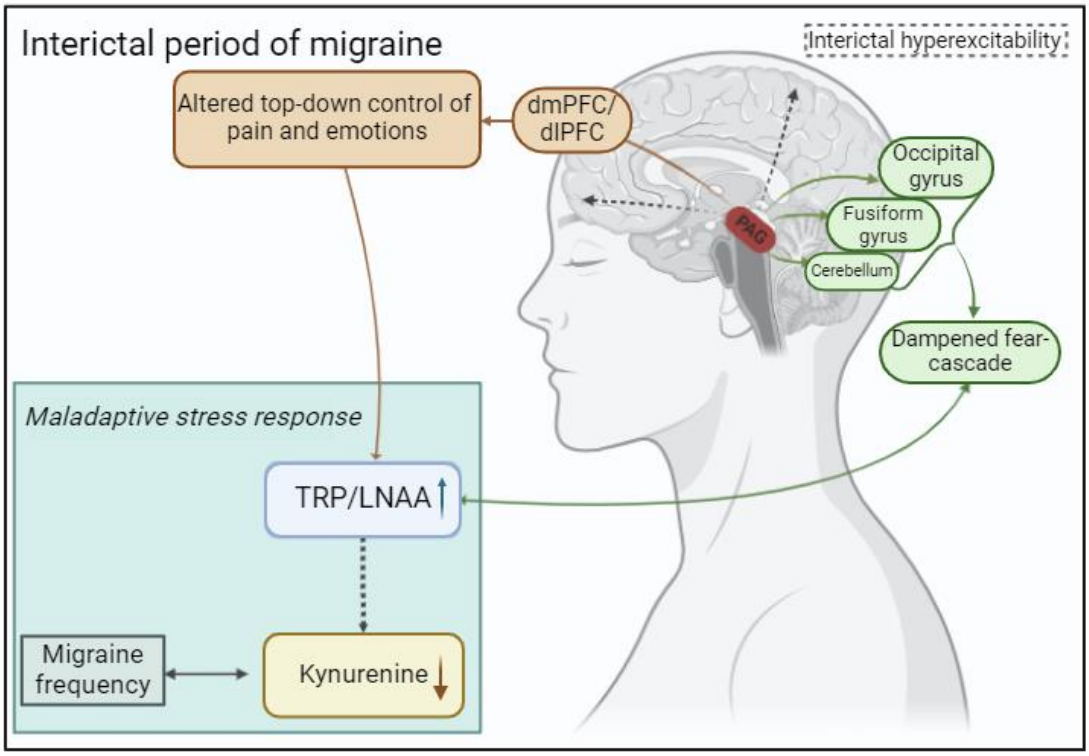

Figure 7. *The visual summary of the results: association between TRP pathway and allostatic load in interictal period of episodic migraine.*

Note: Migraine patients have maladaptive stress response even in interictal period of migraine. The decreased breakdown of TRP via kynurenine pathway interferes with migraine attack frequency. Broken line: Increased connectivity within the pain-matrix in interictal migraine patients compared to healthy controls. Green line: The dampened fear-cascade network is associated with TRP concentration in migraine patients. Brown line: The connectivity between PFC and PAG showed association with TRP concentration and depression symptoms and trait-anxiety in migraine patients. Underlying the role of TRP pathway in altered top-down pain and emotions control of migraine patients. PAG: periaqueductal gray matter, PFC: prefrontal cortex, TRP: Tryptophan, TRP/LNAA: Tryptophan/large neutral amino acids. The figure was created in BioRender.com.

8. Conclusions

Considering the results of our analysis, 1) we demonstrated an increased TRP/LNAA ratio in migraine patients for the first time. Our study provided further evidence for downregulated KYN pathway that showed association with migraine frequency. 2) The maladaptive stress response of migraine patients was observed during acute citalopram challenge that affected the TRP and KYN metabolism. Furthermore, migraine frequency was associated with KYN pathway responsivity during acute stress highlighting its importance in maladaptive stress response. 3) We also demonstrated that TRP pathway plays a significant role in migraine brain hypersensitivity and in the interictal dampened fear-cascade. The correlation between TRP concentration and PAG-PFC connectivity showed association with emotional symptoms of migraine, but not with migraine attack generation. These mechanisms may interfere with stress coping strategies of migraine brain and support the hypothesis considering migraine as an allostatic load disorder (**Figure 7.**).

Given these points, the KYN pathway could serve as possible target for influencing hyperexcitability in migraine, but further studies are needed to investigate their relevance in migraine treatment or prophylaxis. Promising results of Phase II. clinical trials with glutamate receptor (mGLUR5) modulators (e.g.: raseglurant), AMPA/kainate receptor antagonist (e.g.: tezampanel) and real-world data with NMDA receptor antagonists (e.g.: ketamine) might uncover future perspectives for individual migraine treatment.

9. Summary

Migraine has been one of the most debilitating diseases in the world since thousands of years. Several hypotheses have emerged regarding its pathophysiology, involving the role of tryptophan (TRP) metabolic pathway. The literature presents conflicting results concerning the blood levels of TRP in migraineurs, reporting both decreased and increased concentrations compared to healthy controls. In our study, we found elevated TRP concentration and TRP/large neutral amino acids (LNAA) ratio in the plasma of episodic migraine patients without aura in two independent samples. The TRP/LNAA ratio is a reliable marker of TRP uptake into the brain, which our study investigated first in migraine. A decreasing kynurenine (KYN)/TRP ratio with increasing migraine frequency suggested a reduced activity of the kynurenine pathway.

In the past decades, the perspective has emerged considering migraine attacks as the consequence of maladaptive stress response of the central nervous system. Recurrent attacks further worsen the adaptive changes to stress. In our study, we demonstrated that TRP pathway of migraine patients failed to adapt during citalopram challenge, an acute neuroendocrine stress, while healthy controls showed physiological adaptive changes. The frequency of migraine attacks was associated with citalopram infusion induced KYN concentration changes, thus confirming the role of the KYN pathway in the maladaptive stress response and the development of recurrent migraine attacks.

Alterations in the resting-state network of the periaqueductal gray matter contribute to the increased sensitivity of the interictal migraine brain to environmental stimuli. In our study, we found that plasma TRP concentration (corrected for LNAA) is associated with functional connectivity between PAG and fear-cascade regions. Furthermore, TRP concentration affected the functional connectivity of the PAG with the prefrontal cortex, which connection was associated with depressive symptoms and trait-anxiety. These psychological traits are often co-occurring with migraine.

Further studies are needed to determine whether influencing the TRP-KYN pathway could prevent migraine attacks. In ongoing clinical trials, receptors under the influence of KYN metabolites are emerging as promising drug targets for both migraine attack treatment and prophylaxis.

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