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THE ROLE OF CIRCADIAN RHYTHM IN MIGRAINE

PhD thesis

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List of abbreviations

$5HT_{1F}$: 5-hydroxytryptamine (serotonin) 1F receptor

5HTTLPR: serotonin transporter-linked promoter region

A1: minor (and effect) allele

ADD: additive genetic model

aMT6s: 6-sulphatoxymelatonin

BD: bipolar disorder

BDNF: brain-derived neurotrophic factor

BMAL1: brain and muscle ARNT-like 1 gene

BOLD: blood oxygen level dependent

BP: Budapest subsample

CGRP: calcitonin gene-related polypeptide

CHA: childhood adversity

CK1δ: casein kinase Iδ gene

CLOCK: circadian locomotor output cycles kaput gene

CNR1: cannabinoid receptor 1 gene

COMT: catechol-O-methyltransferase gene

CRY: Cryptochrome gene family

CSD: cortical spreading depression

CTQ: Childhood Trauma Questionnaire

DALYs: disability-adjusted life years

DOM: dominant genetic model

DRD4: dopamine receptor D4 gene

EEG: electroencephalography

FASPS: familial advanced sleep phase syndrome

FC: functional connectivity

FHM: familial hemiplegic migraine

FINANC: financial hardship

FWE: family wise error rate

fMRI: functional magnetic resonance imaging

GABA: gamma-aminobutyric acid

GWAS: genome-wide association study

GxE: gene-environment interaction

ICHD-3: The International Classification of Headache Disorders, 3rd edition

IL1β: interleukin-1 β gene

IPL: inferior parietal lobule

L95: lower endpoint of the 95% confidence interval

LD: linkage disequilibrium

LR+: positive likelihood ratio

MA: migraine with aura

MAN: Manchester subsample

MAF: minor allele frequency

MAOA: monoamine oxidase A gene

MCC: middle cingulate cortex

M_{circ} subgroups: subsamples with different typical circadian attack onset peaks

MDD: major depressive disorder

MigraineID: positive for migraine according to the ID-Migraine questionnaire

miRNA: microRNA

MNI space: Montreal Neurological Institute space

MO: migraine without aura

mRNA: messenger RNA

MTHFR: methylenetetrahydrofolate reductase gene

NO: nitric oxide

NOS1: nitric oxide synthase 1 gene

OECD: Organisation for Economic Co-operation and Development

OR: odds ratio

PCC: posterior cingulate cortex

PCL: paracentral lobule

PER: Period gene family

PER2: Period circadian protein homolog 2

PET: positron emission tomography

REC: recessive genetic model

REM: rapid eye movement

RLE: recent negative life events

RORs: retinoic acid receptor-related orphan receptors

SCN: suprachiasmatic nucleus

SD: standard deviation

SEM: standard error of mean

SLC6A4: serotonin transporter gene

SMA: supplementary motor area

SMG: supramarginal gyrus

SNP: single nucleotide polymorphism

STG: superior temporal gyrus

U95: upper endpoint of the 95% confidence interval

VNTR: variable number of tandem repeats

YLDs: years lived with disability

1. Introduction

Migraine affects more than 1 billion people worldwide (1, 2). It is a serious neurological disorder consistently representing the second strongest nonfatal burden captured by age-standardized years lived with disability (YLDs) (2, 3). The recurrent, 4-72 hours long headache attacks are typically characterized by unilateral location, moderate or severe pain intensity, pulsating pain quality, aggravation by routine physical activity and also accompanying nausea or vomiting and/or photo- and phonophobia – as defined by The International Classification of Headache Disorders, 3rd edition (ICHD-3) (4). Although, migraine attacks represent only a section of a cyclic, multiphasic disorder with three main phases which typically follows a timely order: 1) the prodrome – preceding headache attacks by up to 48-72 hours with symptoms including tiredness, yawning, irritableness, reduced attention, sensitivity to light, stiffness of the neck, changes in mood, activation, hunger and sleep-wake rhythms; 2) the headache attack; and 3) the postdrome – lasting for 24-48 hours after the attack with symptoms reflecting the prodromal ones (5-7). The two major subtypes of migraine are episodic migraine without aura (MO) and episodic migraine with aura (MA) – in case of the latter one, attacks are preceded and/or accompanied by transient and fully reversible neurological deficits, typically visual and other sensory or central nervous system symptoms, collectively named migraine aura (4, 5). MO is 3-4 times more frequent than MA (5, 8). Women are three times more affected by migraine than men (8). In both sexes, global migraine prevalency peaks in their thirties (2, 8) which significantly overlaps with the peak years of productivity (5). Indeed, besides significant personal burden migraine also causes high economic costs for migraineurs themselves and the society in direct (e.g. healthcare resource utilization, medication use) and indirect (e.g. decreased productivity, work/school absenteeism) forms (9-11). A recent study in Sweden identified a total annual cost of migraine per patient between €5000-24000 and about 80% of this cost represented indirect costs (12). So far, in Hungary no such an estimation of migraine cost was calculated but headache disorders were included in the top 10 causes of death and disability measured by age-standardized disability-adjusted life years (DALYs) according to the Institute for Health Metrics and Evaluation (13) based on data from the Global Burden of Disease Study 2019 (3).

All these data justify the extended research on migraine pathophysiology which is far from understood and may contain elements that are often overlooked including circadian factors. As we will see, circadian rhythm-related associations of migraine were demonstrated from the fields of genetic and functional brain imaging studies, and further indirect chronobiologic links (e.g. with melatonin level, chronotype, sleeping problems) were also found. The main focus of this thesis is to show our results indicating a role for circadian rhythm-related phenomena in migraine. But first, we discuss what we already know about migraine pathophysiology to reveal the gaps in the literature which lead us to our investigations.

1.1 Pathophysiology of migraine

After a centuries-long debate on migraine pathophysiology which centered on neural versus vascular sources of migraine attack generation, nowadays migraine is frequently described as a neurovascular disease and we can conclude that migraine is definitely a disorder of the brain, at its core (5). Involvement of the trigeminovascular system in migraine is widely accepted (5, 14) – it can be considered as the anatomical and physiological basis where nociceptive transmission originates and generates migraine pain perception (15). To explain the primary dysregulation of sensory processing among migraineurs, migraine is often characterized as a brain state of altered excitability (5). Migraine attack initation involves dysfunction of brain stem and diencephalic nuclei (15). The non-nociceptive nature of premonitory neurological symptoms also suggests an origin in the brain (5). A strong genetic component in migraine is also obvious (16). Furthermore, certain environmental factors, especially stress are known migraine triggers (17, 18).

As we can see, migraine is a multifactorial disease. A detailed elaboration on migraine pathophysiology is beyond the scope of this work. To capture the potential role of circadian rhythm in migraine which lead us to our studies, here we would like to focus on two main aspects related to migraine pathophysiology: 1) genetics and 2) functional brain mechanisms.

1.1.1 Genetic factors in migraine

Heritability of common migraine is between about 30 and 60% according to family and twin studies (16, 19, 20). Familial aggregation was found to be more profound in case of early onset, higher pain severity and MA subtype (21-23). Genetic heterogeneity of common migraine seems to be more and more obvious and this was clearly demonstrated by both candidate gene and genome-wide association studies (GWASs). To mention a few hypothesis-driven candidate gene association study results, polymorphisms in serotonin- (24-34), dopamine- (35-44), potassium channel- (45-47), gamma-aminobutyric acid (GABA)- (48) and brain-derived neurotrophic factor (BDNF)-related genes (49, 50), furthermore catechol-O-methyltransferase (*COMT*) (51-53) and the cannabinoid receptor 1 gene (*CNR1*) (54) among many other genetic variants associated with migraine phenotypes. In 2016, Kondratieva et al. (55) reviewed genetic biomarkers of migraine and concluded that the identified genes based on their functions can be classified into eight major categories. The two largest categories were: 1) homeostasis of blood vessels and 2) transport and reception of neurotransmitters. Other categories represented, in order of magnitude: metabolism of neurotransmitters, ion channels and membrane potential, inflammation, „other", neurogenesis and sex hormones.

GWASs in the last decade identified a large number of genetic variants with small effect sizes to associate with migraine which unambiguously demonstrates the polygenic nature of the disease (56-62). Here, we would like to point out the outcomes of the three most recent GWAS metaanalyses. Gormley et al. (60) identified 38 loci for migraine and concluded that migraine-associated genes are mostly involved in vascular and smooth muscle functions. Although, they still highlighted the possible role of neurogenic mechanisms in migraine pathophysiology since several of the identified genes were active in brain tissues. Other genes, in much lesser extent, represented functions related to ion channels and ion homeostasis – supporting the previous notion that ion channel dysfunction is not the major mechanism in pathophysiology of common migraine (unlike to familial hemiplegic migraine (FHM), a rare, severe MA subtype with accompanying fully reversible motor weakness symptoms (4)). Some further genes were related to oxidative stress and nitric oxide (NO) signaling approving the NO hypothesis of migraine which postulates NO as a causative molecule in migraine pain (63, 64).

Another recent GWAS metaanalysis by Choquet et al. (61) used a multiethnic sample and reported 79 loci for migraine of which 45 were previously unidentified. The results validated 78% of the loci found by Gormley et al. (60) and for the first time, some novel female-specific loci were identified which were previously related to other diseases and conditions including psychiatric disorders (supporting known comorbidities of migraine (65-68)), metabolic deficiencies and Alzheimer's disease. Novel loci pointed to some additional functions related to early development, proinflammatory mechanisms, calcitonin gene-related polypeptide (CGRP), a recent migraine-specific drug target (69) and two conditions that were previously connected to migraine: motion sickness (70) and patent foramen ovale (71).

Finally, the most recent GWAS metaanalysis (62) identified 123 risk loci of migraine including some MO- and MA-specific variants. According to the authors, 86 novel loci were discovered – however, they did not consider the results of Choquet et al. (61). Nevertheless, they highlighted that the new risk loci contain target genes of recent migraine-specific drugs acting on CGRP pathway (similarily to Choquet et al. (61)) and 5-hydroxytryptamine 1F receptor ($5HT_{1F}$). Regarding subtype-specific variants, the authors emphasized that migraine risk is clearly provided both by MO- or MA-specific variants and also by loci that are shared among the two subtypes. Finally, the authors concluded that their results supported the involvement of both neuronal and vascular genetic variants in migraine – strenghtening the neurovascular nature of the disease.

Some general conclusions can be obtained from these genetic studies. With the accumulation of more and more data, the number of migraine-associated loci constantly growed – clearly demonstrating the highly polygenic nature of migraine (62). Functional analyses supported that migraine can be described as a neurovascular disease. The identification of target genes of recent migraine-specific drugs is also promising and raises the possibility of other potentional drug targets among recently found loci (62). Furthermore, the latest GWAS metaanalyses (61, 62) were even able to capture migraine heterogeneity by identifying variants specific to migraine subtypes and females. However, besides these advantages of the GWAS approach, its main technical limitations also need to be taken into account. First, it is unable to identify rare genetic variants. Second, the addition of environmental factors to capture gene-environment interactions (GxE) is missing from GWASs. Third, samples of the mentioned GWAS metaanalyses (60-62) highly overlap. Based on all these drawbacks, conclusions of GWAS findings regarding pathophysiological mechanisms of migraine should be interpreted with caution and independent case-control studies are needed to confirm the identified associations (5).

So far, no circadian genes were detected in population genetic studies. However, in two families showing familial migraine and also familial advanced sleep phase syndrome (FASPS) (a circadian rhythm sleep-wake disorder with an unusually early bedtime and awakening time), a mutation in the casein kinase Iδ gene (*CK1δ*) cosegregated with both migraine and FASPS (72). Further results with mice carrying the CK1δ mutation showed an increased sensitivity to pain induced by nitroglycerin, a migraine trigger and also a decreased threshold for cortical spreading depression (CSD), the believed physiological correlate of migraine aura (73) and a greater arterial dilation during CSD (72). Additionally, astrocytes of the mutation carrying mice showed increased calcium signaling which appears in conjunction with CSD (72). CK1δ, a ubiquitous serine-threonine kinase plays an important regulatory role in the circadian clock by phosphorylating Period circadian protein homolog 2 (PER2) (72, 74). Cell-autonomous clocks are ubiquitous but at the top of a hierarchical system, there is a central circadian pacemaker, the suprachiasmatic nucleus (SCN) which is located in the hypothalamus and its 24-h time-keeping capacity originates from a complex transcriptional / posttranslational negative feedback loop with rhythmically expressed clock genes (75-77). Among the core clock genes, circadian locomotor output cycles kaput (*CLOCK*) and brain and muscle ARNT-like 1 (*BMAL1*) encode transcriptional activators, while the Period (*PER*) and Cryptochrome (*CRY*) gene families encode repressors (75). Briefly, the transcription of *PER* and *CRY* genes is activated by an E-box-binding basic helix-loop-helix transcription factor containing the CLOCK-BMAL1 complex, while the resulting PER and CRY proteins dimerize and repress the transactivation of the CLOCK-BMAL1 complex (76). Besides this negative feedback loop, PER and CRY proteins are also repressed by the interaction with casein kinases which express phosphorylating effects (75-77). Furthermore, rhythmic expression of BMAL1 is influenced by nuclear receptors REV-ERBα/β and retinoic acid receptor-related orphan receptors (RORs) (75, 77, 78). The various combinations of these components produce different phases of transcriptional rhythms (75). In light of the above, the association between *CK1δ* and migraine suggests that other elements in the circadian clock, including clock genes might also play a role in migraine.

Although, even in the investigated two families some members had migraine without carrying a *CK1δ* mutation suggesting different predispositions to the disease (72)

– and again, demonstrating the heterogeneic nature of migraine. This heterogeneity could be also captured by GxE studies since the inclusion of environmental factors can reveal further pathomechanisms – for instance, by unfolding epigenetic mechanisms.

Environmental factors clearly play a role in migraine, especially stress. Sauro & Becker (79) highlighted the various roles of stress in migraine. Migraineurs report stress as the primary triggering factor of their attacks. Stress can promote amplification of migraine attack intensity, duration and frequency – and these effects probably contribute to migraine chronification. A recent cross-country study showed that external stress factors (e.g. work- and insecurity-related stressors) associate with migraine-related YLD in both developed and developing countries (80). Further non-specific stress factors of various psychiatric and somatic disorders were also connected to migraine, including childhood maltreatment (81), recent negative life events (82) and socioeconomic status (83). Stress-related psychiatric disorders, mostly anxiety and mood disorders show high comorbidity with migraine (65, 66, 84) – and stress may have a direct effect on these associations: for example, a prospective cohort study found that the bidirectional relation between migraine and depression vanishes after adjusting for the effects of stress factors (85). Although, this latter study measured migraine only with a self-reported question which limits the generalizability of its conclusions, but the possibility that stress, especially chronic stress might explain much of the association between migraine and depression highlights that frequent stress experienced by migraineurs may contribute to comorbidities with other stress-related disorders such as major depression. Another study also suggested that stress can cause relapse and even make previously effective pharmacological migraine treatment ineffective (86). These examples illustrate that stress is involved in migraine in many ways, however a novel review concluded that the exact causal relationship between stress and migraine is unclear but still, many migraine patients can benefit from stress-oriented therapies (87). A useful theory explaines the strong stress-migraine connection through the concept of maladaptive stress response: among migraineurs, recurring stress can lead to allostatic load resulting in changes in normal homeostatic processes, deficiency in habituation and shutting down the stress response (88).

Thus, it seems to be plausible to test GxE effects (especially using stress factors) in migraine to explore novel candidates and pathways in its pathophysiology. Similarly to migraine, depression represents a frequent, highly polygenic and stress-related disorder and multiple GxE effects were found in association with depression phenotypes (89, 90) – the most known example is the interaction between the short allele of the 5HTTLPR polymorphism of the serotonin transporter gene (*SLC6A4*) and childhood maltreatment and negative life events (89). But interestingly, in case of migraine GxE studies are notably missing. In 2012, Ishii et al. (91) highlighted that most studies focused on a single contributing factor despite the multifactorial nature of migraine. Some studies showed that different genetic variants (e.g. in *SLC6A4,* monoamine oxidase A (*MAOA*) and methylenetetrahydrofolate reductase (*MTHFR*)) and personality traits (e.g. harm-avoidance, neuroticism, conscientiousness) independently contributed to migraine (91, 92) – although these studies not tested directly interaction effects. A twin study also showed that the association between migraine and neuroticism could be partly explained by the same genetic and environmental factors (93). During the last decade, some authors highlighted epigenetic mechanisms and GxE interactions as a promising avenue in migraine research (81, 94-96) but since then – according to our knowledge – surprisingly only one study addressed this topic directly by testing GxE effects on migraine: Juhasz et al. (82) found that single nucleotide polymorphisms (SNPs) in *CNR1* showed no main effect on migraine, but a *CNR1* variant interacted with recent negative life events to affect headache with nausea (a migraine-like phenotype). This study sets a good example: although no genes within the endocannabinoid system were found in previous GWASs, considering the complex associations between migraine, negative life events, the endocannabinoid system, and specifically *CNR1*, and also other phenotypes (neuroticism, depression), the authors were able to detect a GxE effect on migraine by selecting relevant tag SNPs of *CNR1* and using recent negative life events as environmental factors.

In our first study, a similar strategy was used to capture a GxE effect on migraine by using a circadian gene variant which will be discussed in details later on. Before that, we highlight the most important previous results regarding functional brain mechanisms, another important component to understand migraine which directed us to our second study.

1.1.2 Functional brain mechanisms related to migraine

As we already stated, migraine can be considered as a brain disorder, at its core (5). So far, no clear-cut neuroimaging biomarkers were found for migraine (97-99) but the „migraine brain" consistently shows structural and functional changes even in headache-free states compared to healthy controls (5, 99). Migraine-related changes in the brain are present at multiple levels: cerebral, cerebellar and brainstem structures show altered morphology, furthermore alterations of functional and structural connectivity and function are also typical (99). The most known structural changes in migraine include white matter abnormalities, silent infarct-like lesions and volumetric changes in white and gray matter regions (99, 100). A recent narrative review (101) suggests that these structural abberations are involved in regions of pain and sensory processing (e.g. somatosensory cortex, middle frontal gyrus, fusiform gyrus, postcentral gyrus), and some structural alterations might reflect an inherent trait of the migraine brain, while others might be a consequence of recurrent migraine attacks. The latter type represents plastic changes that may underlie disease progression (5) but also raises the promising possibility of reversibility with effective therapy (101).

Here, we would like to focus on results of functional brain imaging studies which complement structural imaging (5). Functional magnetic resonance imaging (fMRI) studies include resting state studies where functional neuronal connections (i.e. networks) can be measured without any external stimuli based on coupled spontaneous fluctuations in the blood oxygen level dependent (BOLD) signal and task-based studies where brain activity is captured during a task performance (99, 102). In 2019, Skorobogatykh et al. (98) systematically reviewed resting state functional connectivity (FC) studies of migraine. More than 20 altered FC networks were detected in case of studies of interictal migraine compared to headache-free controls. Only a few FC studies compared the ictal state to interictal state and altered FC during migraine attack was found in the following networks and regions: salience, somatosensory and default mode networks, furthermore left pons and right thalamus. The authors identified a low level of reproductibility and highlighted that the reported FC alterations may not be specific to migraine since highly similar FC changes were detected in many other conditions. They also emphasized the heterogeneous nature of migraine as a potentional cause of high variability in the results (98) – this might be supported by a study showing associations between FC changes and migraine severity variables as headache frequency and allodynia symptoms (103) and also by a study capturing differences in FC according to migraine subtypes (MO versus MA) (104).

Since migraineurs show hypersensitivity to sensory (including visual, auditory and olfactory) and somatosensory stimuli during migraine attacks and although, to a lesser extent, but often even interictally (105, 106), task-based fMRI studies give the opportunity to measure brain activity in situations where migraineurs are exposed to typical trigger factors (106, 107) of their attacks. This type of sensory hypersensitivity, which can function as a trigger and is present ictally and also interictally, is specific to migraine among other headache and pain disorders (105). So, it is not suprising that task-based fMRI studies consequently show an altered cerebral response to sensory stimuli among migraineurs in interictal state compared to healthy controls (105). Several brain areas have been found to contribute to altered sensory processing in migraine. Studies using painful stimuli (e.g. heat or ammonia) identified many pain processing regions including for example the thalamus, middle cingulate cortex, anterior cingulate cortex, amygdala, pre- and postcentral gyri, cerebellum, supramarginal gyrus, middle temporal gyrus, temporal pole, fusiform gyrus, hippocampus, and dorsolateral cortex among many others (105, 108). These areas are involved in affective, cognitive and modulatory aspects of pain processing and the results suggest an inadequate facilitation and inhibition of pain signals and also a decreased habituation (105, 108).

Several task-based fMRI studies demonstrated the hyperresponsivity to visual stimuli among migraineurs by showing increased activation mostly in the visual cortices (105) but also in the middle temporal complex (109), furthermore red nucleus, substantia nigra and occipital cortex among MA patients (110). A study with olfactory stimuli detected elevated activation during spontaneous migraine attacks in limbic regions including the amygdala, insular cortices, and specifically rostral pons (111) – showing evidence for a strong physiologic association between the olfactory and trigemino-nociceptive pathways in migraine (108, 111). These studies with visual or olfactory stimuli show the importance of brainstem structures in the early phases of and during migraine attack initiation – suggesting a pathway for migraine attack generation by external stimuli (105).

Besides pain and sensory processing, emotional factors also play an important role in migraine. Emotional stress is often reported as a trigger for migraine attacks (112), for migraineurs elevated emotionality as a premonitory symptom is one of the best predictors for a forthcoming headache attack (6), neuroticism (emotional lability) associates with migraine risk (93, 113, 114) and among childhood maltreatment types, emotional abuse showed the strongest association with migraine (even after adjusting for the effects of anxiety and depression) (115). In consort with these results, fMRI studies using emotional stimuli certified altered neural processing of emotions, especially negative emotions among migraineurs in interictal phase compared to headache-free controls in regions including for instance the posterior cingulate cortex, cerebellum, superior frontal gyrus, middle frontal gyrus, lingual gyrus, precuneus, cuneus, amygdala and brainstem (116-118). Here, we would like to also highlight that it has been suggested that emotional processing, including emotional face perception may be affected by circadian rhythm (119) – for example, previous findings showed that sleeping problems impair facial emotion recognition among adults and adolescents (120) and late chronotype associated with negative biases in emotional face processing (e.g. an increased sensitivity to sad faces) (121). It might be a reasonable research topic to address whether the altered neural processing of emotions among migraine patients is mediated by circadian rhythm-related factors.

Among the identified brain areas, hypothalamus may represent a more direct circadian rhythm-related association with migraine. Recently hypothalamus is getting more and more attention because of its various roles in migraine pathophisiology. The hypothalamus is a small area which is located under the thalamus and above the adenohypophysis and serves as a central homeostatic regulator of various physiological processes including body temperature, sleep, circadian rhythms, food and liquid intake, furthermore it regulates the autonomic nervous system (122, 123). Through its direct contact with the vascular system, the hypothalamus also provides a brain-hormonal interface through which a top-down control of peripheral body functions can be achieved (123). Because its responsiveness to circulating metabolites and hormones, the hypothalamus is particularly positioned to go through plastic changes by replying to long-term environmental changes (122). Hypothalamic nuclei are also densely interconnected with the trigeminal pain processing and modulating systems (123, 124). Hypothalamic neurotransmitters include the orexins which have a control over arousal, nociception, thermoregulation and autonomic functions and became a potential target in migraine research and therapy (123, 125, 126). The central dopaminergic system also involves the hypothalamus and has a role in migraine-related phenomena including yawning, fatigue, nausea, craving and appetite changes (123). Some clinical features of migraine, especially the cyclic nature of attacks, premonitory and postdromal symptoms (e.g. changes in mood, activity and appetite, yawning, fatigue), autonomous symptoms before and/or during the attack (e.g. nausea and/or vomiting) and the effects of sex hormones and the menstrual cycle (127) on migraine attacks suggest a direct hypothalamic involvement in migraine pathogenesis (123, 128).

In 2007, a positron emission tomography (PET) study was the first one to detect increased cerebral blood flow in the hypothalamus during spontaneous migraine attacks (129). In accordance with previous concepts, in fMRI studies the premonitory phase has been associated with increased hypothalamic activation both in case of spontaneous and nitroglycerin-induced attacks (130, 131). A high-resolution fMRI study comparing episodic and chronic migraineurs with healthy controls (132) identified various functions for the hypothalamus in migraine: 1) migraine attack initiation, 2) elevated activation during acute pain and 3) a possible role in migraine chronification. Novel longitudinal fMRI studies gave further support for a hypothalamic dysfunction in migraine. In a study with nociceptive, olfactory and visual stimulation, 7 migraine patients were scanned on a daily basis for a period of 30 days and increased hypothalamic activity appeared up to two days before attack onset but not during the attack or the postdrome (124). The same research group measured resting state activity of 8 patients similarly on a daily basis for a period of 30 days and found an increased FC between hypothalamus and dorsal rostral pons in the ictal phase compared to the interictal phase (133). Another study (128) covered the brain activity changes of 12 migraine patients throughout a whole migraine cycle (i.e. from a spontaneous migraine attack to the subsequent attack). The authors showed that the FC of hypothalamus with insula and nucleus accumbens increased during the interictal phase, reaching its peak shortly before the attack onset, while during the attack the FC was low – proposing a hypothalamo-limbic FC collapse, ictally. Similar patterns were found for hypothalamic FCs with basal ganglia and cerebellar regions.

So, the hypothalamus represents a region that is clearly involved in both migraine and the circadian clock – and potientally, it can serve as a bridge between migraine and circadian rhythm-related phenomena. Interestingly, fMRI studies consistently found activation within the lateral anterior part of the hypothalamus (124, 130-132) and the anterior hypothalamus contains the SCN, the main circadian oscillator which has been hypothesized to play an important role in migraine attack initiation a long time ago (5, 134).

So far, we have introduced what motivated us to approach the association between migraine and the circadian clock from a genetic, more specifically a gene-environment interactional standpoint and from the field of functional neuroimaging. In the followings, we also present some other factors indicating associations between migraine and circadian rhythm-related mechanisms which we need to consider before discussing our research goals.

1.2 The potential role of circadian rhythm in migraine

Circadian rhythms represent a form of predictive homeostasis which helps the organism to adapt to around 24 hours long periodic environmental changes arising from the rotation of the Earth including for example the light-dark cycle and shifts between high and low temperature. This strong synchronization between the organism and the environment is based on a complex hierarchical biological system in which the SCN constitutes the main circadian pacemaker – as we previously discussed. Virtually all cells are able to express the clock genes and to generate circadian oscillations and the SCN synchronizes or entrains these peripheral clocks (75). Since these cell-autonomous clocks are ubiquitously distributed throughout the body (75), it is not suprising that many of our physiological and behavioral functions show circadian rhythmicity including the sleep-wake cycle, locomotor activity, feeding, glucose and hormon production, fat storage, body temperature, cardiac, pulmonary and nervous system activity, attention, memory and executive functions among others (77, 135, 136). The importance of circadian rhythms in health is obvious and circadian disruption can lead to various adverse outcomes such as premature aging and associates with several disorders including obesity, dementia, cardiovascular disease and further neurologic, psychiatric and immune disorders (77, 137-143). Furthermore, considering the complex interconnection between circadian rhythms and stress throughout the strong circadian inputs to stress regulatory systems including the hypothalamus-pitiutary-adrenal axis and autonomic nervous system (144), it is plausible to propose a role for the circadian system in stress-related disorders (145) such as migraine. Based on this rationale (and the previously detailed aspects of GxE studies), it might be also meaningful to test clock gene variants in interaction with stress factors in association with such diseases.

Some clinicians and also migraine patients have indicated a diurnal variation in the appearence of migraine attacks using different terms including cyclical, weekend, morning or nocturnal migraine suggesting that the attacks do not start randomly but may follow distinct temporal patterns (146, 147). Authors generally suggest that a morning or dawn start of attacks is the most frequent one but two recent reviews (146, 148) show a more mixed picture: indeed, an attack onset peak early in the morning or late at night is found in most studies, but others also showed an afternoon peak or even two peaks during the day, furthermore a study concluded that most migraine patients do not report a constant circadian attack onset peak. These inconsistent results suggest that migraine patients may show heterogeneity even in diurnal attack onset peaks. Environmental factors may also have an impact: morning migraines may associate with sleeping problems, while attacks in the afternoon may correlate with stress linked to school or work (147). Furthermore, the diversity in diurnal attack onset distribution might also suggest a role for biological factors including genetics and altered functional brain activity or an interaction between external and endogenous factors – however, the investigation of such mechanisms in the background of circadian variation of migraine attack onset is lacking.

Chronotype may also contribute to specific circadian attack onset peaks among migraineurs: early chronotype associated with an earlier peak, whereas late chronotype with a later peak (149). A recent study (150) detected that migraineurs with a specific diurnal attack onset peak were more likely to show an early chronotype in comparison with migraineurs without a specific onset peak, furthermore late chronotype associated with later onset peak and higher attack frequency – and these results appeared uniquely among migraine patients, but not tension-type headache patients. Others also showed an association between late chronotype and higher migraine attack frequency, additionally migraineurs with late chronotype searched for medical help at an earlier time (i.e. reported a shorter disease duration at migraine diagnosis) (151). Further self-reported data indicated that migraine patients are more susceptible to have an extreme (either an early or late) chronotype, and showed lower flexibility in adjusting to circadian rhythm-related changes (e.g. higher difficulty to cope with changes in sleep-wake pattern) compared to headache-free controls (149). Explaining the results regarding circadian variation of migraine attack onset and the associations with chronotype, some authors highlighted a possible hypothalamic dysfunction (152) or a potentionally different setting of the circadian clock among migraineurs (149).

Interestingly, a novel study showed that bipolar disorder (BD) patients with a lifetime history of comorbid migraine reported a higher ratio of evening chronotype compared to BD patients without migraine, furthermore the evening chronotype was a positive predictor of migraine (153). The association between unipolar depression and migraine is generally more known, but many studies also show the co-occurence of migraine and BD (153, 154) and some authors even suggest that among unipolar depressed patients, the onset of migraine might reflect a bipolar spectrum trait since the symptom profile of these patients with depression-migraine comorbidity is more similar to the one of BD patients compared to major depressive disorder (MDD) patients (65, 155). Since comorbid diseases do not occur randomly, rather they reflect shared pathomechanisms (156-158), the importance of circadian factors in mood disorders might also suggest a role for the circadian system in migraine or at least in some migraine patients. To briefly highlight the relevance of circadian rhythms in mood disorders, we would like to mention that MDD depressive symptoms show a diurnal variation (typically, the worst state is reported in the morning and symptoms are less severe during the afternoon and the evening); circadian misalignment correlates with depressive symptom severity (159); sleeping problems are among the earliest and most frequent symptoms of mood disorders, specifically insomnia is more characteristic to MDD, while hypersomnia or the co-occurence of insomnia and hypersomnia to BD (160); and evening chronotype is more frequent both in MDD and BD patients (161, 162). A circadian dysfunction seems to be a general feature of depression which is further supported by associations of clock genes with MDD and BD (163-166).

Sleeping problems represent a shared circadian rhythm-related factor between mood disorders and migraine. Sleep disturbance is among the most frequently reported migraine attack triggers (18, 107, 112). A recent study (167) reviewing the last two decades reported that among migraine-associated sleep disorders, insomnia, parasomnias (e.g. somnambulism), bruxism, restless legs syndrome and increased nightmare frequency are the ones that show more convincing evidence. The authors concluded that migraine and sleep problems seems to share underlying pathomechanisms involving common mediating neurotransmitters (mostly dopamine and serotonin) and subcortical regions, especially the hypothalamus. A study in an arctic population interestingly found that insomnia-related migraine attacks showed a biphasic diurnal cycle of attacks, specifically a peak early in the morning and another one just after noon, while attacks unrelated to insomnia showed just one peak in the afternoon (168). Early morning attacks seem to increase with age probably in association with the decreased slow wave sleep and increased nighttime arousals among older adults (167). Additionally, some studies detected an association between alterations of sleep stages and migraine. An electroencephalography (EEG) study (169) identified a minimal sleep disturbance among migraineurs mostly involving the rapid eye movement (REM) phase: decreased REM sleep quantity and latency among migraineurs suggesting shared monoaminergic mechanisms including a decreased serotonin level which can be detected both during the REM phase and migraine attack (146, 170, 171). A study with eight migraine patients detected a decrease in arousals, REM density, alpha power in first REM phase and beta power in slow wave sleep during the nights preceding a migraine attack (172). Another study (173) compared the polysomnographic profiles of MO patients and controls and found a lower level of sleep efficiency (e.g. lower total sleep time, higher wake time), stage 4 and non-REM sleep, furthermore a prolonged sleep onset and stage 1 latency among migraineurs. The possible roles of alterations in sleep phases in migraine might suggest a brainstem dysfunction in switching between sleep stages (146, 167).

There are still many gaps in our understanding regarding the relationship between migraine and sleep. Prospective longitudinal studies with detailed data on migraine and sleep characteristics might add further knowledge – such a study recently revealed that short sleep duration (less than 6.5 hours) and self-reported poor sleep quality showed no temporal association with migraine attacks, however low sleep efficiency (captured by high fragmentation) associated with attack onset: not on the day that immediately followed the sleep, but on the next day (174). A previous result might support this assocation: among healthy females, a decreased pain threshold correlated only with frequent awakening (i.e. sleep continuity disturbance) but not with partial sleep deprivation (175).

Among the postulated shared biochemical mediators of migraine and sleep (176), melatonin is a clear indicator of the circadian clock. Melatonin is produced by the pineal gland in sync with the light-dark cycle in all mammals: it is only secreted during the night, in absence of light (177). It represents a chronobiotic hormonal signal which has an important role in the adaptation to environmental changes during the day and the season by mediating circadian and circannual rhythm-related physiological and behavioral functions (177). A retino-hypothalamic-pineal axis dyfunction in migraine has been already proposed in 2006 (178). A recent metaanalysis detected a lower level of nocturnal serum, as well as urinary melatonin and urine 6-sulphatoxymelatonin (aMT6s) among adult migraineurs compared to healthy controls, however after excluding patients with comorbid insomnia or depression, the difference between migraineurs and controls in serum melatonin disappeared (179). Regarding the latter result, the authors concluded that the shared pathomechanisms of migraine, sleep and mood disorders include disturbed melatonin secrection – at least, among some migraine patients. Furthermore, two meta-analyses confirmed the efficacy of exogenous melatonin in migraine prophylaxis (179, 180).

Finally, we would like to highlight that shift and night work, clearly affecting circadian rhythms, has been also suggested to associate with migraine, however recent reviews showed conflicting results (181, 182). The authors highlighted methodological problems, especially in case of earlier studies. One review also included two case studies which demonstrated an association of shift work with migraine chronification and higher headache-related disability (182). At the moment, it is too early to extrapolate clear conclusions regarding the relationship between migraine and shift work, although some data suggest that it is still important to identify shift workers among migraineurs to optimize their lifestyle factors and prevent migraine chronification and an increase in headache-related disability (181, 182).

All in all, multiple results suggested a connection between migraine and circadian rhythms, although most of them can be described as indirect chronobiologic associations (179) which motivated us to further investigate this topic.

2. Objectives

Based on the above, considering the multifactioral nature of migraine including genetic, possibly gene-environment interactional and functional brain mechanism-related factors in its pathophysiology which partly involved circadian rhythm-related factors (namely *CK1δ* from the field of genetics and hypothalamus from the field of fMRI), and also taking into account other indirect chronobiologic associations (specifically the circadian variation of migraine attack onset, correlation with chronotype, associations with sleeping problems, melatonin and shift work), we performed two studies to further investigate the relationship between migraine and circadian rhythms.

In our first study (183) which will be referred as the Genetic study, our goal was:

1. to test whether a variant in a circadian gene, namely *CLOCK* shows a main effect on migraine;
2. to determine whether the association between *CLOCK* and migraine is dependent on stress factors;
3. and to identify whether different stress factors show different effects on the *CLOCK* – migraine association.

For this purpose, we selected a tag SNP of the *CLOCK* gene, rs10462028 which associated previously with bipolar disorder (166), a comorbid disorder of migraine with shared genetic factors (184). Since, previously no main effect was found for common clock gene variants, we aimed to include a representative SNP of an often investigated (166, 185, 186), core clock gene such as *CLOCK*, a transcriptional activator. Investigated stress factors included childhood adversity, recent negative life events and financial hardship which were previously connected to migraine (81-83).

In our second study (187) which will be referred as the fMRI study, we aimed:

4. to test whether circadian variation of migraine attack onset affects interictal functional brain activation patterns during emotion processing among episodic migraine without aura patients;
5. to investigate whether the processing of negative and positive emotions correlates with similar functional brain activation patterns in association with circadian variation of migraine attack onset;

6. and to determine whether morning migraine attack onset associates with different functional brain activation during emotion processing compared to other typical circadian attack onset peaks.

To fullfill this goal, we compared whole brain activitation differences between migraine subgroups showing different typical circadian attack onset peaks using an implicit emotional face processing fMRI task with fearful, sad, happy and neutral stimuli. Two studies with the same fMRI task were conducted with episodic MO patients.

3. Methods

3.1 Methods of the Genetic study

3.1.1 Participants

Our participants „were recruited through general practices and advertisements from Greater Manchester, UK (n=1277) and Budapest, Hungary (n=880; aged between 18 and 60)” (183, p. 2) resulting in a final sample of n=2157 participants who provided valid data on consent to DNA analysis, ethnicity, sex, age, migraine status and *CLOCK* rs10462028 genotype. All our subjects were of European white origin. „Our study was approved by the local Ethics Committees (Scientific and Research Ethics Committee of the Medical Research Council, Budapest, Hungary, ad.225/KO/2005; ad.323-60/2005-1018EKU and ad.226/KO/2005; ad.323-61/2005-1018 EKU; North Manchester Local Research Ethics Committee, Manchester, UK REC reference number: 05/Q1406/26) and was carried out in accordance with the Declaration of Helsinki” (183, p. 2-3).

3.1.2 Self-report measures

English and Hungarian versions of brief standard questionnaires were used. Our previously validated background questionnaire (188) included data on ethnicity, sex, age, lifetime depression and bipolar disorder.

We used the ID-migraine questionnaire, „a validated screening tool for migraine (189), which includes three items of the main migraine symptoms: nausea, photophobia, and disability (experienced in the past 3 months)” (183, p. 3). Migraine was defined as at least two positive answers to the symptom questions – this designation previously reached a 0.93 positive predictive value (189).

The three measured stress factors were captured by the following tools. Childhood adversity (CHA) was measured with the Childhood Trauma Questionnaire (CTQ) (190). We used a shortened version of CTQ (188) which contained items about emotional and physical abuse, neglect and possible loss of parents during childhood. Recent negative life events (RLE) were assessed with the List of Threatening Experiences questionnaire (191). Financial hardship (FINANC) was obtained from our background questionnaire and the related question was used in a previous GxE study (192) of our research group. This variable captures a personal experience of financial status (and not directly income). Originally, it is a five-level variable, but we decided to use three categories by separately merging „the first two and the last two categories to gain more appropriate sample sizes: (1) living very/quite comfortably; (2) just getting by; and (3) finding it difficult/not able to make ends meet" (183, p. 3).

3.1.3 Genotyping

„Genomic DNA was derived from buccal mucosa cells (193). After extraction of DNA the Sequenom® MassARRAY technology (Sequenom®, San Diego, CA, USA (194)) was used to normalize and genotype the samples. Rs10462028 SNP was selected as a haplotype tag of the 3'-UTR region of the *CLOCK* gene (Haploview (195)). Genotyping was performed according to the ISO 9001:2000 requirements and kept blinded with regard to phenotypic data" (183, p. 3).

3.1.4 Functional prediction of *CLOCK* rs10462028

„According to the 1000 Genomes database (196), rs10462028 is in high linkage disequilibrium (LD; $r^2 > 0.8$) with 70 SNPs covering a region of 132,043 base pairs (from base position 56,288,743 to 56,420,786)" (183, p. 3-4).

As a functional prediction of *CLOCK* rs10462028, we decided to predict its potential effects on microRNA (miRNA) binding. For this purpose, we identified miRNAs with seed regions which contain rs10462028. „Further miRNA binding sites were predicted and examined near the SNP (as the altered mRNA sequence/structure can affect the accessibility of the region) and additionally around rs1801260 polymorphism that is in high LD (r^2=0.9, in the same LD-block – according to the 1000 Genomes database (196); and r^2=0.802 in the CEU population according to SNP Function Prediction (197)) with rs10462028. Rs1801260 is a frequently examined SNP of *CLOCK* because of its proposed effect on activity, sleep onset, and sleep quantity (185, 186, 198).

The sequence of the Homo sapiens CLOCK 3-UTR (NM_001267843.1) transcript variant was obtained from the Nucleotide database of NCBI (199). SNPs in LD with rs10462028 and rs1801260 have been collected using NIH SNP Function Prediction (197) with the following parameters: LD≥0.8 in Population CEU, based on Genotype Data from HapMap CEU, based on Genotype Data from dbSNP: European. We only included 3´-UTR polymorphisms in our further investigation and focused on the *CLOCK* gene in this study" (183, p. 4).

The following tools were used to predict miRNA binding sites around the investigated polymorphisms (with reference and alternative alleles): „TargetScan (200), miRanda predictions on NIH SNP Function Prediction (197), and MicroSNiPer (201)" (183, p. 4). Furthermore, GeneCards (202) and miRiAD (203) databases served as our sources to select miRNAs showing known expression in the central nervous system.

3.1.5 Statistical analysis

PLINK v1.07 (204) was used „to calculate Hardy-Weinberg equilibrium (HWE) *p* and LD r^2-values, to run logistic and linear regression analysis with additive, dominant, and recessive genetic models" (183, p. 5).

First, we ran a main effect analysis of *CLOCK* rs10462028 on migraine in the total sample. Next, the interaction effect was tested between the SNP and each stress factors (CHA, RLE, FINANC) separately, similarly on migraine in the total sample. Age and sex were added as covariates to all analyses. To manage potential ancestral differences, subjects were screened for European white origin (according to self-reported data), furthermore, population (i.e. Manchester or Budapest) was also added as a covariate to the total sample analyses. The following three approaches were used to evaluate our nominally significant findings: „(a) the gold standard Bonferroni correction to adjust *p*-values for multiple testing in our 12 main analyses (additive, dominant and recessive models of *CLOCK* SNP main effects and SNP x stress factor (CHA, RLE, or FINANC) interactions on migraine in the total sample) with a Bonferroni-corrected threshold of $p \leq 0.004$ (0.05/12); (b) as Bonferroni correction is overly conservative and does not take into account the interdependences between the three genetic models of the same SNP, we used another more lenient corrected *p*-value taking into account our four hypotheses $p \leq 0.0125$ (0.05/4); and (c) finally, effects were considered statistically significant in case of reaching a significance value below 0.05 not only in the total sample but also in the two subsamples (Budapest and Manchester, separately)" (183, p. 5).

Additionally, in case of significant results, potential confounding effects of lifetime depression and bipolar disorder were also tested by separately adding them as covariates to the models.

Moreover, main effects of *CLOCK* rs10462028 were also tested on the measured stressors (CHA, RLE, FINANC), lifetime depression and bipolar disorder – to screen for its potential effect on these elements that may alter the association between the SNP and migraine.

For displaying purposes, positive likelihood ratios (LR+) were calculated: the frequency of migraine cases were divided by the frequency of controls in every categories of all the stressors which showed significant GxE effects with *CLOCK* rs10462028. We also performed post hoc tests with IBM SPSS Statistics 23 (with a two-tailed p=0.05 threshold). Chi-squared test was used to estimate the association between migraine frequency and the *CLOCK* variant in the categories of each stressors. Phenotypic data of the two subsamples (Manchester and Budapest) were compared with chi-squared test or unpaired t-test. The main effect of the stressors on migraine were tested with logistic regression (covariates in the models: sex, age, population) to replicate these previously found associations.

Statistical power of our study were quantified with Quanto 1.2 (205) with the following settings: case-control design with a control-to-case ratio of 3; additive genetic model; minor allele frequency (MAF): 32-33% according to our study (n=2157). The calculation resulted in 96% power to show a genetic main effect (1.3 odds ratio (OR)), and 80% to show a GxE effect (p=0.05, two-tailed; 1.5 OR).

3.2 Methods of the fMRI study

Two fMRI studies were conducted with different subjects and MRI scanners. Here, we describe the details of our methods pointing out the differences between fMRI Study 1 and 2. In fMRI Study 1, a self-reported questionnaire, while in fMRI Study 2, a more thorough tool, namely a headache diary was applied to operationalize typical circadian attack onset peak. We considered fMRI Study 1 as an exploratory study and fMRI Study 2 as a replication study.

3.2.1 Participants

Episodic MO patients were recruited through advertisings at universities, neurological clinics and in articles. Episodic migraine without aura diagnosis was determined by headache specialists applying International Classification of Headache Disorders-III criteria (4). Our inclusion criteria contained: „(1) right handedness according to the Edinburgh Handedness Inventory (206); (2) normal or corrected to normal vision; (3) lack of history of any chronic medical, neurological (except migraine) or psychiatric disorders diagnosed by senior neurologist and psychiatrist researcher colleagues; (4) lack of daily medication use (except oral contraceptives). Selected migraineurs agreed to avoid to take any prophylactic medication for 3 months and any analgesics or migraine attack medication 48 h before the scan sessions" (187, p. 3).

After applying the inclusion criteria and exclusion due to missing data, 31 MO patients were included to fMRI Study 1, and 48 migraine patients to fMRI Study 2.

„Written informed consent was provided by all participants, in accordance with the Declaration of Helsinki. The studies were approved by the Scientific and Research Ethics Committee of the Medical Research Council (Hungary)" (187, p. 3).

3.2.2 Self-report measures

In fMRI Study 1, typical circadian attack onset peak was measured with a self-reported retrospective question: „Typically, when does your migraine headache start? Please, choose one answer from the options of (1) always in the morning, (2) rather in the morning, (3) in the forenoon, (4) in the afternoon, (5), rather in the evening, (6) always in the evening, (7) at night, during sleep (waking up because of it), (8) varying, and (9) other. Options number (1), (2), (3), and (7) represent morning or dawn start (collectively the first half of day) and were combined as Morning start; and options (4), (5), and (6) capture afternoon or evening start (covering the second half of the day) and were combined under the name of Evening start. A similar categorization to assess a circadian pattern of migraine headache start (namely: "usually before noon" and "usually after noon") was used in a previous study (207). Furthermore, a Varying start group was defined: based on options (8) and (9) representing migraineurs without a typical circadian attack onset peak" (187, p. 3).

In fMRI Study 2, a prospective paper headache diary was used to measure typical circadian attack onset peak. Regarding the headache diary, we expected minimum two reported migraine attacks (similarly as de Tommaso and Delussi (208)) which were separated by a headache-free period which lasted for more than 24 hours (as in Alstadhaug et al. (168)). Each reported headaches were separately reviewed. „A migraine-type headache was classified in case of showing at least four of the six migraine attack features listed by ICHD-III (4): (1) 4–72 h long duration, (2) unilateral pain, (3) pulsating pain quality, (4) moderate or severe intensity, (5) aggravation by routine physical activity, and (6) any of the concomitant symptoms (nausea or vomiting, photo- and/or phonophobia). In case of use of an acute migraine treatment, we expected the fulfillment of at least three of the six features" (187, p. 3). Further details on criteria for the headache diary and migraine attack classification can be found in Supplementary Appendix 1 of our article (187). After applying these inclusion criteria, selected „headache diaries covered an average time-span of 2.15 months (minimum: 1, maximum: 6, SD: 1.08 months) with 255 migraine-type headaches for the 48 participants. Each patient was included to a typical circadian attack onset peak group based on at least 60% of his/her attack occurrence in the two time slots: from 0:00 to 11:59 (Morning start); 12:00–23:59 (Evening start). Varying start group category was used if someone's attacks were below 60% in any of the two categories" (187, p. 3).

Five variables were used as covariates in both studies to control their potential confounding effects based on their associations with either migraine, and/or circadian rhythm. Relationships of migraine with age and sex are known (209, 210). Migraine attack frequency per month is a clinically relevant indicator of migraine severity and previously associated with the extent of functional brain changes (211). Furthermore, it was also shown to be a quite accurate and reliable self-estimated characteristic of migraine (212). It was measured with the following retrospective question: „How many migraine attacks do you have per month?" (187, p. 3). As we already discussed, chronotype and sleeping problems may influence circadian variation of migraine attack onset. „Chronotype was measured with the following question: Do you consider yourself as a morning or an evening type of person? with the options of (1) definitely morning, (2) rather morning, (3) rather evening, (4) definitely evening, (5) I don't know. To gain bigger sample sizes, we combined the first two categories (definitely/rather morning) and also categories number (3) and (4) (definitely/rather evening). Sleeping problems was captured in the following way: Do you have problems falling asleep or waking up in the middle of the night? with the options of (1) never or rarely, (2) sometimes, (3) frequently or usually" (187, p. 3-4).

3.2.3 Experimental task

An implicit emotional processing task was used to measure functional brain activity. A standard set of images (213) depicting fearful, sad, happy and neutral facial expressions was shown in block design, and participants were instructed to identify the sex of faces. This implicit approach, ensuring attention to stimuli, was succesfully used in previous neuroimaging studies of emotional face processing evoking activation predominantly in limbic system and extrastriate cortical regions (214-217).

Images of six adult faces (50-50% males and females) were presented without non-facial features. „Three 20 s long rest blocks (white fixation cross at the center) separated the three 20 s long blocks of each emotional expression (happy, sad, and fearful) in a pseudo-random order, distributed with twelve neutral blocks. One block contained six faces. During the 8 min long task, the presentation time for each faces was 3000 ms, and for the interstimulus interval 333 and 334 ms. The task was presented with the E-Prime 2.0 software (Psychology Software Tools, Inc., Pittsburgh, PA, United States). In the MRI scanner, participants in lying position viewed the face stimuli on a screen through a mirror fixated to the head coil" (187, p. 4). The participants were instructed to handle a two-button response device to denote the sex of the faces by finger-pressing: the index finger was used to indicate female faces with one button, and the thumb to indicate male faces with the other button. Before attending the scanner room, the participants executed a short practice session with neutral faces using a laptop. Our research group succesfully employed the task in multiple studies (118, 218-220).

3.2.4 fMRI data acquisition

MRI scans started after 3:00 p.m, and were conducted in the late afternoon and early in the evening. Participants were instructed to abstain from eating, smoking and caffeine consuming 4 h prior to the scan session.

In fMRI Study 1, „fMRI data acquisition was performed on a 3 T MRI scanner (Achieva 3 T, Philips Medical System) using a BOLD-sensitive T2*-weighted echo-planar imaging sequence (repetition time TR=2500 ms, echo time TE=30 ms, field of view FOV=240x240 mm) with 3x3 mm in-plane resolution and contiguous 3 mm slices providing whole-brain coverage. A series of high-resolution anatomical images were also acquired during the imaging session using a T1-weighted 3D TFE sequence with 1x1x1 mm resolution.

In fMRI Study 2, a 3.0 T MAGNETOM Prisma Siemens Syngo scanner was used, with the following parameters: TR=2220 ms, TE=30 ms, FOV=222x222 mm, with a 3x3x3 mm resolution. High-resolution anatomical images were acquired similarly with a 1x1x1 mm resolution, using a 3D MPRAGE sequence" (187, p. 4).

3.2.5 Self-report data analysis

IBM SPSS Statistics 23 was used to analyze self-report data. Migraine subgroups were compared with non-parametric tests „because of the failure of normality: Kruskal–Wallis test and post hoc pairwise Mann–Whitney test with a two-tailed $p<0.05$ threshold. In case of categorical variables, Freeman–Halton extension of the Fisher exact probability test was performed for two-rows by three-columns and three-rows by three-columns contingency tables at VassarStats website (221). Similarly, a two-tailed $p<0.05$ threshold was set" (187, p. 4).

3.2.6 fMRI data analysis

For fMRI data analysis, „Statistical Parametrical Mapping (SPM12) software (The Wellcome Centre for Human Neuroimaging, UCL Queen Square Institute of Neurology, London, United Kingdom) was used in Matlab R2016a (Mathworks). Standard preprocessing steps were implemented: (1) realignment of functional images; (2) coregistration of the mean functional image to the structural image; (3) segmentation; (4) normalization to the Montreal Neurological Institute (MNI) space; (5) smoothing with an 8 mm fullwidth-at-half-maximum (FWHM) Gaussian kernel. Artifact Detection Tools (ART) were used to screen for motion outliers with the following deviation thresholds: more than 3 standard deviations for the global signal; and more than 1 mm in case of scan-to-scan motion. Exclusion criteria was: higher than 15% of volumes registered as outliers. Motion outliers were included as regressors with no interest to the fMRI model.

For first-level analysis, a general linear model (GLM) was applied in SPM12 to measure BOLD-responses to emotional facial expressions with three contrasts: fear-neutral, sad-neutral, happy-neutral – the same method" (187, p. 4). were used in previous publications of our research group (118, 218-220). In the next step, „the created contrast maps were entered into second-level analysis. To compare the task-related activation in the whole brain between groups with different typical circadian peak of attack onset, we used one-way ANOVA with five covariates: age, sex, migraine attack frequency per month, chronotype, and sleeping problems" (187, p. 4). Post hoc independent samples t-tests were used to reveal effect directions with the same five covariates. All imaging data analyses were carried out „with an initial threshold of $p<0.001$ (uncorrected) with a cluster size of k≥10 voxels. To adjust for multiple testing, results with a cluster level family-wise error corrected threshold of $p_{FWE}<0.05$ were considered as statistically significant. Significantly activated clusters were identified with the Automated Anatomical Labeling atlas (aal) (222). For visualization of statistical maps, the MNI 152 template brain in MRIcroGL was used" (187, p. 4).

4. Results

4.1 Results of the Genetic study

4.1.1 Descriptive statistics of the study sample

Descriptive statistics of the sample and the genetic variable can be found in Table 1. Females represented the majority of our sample (69.9%), and the average age was 32.9 years (SEM: +/-0.22). 27.8% were assigned to have migraine based on the ID-Migraine questionnaire (189). The MAN subsample were significantly older, and showed higher prevalence of migraine, lifetime depression and bipolar disorder, furthermore higher rates in more stressful categories of CHA, RLE and FINANC compared to the BP subsample. Rs10462028 was in Hardy-Weinberg equilibrium (total: p=0.36; MAN: p=0.57; BP: p=0.6).

4.1.2 Main effects of stress factors on migraine

All measured stress factors (CHA, RLE, FINANC) showed a significant (p<0.0001) positive main effect on migraine validating the use of these variables in our study. For details, see Supplementary Table S1 of our article (183).

4.1.3 Main effect of *CLOCK* rs10462028 on migraine

Rs10462028 showed no main effect on migraine – for details, see Table 2.

4.1.4 Interaction effect of *CLOCK* rs10462028 with stress factors on migraine

Rs10462028 was tested in three separate regression analyses in interaction with the stress factors on migraine. Results are summarized in Table 2. A significant interaction effect was found between rs10462028 and FINANC on migraine (both in ADD and REC models), but not in case of CHA and RLE. The SNP x FINANC effect is shown in Figure 1.

Carriers of AA genotype showed a significantly lower frequency of migraine at the most adverse FINANC status compared to GG (χ^2=3.916, p=0.048) and AG (χ^2=5.259, p=0.022) genotype carriers. Furthermore, the AA genotype significantly associated with a higher migraine frequency at the most favourable FINANC status compared to GG genotype (χ^2=7.279, p=0.007) and at a tendency level compared to AG genotype (χ^2=2.98, p=0.084).

Table 1. Details of the study sample and results of statistical analyses comparing the Manchester (MAN) and Budapest (BP) subsamples (183). Table 1A describes phenotype data of our sample and shows results of statistical analyses comparing the subsamples. Table 1B summarizes data on rs10462028.

A. Phenotypic description	**Total sample**	**MAN**	**BP**	**Difference (MAN vs. BP)**
Participant number (n)	2157	1277	880	
Female (n, %)	1503 (69.7%)	893 (69.9%)	610 (69.3%)	χ^2=0.092, *p*=0.761
Age (mean +/- SEM)	32.9 (+/-0.22)	34.02 (+/-0.29)	31.3 (+/-0.36)	**t=-5.982, *p*<0.0001**
MigraineID (n, %)	600 (27.8%)	399 (31.2%)	201 (22.8%)	**χ^2=18.326, *p*<0.0001**
Reported lifetime depression (n, %)	907 (42%)	713 (55.8%)	194 (22%)	**χ^2=244.087, *p*<0.0001**
Reported lifetime bipolar disorder (n, %)	69 (3.2%)	51 (4%)	18 (2%)	**χ^2=6.386, *p*=0.012**
Recent negative life events categories (n, %)				
No or mild	1435 (66.5%)	821 (64.3%)	614 (69.8%)	**χ^2=14.654, *p*<0.0001**
Moderate	400 (18.5%)	237 (18.6%)	163 (18.5%)	
Severe	318 (14.7%)	219 (17.1%)	99 (11.3%)	
Missing data	4 (0.2%)	-	4 (0.5%)	
Childhood adversity categories (n, %)				
No or mild	1398 (64.8%)	780 (61.1%)	618 (70.2%)	**χ^2=29.375, *p*<0.0001**
Moderate	394 (18.3%)	238 (18.6%)	156 (17.7%)	
Severe	355 (16.5%)	254 (19.9%)	101 (11.5%)	

Missing data	10 (0.5%)	5 (0.4%)	5 (0.6%)	
Financial hardship categories (n, %)				
Living very/quite comfortably	1267 (58.7%)	689 (54%)	578 (65.7%)	**χ^2=50.215, *p*<0.0001**
Just getting by	656 (30.4%)	409 (32%)	247 (28.1%)	
Finding it difficult/not able to make ends meet	225 (10.4%)	177 (13.9%)	48 (5.5%)	
Missing data	9 (0.4%)	2 (0.2%)	7 (0.8%)	
B. Genetic data	**Total sample**	**MAN**	**BP**	
Minor allele frequencies (MAF)				
rs10462028 (A/G)	33.01%	33.27%	32.66%	

MAN: Manchester; BP: Budapest. „MigraineID: positive for migraine according to the ID-Migraine questionnaire. SEM: standard error of mean. Childhood adversity categories: (1) *no or mild* (score: 0-3), (2) *moderate* (score: 4-6), (3) *severe* (score: 7 or more). Recent negative life events categories: (1) *no or mild* (score: 0 or 1), (2) *moderate* (score: 2), (3) *severe* (score: 3 or more). χ^2: Pearson χ^2; *p*: significance – bold: significant effects" (183, p. 4).

Table 2. Statistical results of the main effect of *CLOCK* rs10462028 and its interaction effect with the stress factors on migraine in the total sample (183).

			Total sample				
Main effect							
SNP	A1	Model	OR	L95	U95	*p*
rs10462028	A	ADD	1.109	0.962	1.278	0.152
		DOM	1.144	0.942	1.389	0.175
		REC	1.146	0.854	1.538	0.364
Interaction						
SNP x CHA		Model	OR	L95	U95	*p*
		ADD	1.011	0.971	1.053	0.589
		DOM	1.026	0.971	1.084	0.363
		REC	0.988	0.909	1.075	0.784
SNP x RLE		Model	OR	L95	U95	*p*
		ADD	0.942	0.849	1.044	0.255
		DOM	0.930	0.805	1.076	0.332
		REC	0.908	0.738	1.117	0.361
SNP x FINANC		Model	OR	L95	U95	*p*
		ADD	**0.779**	**0.634**	**0.956**	**0.017**
		DOM	0.815	0.617	1.075	0.148
		REC	**0.54**	**0.348**	**0.836**	**0.006**

„SNP: single nucleotide polymorphism; A1: minor (and effect) allele; CHA: childhood adversity; RLE: recent negative life events; FINANC: financial hardship; OR: odds ratio; L95-U95: 95% confidence interval; ADD: additive model; DOM: dominant model; REC: recessive model; *p*: significance – bold: significant effects. Covariates in the model: age, gender and population" (183, p. 6).

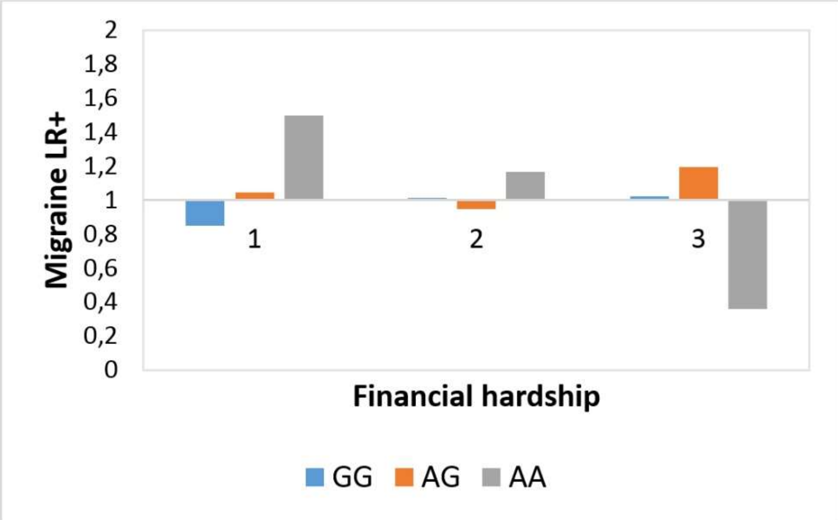

Figure 1. Interaction effect between *CLOCK* rs10462028 and financial hardship on migraine (183). LR+: positive likelihood ratio. Number of cases at Financial hardship levels: 1 (*living very/quite comfortably*): GG=575, AG=550, AA=142; 2 (*just getting by*): GG=294, AG=282, AA=80; 3 (*finding it difficult/not able to make ends meet*): GG=102, AG=100, AA=23 (183).

The SNP x FINANC effect on migraine was also tested in the subsamples – for details, see Table 3. In the BP subsample, both the ADD and REC model survived (Figure 2A), but in the MAN subsample, only the REC model (Figure 2B). Thus, only the recessive model could be replicated at a significant level in both subsamples – we consider this as our main result at a p=0.006 level (see Table 2) which is nominally significant, but after Bonferroni-correction, only a trend effect could be reached. However, with the use of the previously detailed, reasonably more lenient corrected p-value (considering the interdependency between the ADD, DOM and REC genetic models), our result survives.

Table 3. Statistical results of the interaction effect of *CLOCK* rs10462028 with financial hardship on migraine in the subsamples (183).

Interaction		**MAN**				**BP**			
SNP x FINANC	Model	OR	L95	U95	*p*	OR	L95	U95	*p*
	ADD	0.811	0.632	1.042	0.101	**0.612**	**0.408**	**0.916**	**0.017**
	DOM	0.9	0.643	1.26	0.539	*0.596*	*0.353*	*1.006*	*0.053*
	REC	**0.525**	**0.312**	**0.88**	**0.015**	**0.279**	**0.083**	**0.939**	**0.039**

MAN: Manchester; BP: Budapest; „SNP: single nucleotide polymorphism; FINANC: financial hardship; OR: odds ratio; L95-U95: 95% confidence interval; ADD: additive model; DOM: dominant model; REC: recessive model; *p*: significance – bold: significant effects, italic: trend effects. Covariates in the model: age, gender" (183, p. 7).

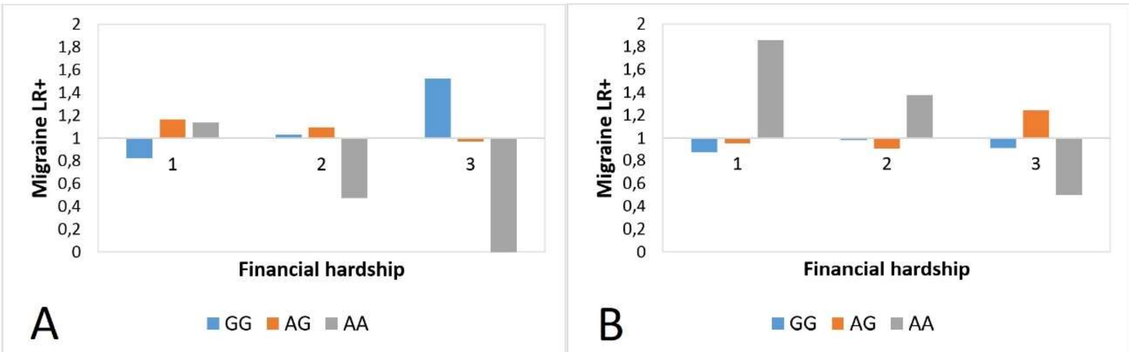

Figure 2. The interaction effect between *CLOCK* rs10462028 and financial hardship on migraine in the subsamples (183). Figure 2A shows the *CLOCK* rs10462028 x FINANC effect on migraine in the BP subsample. Number of cases at Financial hardship levels: 1 (*living very/quite comfortably*): GG=269, AG=242, AA=67; 2 (*just getting by*): GG=106, AG=117, AA=24; 3 (*finding it difficult/not able to make ends meet*): GG=23, AG=19, AA=6 (183). Figure 2B shows the *CLOCK* rs10462028 x FINANC effect on migraine on migraine in the MAN subsample. Number of cases at Financial hardship levels: 1 (*living very/quite comfortably*): GG=306, AG=308, AA=75; 2 (*just getting by*): GG=188, AG=165, AA=56; 3 (*finding it difficult/not able to make ends meet*): GG=79, AG=81, AA=17. BP: Budapest. MAN: Manchester; LR+: positive likelihood ratio (183).

To control for potentional confounding effects of lifetime depression and bipolar disorder on migraine, they were added separately to the regression models as covariates. The SNP x FINANC effect on migraine remained significant with similar OR-values (for details, see Supplementary Table S2 in our article (183).

4.1.5 *CLOCK* rs10462028 x financial hardship effect on migraine according to alternative FINANC definitions

Additionally to the original regression analyses with a three-level FINANC variable, considering that the rather low subject number at the most severe FINANC level could potentionally affect our results, we ran further analyses using a two-level FINANC variable: 1. either living very comfortably or quite comfortably (n=1309); 2. either just getting by or finding it difficult or not able to make ends meet (n=904). With these models, we were also able to test the a priori suggested differential effects of rs10462028 at the positive and negative ends of the financial hardship spectrum in a more direct way. In the total sample and the BP subsample, the additive SNP x FINANC effect replicated at a significant level, but only at a tendency level in the MAN subsample (see Supplementary Table S3 (183)). Furthermore, analyses with the original FINANC variable containing five levels produced significant SNP x FINANC effect on migraine in the total sample and in the BP and MAN subsamples with comparable OR-values (Supplementary Table S3 (183)).

4.1.6 Main effects of *CLOCK* rs10462028 on CHA, RLE, FINANC, lifetime bipolar disorder and unipolar depression

No main effects were found for rs10462028 on the measured stress variables (CHA, RLE, FINANC) and on lifetime bipolar disorder and unipolar depression (for details, see Supplementary Table S4 (183)) confirming that our significant interaction results were not confounded by gene-environment correlations.

4.1.7 Results of the in silico functional analysis of *CLOCK* rs10462028 and rs1801260

For the in silico functional analysis, besides rs10462028, we also used another *CLOCK* SNP, rs1801260 which is in high LD with rs10462028. We detected multiple potential miRNA binding sites around the two SNPs with predicted modifications in binding due to rs10462028 and rs1801260. Significantly predicted miRNA-s included: miR-409-5p in case of rs10462028; miR-365b-3p, miR-365a-3p and miR-664a-5p in case of rs1801260. Detailed results can be found in Supplementary Table S5 (183).

4.2 Results of the fMRI study

4.2.1 Results of fMRI Study 1

4.2.1.1 Results of self-reported data

Three subgroups emerged according to typical circadian attack onset peak: „(1) Morning start (n=8), (2) Evening start (n=9), and (3) Varying start (n=14)" (187, p. 5). In the followings, we will use the term of M_{circ} subgroups to briefly capture these subsamples with different typical circadian attack onset peaks.

Self-reported data of the fMRI Study 1 sample and the M_{circ} subgroups are collected in Table 4. Females represented the majority of the sample (77.4%) and the average age was 26.97 years (SD: 4.83). The age of the Varying start group was significantly higher compared to the Evening start group, but no other differences were detected between the M_{circ} subgroups in further self-reported data.

4.2.1.2 fMRI results

Results of the main effect of the fMRI task processing different emotional faces are shown in Supplementary Table 4 (187). Whole-brain activation was compared between the M_{circ} subgroups with five covariates "(age, sex, migraine attack frequency per month, sleeping problems, chronotype)" (187, p. 5) in the model which resulted in significant differences in case of fearful (but not happy or sad) faces in one cluster covering left superior temporal and left supramarginal gyri (details in Table 5).

According to the post hoc pairwise group comparisons, the Evening start group showed significantly increased neural activation responding to fearful faces in comparison with the Morning start group. The activated three clusters included "regions of left and right superior temporal gyrus, left supramarginal gyrus, left postcentral gyrus, right Rolandic operculum, right Heschl's gyrus, left middle cingulate gyrus, left posterior cingulate gyrus and right precuneus" (187, p. 6) (see Table 6 and Figure 3).

Table 4. Self-reported data of the fMRI Study 1 sample and results of statistical analyses comparing the M_{circ} subgroups (187).

	Total	**Morning start (M)**	**Evening start (E)**	**Varying start (V)**	**Group comparisons**
Participant number (n)	31	8	9	14	
Female (n, %)	24 (77.4%)	7 (87.5%)	6 (66.6%)	11 (78.6%)	Fisher's exact *p*=0.655
Age (mean, SD)	26.97 (4.83)	26.12 (4.32)	23.67 (2.0)	29.57 (5.1)	H=7.516, *p*=0.023* (V>E; U=21, *p*<0.008)*
Attack frequency per month (mean, SD)	3.34 (3.15)	2.31 (1.13)	4.55 (4.44)	3.14 (2.88)	H=0.139, *p*=0.933
Chronotype (n, %)					
Definitely / rather morning	13 (41.9%)	4 (50.0%)	2 (22.2%)	7 (50.0%)	Fisher's exact
Definitely / rather evening	17 (54.8%)	3 (37.5%)	7 (77.8%)	7 (50.0%)	*p*=0.223
Do not know	1 (3.2%)	1 (12.5%)	0 (0%)	0 (0%)	
Sleeping problems (n, %)					
never/rarely	14 (45.2%)	4 (50%)	4 (44.4%)	6 (42.9)	Fisher's exact
sometimes	14 (45.2%)	4 (50%)	4 (44.4%)	6 (42.9)	*p*=0.953
often/usually	3 (9.7%)	0 (0%)	1 (11.1%)	2 (14.3)	

„H: Kruskal-Wallis test statistic; SD: standard deviation; U: Mann-Whitney test statistic; *: significant effect; M_{circ} subgroups: M: Morning start; E: Evening start; V: Varying start" (187, p. 5).

Table 5. Brain regions showing significant activation differences between the three M_{circ} subgroups in response to fearful faces in fMRI Study 1 (187).

Contrast	Cluster size	Cluster *p* (FWE)	Region	Coordinates (MNI)			Peak
				x	y	z	F-value
Fear-neutral	51	0.013	L superior temporal gyrus	-57	-37	17	16.61
			L superior temporal gyrus	-45	-37	20	14.54
			L superior temporal gyrus	-51	-40	20	13.15
			L supramarginal gyrus	-63	-34	23	11.04

„Cluster *p* (FWE): cluster level family-wise error corrected p-value; L: Left hemisphere; MNI: coordinates in Montreal Neurological Institute (MNI) space; Peak F-value: peak test-statistic of one-way ANOVA. Covariates in the analysis: age, sex, migraine attack frequency per month, sleeping problems, chronotype” (187, p. 5).

Table 6. Brain regions showing significantly increased activation in response to fearful faces in the Evening start group compared to the Morning start group (fMRI Study 1) (187).

Contrast	Group comparison	Cluster size	Cluster *p* (FWE)	Region	Coordinates (MNI)			Peak t-value
					x	y	z	
Fear-neutral	Evening > Morning	132	<0.001	L superior temporal gyrus	-45	-37	20	5.27
				L superior temporal gyrus	-57	-34	17	4.92
				L supramarginal gyrus	-60	-31	23	4.42
				L postcentral gyrus	-51	-22	29	4.26

		L supramarginal gyrus	-60	-25	23	4.19
		L supramarginal gyrus	-57	-31	32	4.03
		L supramarginal gyrus	-60	-40	29	3.75
63	0.022	R Rolandic operculum	54	-19	11	4.59
		R Heschl's gyrus	45	-25	14	4.37
		R superior temporal gyrus	42	-28	11	4.08
		R superior temporal gyrus	48	-31	14	3.99
71	0.013	L middle cingulate gyrus	-9	-40	35	4.36
		L posterior cingulate gyrus	-9	-34	32	4.32
		L middle cingulate gyrus	-15	-46	35	4.26
		L posterior cingulate gyrus	-18	-43	32	4.09
		L middle cingulate gyrus	-12	-43	38	4.06
		L posterior cingulate gyrus	-6	-43	32	4.02
		R precuneus	3	-46	41	3.73

„Cluster *p* (FWE): cluster level family-wise error corrected p-value; L: Left hemisphere; R: right hemisphere; MNI: coordinates in Montreal Neurological Institute (MNI) space, Peak t-value: peak test-statistic of the two-sample t-test. Covariates in the analysis: age, sex, migraine attack frequency per month, sleeping problems, chronotype" (187, p. 6).

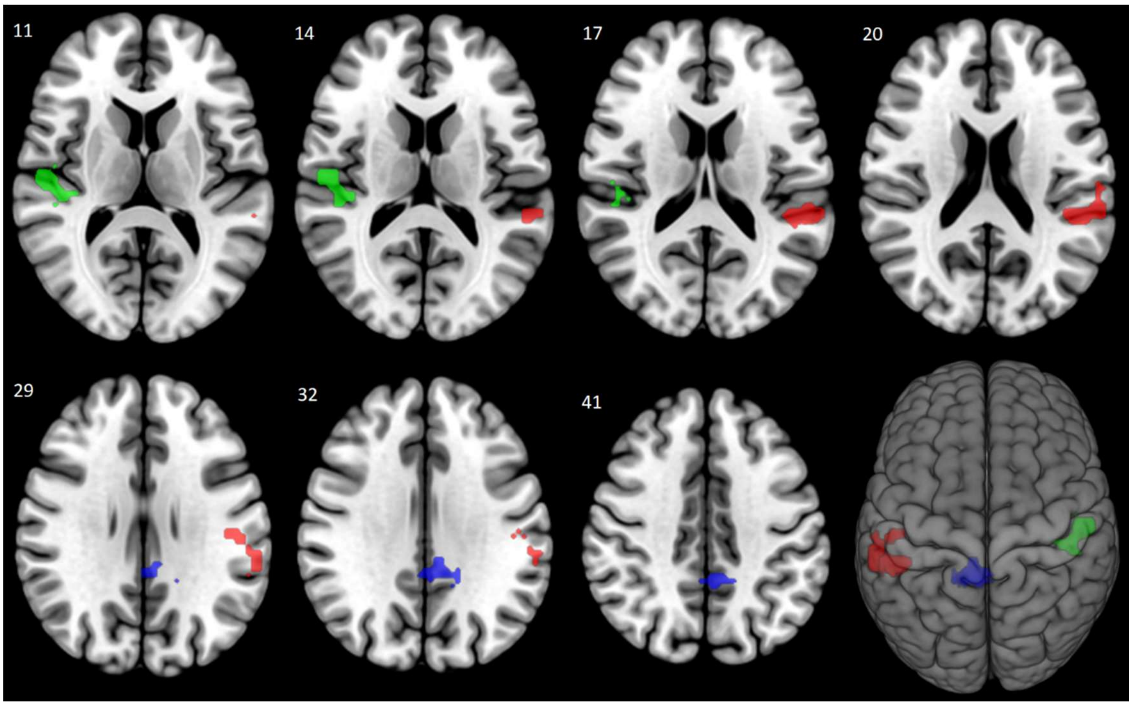

Figure 3. Brain regions showing significantly increased activation to fearful faces in the Evening start group compared to the Morning start group (fMRI Study 1) (187). The three clusters with significantly increased activation ($p_{FWE}<0.05$, with correction for multiple comparisons) are displayed (according to the order shown in Table 6) in different colors: „red (left superior temporal, left supramarginal and left postcentral gyri), green (right superior temporal gyrus, right Rolandic operculum and right Heschl's gyrus) and blue (left middle and left posterior cingulate gyri, right precuneus)" (187, p. 7).

4.2.2 Results of fMRI Study 2

4.2.2.1 Results of self-reported data

Similarly, all three M_{circ} subgroups were identified: „(1) Morning start (n=13), (2) Evening start (n=26), and (3) Varying start (n=9)" (187, p. 6). Self-reported data of the fMRI Study 2 sample and the M_{circ} subgroups are collected in Table 7. Again, females represented the the majority of the sample (89.6%) and the average age was 27.02 years (SD: 6.29). The Morning start group was significantly older compared to the other subgroups, but no other differences were detected between the M_{circ} subgroups in further self-reported data.

Table 7. Self-reported data of the fMRI Study 2 sample and results of statistical analyses comparing the M_{circ} subgroups (187).

	Total	**Morning start (M)**	**Evening start (E)**	**Varying start (V)**	**Group comparisons**
Participant number (n)	48	13	26	9	
Female (n, %)	43 (89.6%)	11 (84.6%)	24 (92.3%)	8 (88.9%)	Fisher's exact *p*=0.822
Age (mean, SD)	27.02 (6.29)	31.23 (7.81)	25.62 (5.2)	25 (4.09)	H=7.354, *p*=0.025* (M>E; U=86, *p*=0.013*; M>V; U=25, *p*=0.025*)
Attack frequency per month (mean, SD)	3.06 (2.68)	2.77 (2.1)	3.02 (2.73)	3.62 (3.46)	H=0.021, *p*=0.99
Chronotype (n, %)					
Definitely / rather morning	18 (37.5%)	6 (46.2%)	7 (26.9%)	5 (55.6%)	Fisher's exact *p*=0.474
Definitely / rather evening	28 (58.3%)	7 (53.8%)	17 (65.4%)	4 (44.4%)	
Do not know	2 (4.2%)	0 (0%)	2 (7.7%)	0 (0%)	
Sleeping problems (n, %)					
never/rarely	26 (54.2%)	6 (46.2%)	17 (65.4%)	3 (33.3%)	Fisher's exact *p*=0.342
sometimes	16 (33.3%)	6 (46.2%)	6 (23.1%)	4 (44.4%)	
often/usually	6 (12.5%)	1 (7.7%)	3 (11.5%)	2 (22.2%)	
Headache diary duration (months) (mean, SD)	2.15 (1.08)	2.23 (0.8)	2.12 (1.2)	2.11 (1.14)	H=0.773, *p*=0.68

„H: Kruskal-Wallis test statistic; SD: standard deviation; U: Mann-Whitney test statistic; *: significant" (187, p. 8).

Additionally, we compared self-reported data of the samples and M_{circ} subgroups of fMRI Study 1 and 2. The Varying start group showed significantly higher age in fMRI Study 1 compared to Study 2. Furthermore, the M_{circ} subgroups showed significantly different distribution: in fMRI Study 1, the Varying start group was represented with higher subject number, while in fMRI Study 2, the other two subgroups showed higher participant number. Details can be found in Supplementary Table 3 (187).

4.2.2.2 fMRI results

Similarly to fMRI Study 1, whole-brain activation was compared between the M_{circ} subgroups with five covariates "(age, sex, migraine attack frequency per month, sleeping problems, chronotype)" (187, p. 6-7). According to the ANOVA, no significant results were found between the three subgroups. Nonetheless, taking into account the highly unequal subject number distributions between the subgroups (see Table 7) and that our main goal was to replicate the results of fMRI Study 1, we still ran pairwise group comparisons which resulted in one nominally significant finding: increased activation to fearful faces was detected in the Morning start group in comparison with the Varying start group. The activated cluster included „bilateral paracentral lobule, right precentral gyrus and right supplementary motor area" (187, p. 7) (see Table 8). However, it has to be noted that this result not survived correction for multiple testing (considering six pairwise tests: p=0.05/6 = 0.008).

Table 8. Brain regions showing nominally significantly increased activation in response to fearful faces in the Morning start group compared to the Varying start group (fMRI Study 2) (187).

Contrast	**Group comparison**	**Cluster size**	**Cluster *p* (FWE)**	**Region**	**Coordinates (MNI)**			**Peak t-value**
					x	**y**	**z**	
Fear-neutral	Morning > Varying	97	0.012	R paracentral lobule	6	-34	65	4.32
				R precentral gyrus	18	-31	74	4.2
				R supplementary motor area	9	-19	62	3.82
				L paracentral lobule	-3	-37	68	3.69

„Cluster *p* (FWE): cluster level family-wise error corrected p-value; L: Left hemisphere; R: right hemisphere; MNI: coordinates in Montreal Neurological Institute (MNI) space, Peak t-value: peak test-statistic of the two-sample t-test. Covariates in the analysis: age, sex, migraine attack frequency per month, sleeping problems, chronotype" (187, p. 8).

5. Discussion

5.1. Discussion of the Genetic study

Our exploratory genetic study identified an association between a circadian gene variant and common migraine: *CLOCK* rs10462028 showed an interaction effect with financial hardship on a migraine phenotype in a European sample from Manchester and Budapest. Previous migraine GWASs (56-62, 223) did not found a main effect for *CLOCK* which is in line with our results. Nevertheless, with the addition of a chronic stress factor, namely financial hardship, we were able to detect a role for rs10462028 in migraine. Interestingly, other stress factors including childhood adversity and recent negative life events have not expressed the same effect. Thus, our results highlight that it is important to involve different stress factors to unfold genetic vulnerability to migraine.

5.1.1 *CLOCK* rs10462028 shows a specific interaction effect with financial hardship on migraine

Circadian rhythms are set up on a daily basis, while the effects of different stress factors and the involved pathways may vary based on their origin and timing. Childhood adversity represents a distant stress factor, while the other two are more recent ones. Recent negative life events might be in a better position to directly contribute to circadian disruption but they represent temporal situations which seems to be a substantial difference from financial hardship. Furthermore, it is a more diversified construct covering multiple stress factors in comparison with financial hardship.

Financial hardship represents a chronic stress variable – a subsitute for deprivations and social problems (192). It might create an effect similar to the chronic mild stress model, an often used paradigm in animal studies of depression (224): a lasting feeling of vulnerability, insecurity and loss of control (225). Socioeconomic disparities are major mediators of multimorbidity and mortality (226) and even experienced financial strain regardless of actual socioeconomic status shows assocations with earlier disability and increased mortality (227). Financial hardship seems to be more related to personal survival than other type of stressors (192). A report from 2018 by the Organisation for Economic Co-operation and Development (OECD) (228) showed that the average number of generations needed by low-income family members to achieve the mean income of their society in OECD countries is 4.5 generations (5 in United Kingdom, and 7 in Hungary). The same report also revealed that the offsprings tend to remain in the same socioeconomic status as their family by a high chance. All these data may suggest that financial hardship might be a persistent, trait-like factor with pervasive detrimental effects. Furthermore, similarly to our results, previous depression studies by our research group using the same database also identified a specific interacting role for financial difficulties with variants in nitric oxide synthase 1 (*NOS1*) and 5-HTTLPR (192, 225). Our identified *CLOCK* rs10462028 x financial hardship interaction affected specifically migraine without any confounding effects by lifetime unipolar depression and bipolar disorder. Additionally, our main result was replicated in both the Manchester and Budapest subsamples despite their significantly different phenotypic characteristics.

Perviously, it was highlighted that stress and sleep might have a bigger impact on the diurnal distribution of migraine attack onset compared to the endogenous circadian system (147) but others also emphasized that lifestyle factors can contribute to a desynchronization with the circadian clock which can lead to stress and more severe migraine symptoms (229). Our results suggest an interaction between a chronic stress factor and the circadian clock (represented by a *CLOCK* variant) in association with migraine.

5.1.2 Crossover-type interaction of *CLOCK* rs10462028 and financial hardship

A crossover-type interaction was found between *CLOCK* rs10462028 and financial hardship on migraine: specifically AA genotype acted as a risk factor at the most favourable financial status, but a protective factor at the most adverse financial status. Crossover-type interaction effects between genetic variants and environmental factors on diseases are not unprecedented. The environmental sensitivity hypothesis (230) postulates that in gene-enviroment studies, we should exceed the simple concept of genetic vulnerability and look at these polymorphisms as „plasticity genes" which are able to enhance the individual's susceptibility to both positive and negative external factors. With this perspective, we are able to understand results suggesting that the same genetic variant can contribute to vulnerability in an adverse situation and advantage in a favorable situation (e.g. lack of adversity). In case of psychiatric disorders, crossover interactions were found between different environmental factors and genes including *MAOA,* 5-HTTLPR, dopamine receptor D4 (*DRD4*) and interleukin-1β (*IL1β*) (230, 231). According to our results, *CLOCK* rs10462028 might also show this plasticity gene effect.

We would like to highlight that in case of crossover interaction, the opposing effects at different levels of the environmental factor can statistically „extinguish" each other if only the main genetic effect is tested. Therefore, we cannot expect the appearence of these genetic effects in GWASs. In conclusion, it is important to include relevant environmental factors in genetic studies, furthermore gene-environment studies are able to significantly contribute to the „missing heritability" occuring in GWASs (232, 233).

5.1.3 Molecular biological and functional characteristics of *CLOCK* rs10462028

CLOCK rs10462028 and another SNP, rs1801260 (in high LD with it) are able to affect miRNA binding according to our functional analysis results. Among the most relevant predicted miRNAs, the majority previously associated with different cancer types (234-237), but none of them were connected to migraine, however chronic stress might contribute to these associations since miRNAs can mediate environmental effects leading to modifications in gene expression (238). It was suggested that clock genes might play a role in cancer development via a loss of circadian homeostatis in control of functions related to energy balance, immune system and aging (239). The same processes can assist functional decline in the brain (240). Additionally, it has been suggested that clock genes control cortical plasticity during key developmental stages (241), thus might contribute to the development of brain disorders including migraine. However, in our study childhood adversity representing early negative effects not interacted with *CLOCK* on migraine.

Our in silico results suggested allele effects impairing miRNA binding (see Supplementary Table S5 (183)) possibly resulting in a higher production of CLOCK protein assuming that attenuated interactions between messenger RNAs (mRNAs) and miRNAs can increase protein levels (242). Since the previously described transcriptional / posttranslational feedback loops in the circadian clock are in strong interactions with each other, a change in any element (such as the higher level of CLOCK) can cause a considerable disturbance in the mechanism and potentially a higher susceptibility for external effects which might clarify the identified crossover interaction in our study. Financial hardship may contribute to changes in lifestyle factors resulting in a circadian desynchronization which may intensify the described process – eventually, leading to migraine susceptibility.

The C allele of rs1801260 (in Supplementary Table S5 (183)), its complementary nucleotide, the G allele is shown) associated previously with evening chronotype, delayed sleep onset and decreased amount of sleep (185, 186, 198) and it is in linkage with the A allele of rs10462028 (according to the web-based LDhap module of LDlink (243)). Thus, these associations might suggest that evening chronotype, delayed sleep onset and decreased amount of sleep might provide an advantage during financial hardship in preventing migraine, while a disadvantage during financial stability. Although, these assumptions are completely hypothetical at this moment, but the integration of chronic stress, the endogenous circadian clock and possible epigenetic processes might provide a meaningful pathway in migraine pathophysiology.

5.1.4 Limitations of the Genetic study

Detailed phenotyping represents the main strength of our study which allowed us to detect a specific gene-stressor interaction effect on migraine. Our study also had some limitations. A relatively small sample was used (especially, the subject number of rs10462028 AA genotype carriers was low), however, power analyses still showed enough power to reveal the investigated genetic. Our main result showed only a trend effect after Bonferroni correction – therefore, it is only nominally significant which should be interpreted with caution. Independent replication studies are needed to validate our findings. A cross-sectional design was used not allowing causative conclusions. Instead of medical diagnosis, we applied a self-reported questionnaire to capture migraine without screening for migraine subtypes (e.g. MO versus MA). However, the ID-migraine queastionnaire is widely used in population-based studies. Females represented the majority of our sample. Women are three times more affected by migraine compared to males (8) and also show a higher risk for sleeping problems (244) and sex-related differences in circadian timing systems are also known (245). Future studies investigating the relationship between migraine and circadian mechanisms should include data on migraine subtypes and more male participants to detect further specific pathways. Finally, we need to admit that the circadian clock system shows a much higher complexity – we only selected one polymorphism of one clock gene. Nevertheless, with this approach we were able to find a possible role for the circadian clock in migraine pathophysiology suggesting a need for further investigations regarding the associations between circadian rhythms and migraine.

5.2 Discussion of the fMRI study

We conducted two fMRI studies to investigate the association between circadian peak of migraine attack onset and interictal brain activity in an implicit version of emotional face processing fMRI task among episodic MO patients. In fMRI Study 1, migraineurs with a later circadian attack onset peak showed increased neural activity in many brain areas responding to fearful faces compared to migraineurs with an earlier circadian attack onset peak. In fMRI Study 2, again only fearful (but not happy or sad) faces induced differences in neural activation. Although this time, higher activation was detected among migraine patients with an earlier diurnal attack onset peak compared to participants with a varying attack onset peak – and this result was only nominally significant, therefore it should be interpreted with caution. Despite some important methodological discrepancies (e.g. measurement of typical circadian migraine attack onset and the used MRI scanners), results of the two studies still showed some relevant overlaps.

5.2.1 Migraine subgroups emerged with different typical circadian migraine attack onset peaks

Our results support the existence of migraine subgroups showing various typical circadian attack onset peaks. In both studies, all predefined M_{circ} subgroups were detectable: groups with a Morning, an Evening and a Varying start. However, these subgroups distributed significantly differently in the two studies. In fMRI Study 1, based on self-reported categorization, the Varying start group (45.16%) showed the highest percentage, followed by the Evening (29.03%) and the Morning start (25.8%) groups. In Study 2, based on headache diary data, the following order occurred: Evening (54.16%), Morning (27.08%) and Varying start (18.75%). As a whole, the Evening start group covered 44.3% of the two study samples, while the other two subgroups showed similar ratios: 29.1% for the Varying and 26.6% for the Morning start groups. Thus interestingly, a later attack onset peak appeared with a much higher frequency compared to an earlier attack onset peak – even with broad categories (the Morning start group represented the first, while the Evening start group the second half of the day). In contrast, most authors mention morning migraines as the most frequent ones, but recent reviews show more mixed results (146, 148). Additionally, the Varying start group showing no specific diurnal attack onset peak represented nearly one third of the samples (and around 45% of the fMRI Study 1 sample). A previous study reported an even higher ratio (almost 60%) of episodic and chronic migraineurs without a specific circadian attack onset peak (208).

5.2.2 Overlaps between results of fMRI Study 1 and 2

Firstly, the Morning start group was involved in both results showing a lower cerebral activity than the Evening start group in fMRI Study 1, while a higher activation than the Varying start group in fMRI Study 2. So, we could not replicate Study 1 results in the same direction but these results are not certainly conflicting. The question regarding the biological and/or environmental nature of this circadian phenomena in migraine still remains unanswered, at least our fMRI results suggest brain activity differences between subgroups of migraine patients showing different typical circadian migraine attack onset peaks specifically in reaction to a threatening environmental stimuli, namely fearful faces.

Secondly, neural activity differences between the M_{circ} subgroups were detected in brain regions showing similar functions including pain processing represented by middle cingulate cortex (MCC), postcentral gyrus (in fMRI Study 1), precentral gyrus and supplementary motor area (SMA) (in fMRI Study 2); and sensory processing via Heschl's gyrus, precuneus (Study 1) and paracentral lobule (PCL) (Study 2). These brain areas are thought to be involved in migraine attacks (105) and many of them also showed associations with circadian rhythm-related phenomena, namely the precuneus, pre- and postcental gyri, and posterior (PCC) and middle cingulate cortices (246-249).

Thirdly, specifically fearful stimuli (but not happy or sad emotional faces) induced brain activation differences between the M_{circ} subgroups in both fMRI studies. Similarly to pain, fear also represents an aversive stimuli, furthermore pain and fear often co-occur implying a strong connection (250) which might be based on a core aversion-related brain network processing both pain and non-painful aversive stimuli (251) involving brain areas showing overlap with our detected regions, namely the MCC, PCC (Study 1) and SMA (Study 2).

To put our findings into broader context, in the followings we discuss them in comparison with previous emotional processing fMRI results.

5.2.3 Emotional processing in migraine

Previously, two fMRI studies (116, 117) detected an increased neural activity selectively to negative (but not positive) emotional stimuli in adult migraine patients, interictally compared to healthy controls in areas of „superior and middle frontal gyrus, frontal medial cortex, frontal pole, PCC, precuneus, cuneal cortex, left cuneus, caudate, thalamus, left amygdala, right hippocampus, brainstem, cerebellum" (187, p. 9) and lingual gyrus. Recently, our research group using the same implicit emotional face processing task similarly found increased neural activation to fearful stimuli among migraineurs compared to healthy controls in right superior, middle and inferior frontal gyri, and in further areas in association with migraine frequency including pre- and postcentral gyri (118). However, these fMRI studies compared migraineurs to healthy controls, while we investigated migraine subgroups, therefore it is hard to make comparisons between our results and those previous ones. Nonetheless, our results still show overlaps with these previous data: 1) we also detected an increased activation selectively in response to a negative emotion in both of our studies, 2) identifying three brain regions, namely left PCC, right precuneus (Study 1) and right precentral gyrus (Study 2) which previously showed hypersensitivity to aversive stimuli. PCC and precuneus are also involved in the default mode network (252) and these cortical midline structures has been connected to self-referential processing (253, 254).

Among the investigated emotions, sadness is also a negative one but exclusively fear evoked an increased neural response in our studies. This specific role of fear is not surprising since fearful faces serve as a threat stimuli and are evaluated unconsciously, furthermore take priority in access to conscious visual processing (255). In Study 1, the identified brain areas showing enhanced activation included superior temporal gyrus (STG) which previously exerted a positive trend of activation responding to facial stimuli expressing growing intensity of fear in healthy controls (but not among schizophrenic patients) (214).

5.2.4 Pain processing in migraine

Among the identified brain regions, many may be also involved in the processing of other aversive stimuli including pain.

The STG (Study 1) is a region typically showing different activation during painful experiences among migraineurs (105). Further pain processing areas, many detected by previous migraine studies, were also identified by us including the MCC (Study 1) which showed enhanced activity in migraineurs in studies applying painful stimuli (256, 257). The PCC (Study 1) is not involved in the processing of direct physical pain but plays a role in secondary psychological pain processing (117, 258). The precentral gyrus (Study 2) and the postcentral gyrus (including Rolandic operculum (Study 1)) take part in the pain processing network and exert different pain-induced activations among migraineurs, interictally in comparison with healthy controls (105). The SMA (Study 2) is involved in the pain matrix, its activation in response to pain is thought to alarm the body to get away from pain (257). The supramarginal gyrus (SMG) (Study 1) can be activated by intranasal ammonia (256) and was detected in many FC studies of migraine (105).

All these data correspond to the numerous fMRI findings suggesting an enhanced interictal pain sensitivity among migraine patients as a result of recurring painful attacks and/or prolonged pain in association with migraine (105). In our study, the interictally increased activation of these pain processing areas without applying any pain stimuli might suggest an elevated sensitivity to threatening emotional stimuli (and not solely pain) among patients showing a later typical circadian migraine attack onset peak in comparison with the Morning start group (Study 1) and an earlier attack onset peak in comparison with the Varying start group (Study 2).

5.2.5 Multisensory integration in migraine

In fMRI Study 1, among the identified regions, the right Heschl's gyrus (also known as temporal transverse gyrus) contains the primary auditory cortex (259). Similarly to pain and other sensory hypersensitivity, phonophobia reaches its climax during migraine attacks but can be detected, to a lesser extent, even interictally among many migraineurs (260). Sensory stimuli are not processed simply according to modality but rather in a simultaneous, integrated way through the process of multisensory integration which might be relevant in migraine (260). For instance, the STG is mainly involved in auditory processing (261), furthermore it was also connected to olfactory processing among migraineurs, along with the PCC (262). Here, we detected elevated activation in the right precuneus which previously also associated with visual processing among migraineurs (263), moreover in the SMG which jointly with adjacent angular gyrus compose the inferior parietal lobule (IPL, or ventral parietal cortex), a center of higher cognitive functions and multimodal sensory integration (264). This higher order function of the IPL was also found to have a role in decoding high level details of dynamic emotional faces (265, 266). Thus, these results suggest that areas involved in various sensory and multisensory processing showed increased activation among migraine patients with later attack onset peak (Study 1) – proposing an enhanced level of sensory processing in response to fearful faces.

In fMRI Study 2, regions of the frontal lobe with motor functions showed increased activation in the Morning start group versus the Varying start group. The PCL includes primary motor and sensory regions controlling lower limbs and genitalia (267), furthermore it takes part in the sensorimotor network (also including precuneus), an associative cortex with important functions in multisensory integration, too (268). Recently, elevated activation of the PCL was found during migraine attack compared to interictal state (268). The revealed enhanced PCL activation might suggest a heightened level of sensory processing of fearful faces among the Morning start migraineurs compared to the Varying start subgroup (Study 2).

5.2.6 Circadian factors in variation of migraine attack onset

The processing of emotional, pain and sensory stimuli may all be under the control of the circadian clock (269, 270) and our results might suggest that diurnal distribution of these stimuli might impact migraine attack onset. Previously, studies using various pain stimuli consistently showed that the highest perceived pain intensity occurs early in the morning, furthermore others also suggested that morning migraine attacks correlate with a more severe symptom profile compared to attacks at other times (152, 271-274). Suprisingly, positive affective states show a circadian variation, while negative ones do not (275-277) suggesting the possibility that negative affect might show higher susceptibility to environmental factors (275). Negative environmental effects, especially stress are triggers of migraine attacks and as we discussed previously, an environment-dependent nature of diurnal attack onset has been already suggested in relation to sleep and stress experienced during work, school or social situations (147, 152, 278). As we also mentioned, an interaction between environmental factors and the circadian clock might also influence migraine attack onset (152).

5.2.7 Limitations of the fMRI study

Our main limitation is the low sample size impairing statistical power and generalizability. Additionally, unequal subgroup sizes (especially in Study 2) also might have impacted our results. Still, we were able to detect differences in cerebral activation between migraine subgroups even after controlling for the effects of many covariates. Our work illustrates that besides case-control studies, it is important to capture migraine heterogeneity by comparing migraine subgroups.

Because of the cross-sectional study design, we cannot draw causative conclusions regarding the effect of diurnal variation of migraine attack onset on brain activation. Self-report measures were used to capture chronotype, sleeping problems and typical circadian attack onset peak in Study 1. In Study 2, we applied a headache diary however, participation time varied between participants. Potentially, seasonal variation in migraine attacks also might have biased diary data. However, we used an exact and quite stringent criteria to differentiate between headache types (i.e. migraine versus nonmigraine type headaches) in the diary, furthermore all our participants were thoroughly screened for medical conditions, hence we controlled for the effects of comorbidities. We also need to highlight that differences between the used methods to capture typical circadian migraine attack onset could have influenced our results and make the comparison of the two fMRI studies more difficult. Namely, the retrospective question (Study 1) is less objective and more vulnerable to biases (e.g. recall bias), and migraine patients might be less able to differentiate between migraines and other headache types; while the prospective headache diary (Study 2) might have provided more reliable and current data, although this method requires much more effort by the participants, hence can increase the dropout rate and the degree of insufficient or incorrect answers.

To gain bigger sample sizes, we used broader M_{circ} subgroup categories by separately merging the first two and the last two 6-h long time slots representing the first (i.e. 00:00-12:00) and second (i.e. 12:00-00:00) halves of the day – similarly as in the study of Shin et al. (207). Future fMRI studies with larger samples could reveal more comprehensive findings with the use of four 6-h long time slots.

Finally, MRI scans were performed during the late afternoon and early evening hours. Future studies with scan sessions during the morning or forenoon hours are required to analyze the potential effect of scan session timing on brain activity of migraine groups with various circadian attack onset peaks.

5.3 A synthesis of the Genetic study and the fMRI study

We approached the association of circadian rhythms and migraine with two different strategies utilizing different methods involving different biological levels and environmental factors. Thus, it is not an easy task to synthesize all our results but identifying some similar points is still possible.

We highlighted multiple times that the association of circadian rhythms and migraine might depend more on environmental factors showing differing circadian distributions which can serve as migraine triggers including stress and sleeping problems (147, 152, 278). According to this concept, we would not expect to see direct biological associations with circadian factors in migraine. Another theory proposes an interaction between environmental migraine triggers and the endogenous circadian clock (152). According to our results, this last option seems to be more plausible. In case of our Genetic study, it is more obvious to draw such a conclusion since we detected an interaction effect between a circadian gene variant (representing the circadian clock) and a chronic stress factor in the form of financial hardship (representing a migraine trigger (83)). We also showed a possible epigenetic mechanism, specifically miRNA binding which can mediate this effect. In the fMRI Study, a similar interaction effect might be reflected in a broader sense. Brain activation differences between the groups with different circadian attack onset peaks were not found generally but specifically in association with fear processing. Although here, we were not able to directly test main and interaction effects as in the Genetic study, but similarly a specific association was found between a negative environmental element and a circadian factor. As we previously stated, the migraine brain might shows some disease-related inherent traits but also alterations through plastic changes as a consequence of recurring attacks (5, 101), furthermore the hypothalamus represents an area showing remarkably high susceptibility to go through plastic changes by replying to environmental factors (122). This brain plasticity might provide a possible pathway in mediating migraine-related environmental effects to the circadian clock.

Financial hardship and fear processing similarly represent potentially threatening situations that may alert the body to cope with negative effects. Thus, both of our studies may add further evidence to a maladaptive stress reponse (88) among migraineurs providing novel pathways involving circadian factors. As we discussed previously, stress shows strong interconnections with circadian rhythms (144) and may also contribute to the comorbidity of migraine and stress-related psychiatric disorders (85). Furthermore, a role for the circadian system in stress-related disorders was also proposed (145). The comorbidity of migraine with stress-related psychiatric disorders, primarily mood disorders and the importance of circadian rhythms in depression and bipolar disorder highly inspired both of our studies. Although, we would like to emphasize that none of our results were a simple consequence of psychiatric disorders: in the Genetic study, our results survived correction for lifetime depression and bipolar disorder, while in the fMRI study a thoroughly screened sample was used.

Finally, we would like to highlight that both of our studies demonstrated the complex multifactorial and heterogeneous nature of migraine. With detailed phenotyping, we showed a gene-environment effect (notably missing from migraine studies) and functional brain activity differences between subgroups of migraine patients. The identification of distinct pathways and specific subgroups might help us on the way to achieve personalized medicine. The inclusion of circadian factors to migraine therapy might be important, at least for some migraineurs – for instance, a drug delivery system with sumatriptan succinate was designed to accomplish a drug delivery during the morning hours with a bedtime administration to prevent morning migraine attacks (279).

6. Conclusions

In conclusion, in both of our studies, we were able to identify specific associations between circadian rhythm-related factors and migraine. Our novel findings are listed below.

Regarding our specific goals in the Genetic study, we can conclude that:

1. *CLOCK* rs10462028 shows no main effect on migraine;
2. the association of *CLOCK* rs10462028 and migraine is stress-dependent;
3. specifically, rs10462028 in *CLOCK* associates with migraine depending on the degree of a chronic stress factor, financial hardship. No other stress factors (childhood adversity, recent negative life events) show a similar interaction effect. Furthermore, the association between *CLOCK* rs10462028 and financial hardship on migraine might be explained through a mechanism in which continuous stress leads to a disturbance in functions of the circadian clock, likely via epigenetic mediation, specifically miRNA binding.

Regarding our specific goals in the fMRI study, we can conclude that:

4. circadian variation of migraine attack onset affects interictal functional brain activation patterns during emotion processing among episodic migraine without aura patients;
5. only the processing of a negative emotional, specifically fearful (but not sad and happy) stimuli evoked functional brain activation differences between migraine groups showing different typical circadian attack onset peaks;
6. migraine patients with a typical Morning attack start show altered brain activation patterns in comparison with the other circadian attack onset peak groups (a lower activation compared to the Evening start group in fMRI Study 1, and a higher activation compared to the Varying start group in fMRI Study 2), specifically in response to fear in brain regions of emotional (e.g. precuneus, posterior cingulate gyrus), pain (e.g. pre- and postcental, middle cingulate and supramarginal gyri) and sensory processing (e.g. superior temporal gyrus, paracentral lobule).

By showing these complex associations between circadian factors and migraine involving various biological and environmental elements, we consider our results as promising first steps towards a better understanding of circadian phenomena in migraine.

7. Summary

Our goal was to investigate the relationship between migraine and circadian rhythms. For this purpose, we performed 1) a Genetic study to test the main effect of a *CLOCK* gene variant and its interaction effect with stress factors on migraine; and 2) an fMRI study comparing whole brain activation differences between migraine subgroups showing different typical circadian migraine attack onset peaks in an implicit emotional faces task.

The Genetic study (n=2157) showed no main effect for *CLOCK* rs10462028 on migraine. However, a significant interaction effect was found between rs10462028 and financial hardship on migraine. This result could be replicated in the Budapest and Manchester subsamples and also survived correction for lifetime depression and bipolar disorder. Albeit, without considering interdependencies, our main result showed only a trend effect after Bonferroni-correction. No other interaction effects were detected between the SNP and childhood adversity and recent negative life events. In silico analysis revealed an effect for the genetic region tagged by the SNP on the binding of several miRNAs. Our study suggests that variation in the *CLOCK* gene associates with migraine depending on the degree of a chronic stress factor, financial hardship – probably through a mechanism in which continuous stress leads to a disturbance in functions of the circadian clock, likely by epigenetic mediation, specifically miRNA binding.

Two fMRI Studies were conducted with the same task with migraine without aura patients. Three subgroups were compared with typical Morning, Evening and Varying attack onset start. In both studies, only fearful (and no happy or sad) faces evoked significantly increased cerebral activation: among the Evening start patients compared to Morning start migraineurs (Study 1, n=31); and at a nominally significant level, among the Morning start migraineurs compared to Varying start patients (Study 2, n=48). The activated regions were mostly involved in emotional (e.g. precuneus, posterior cingulate gyrus), pain (e.g. pre- and postcental, middle cingulate and supramarginal gyri) and sensory processing (e.g. superior temporal gyrus, paracentral lobule). Our results suggest an association between circadian variation of migraine attack onset and interictal brain activity in response to threatening fearful stimuli. Circadian attack onset may be a relevant aspect to attend in future studies and prophylactic therapy of migraine.

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9. Bibliography of the candidate's publications

9.1 Publications related to the PhD thesis

- **Baksa D**, Szabo E, Kocsel N, Galambos A, Edes AE, Pap D, Zsombok T, Magyar M, Gecse K, Dobos D, Kozak LR, Bagdy G, Kokonyei G, Juhasz G. Circadian Variation of Migraine Attack Onset Affects fMRI Brain Response to Fearful Faces. Front Hum Neurosci. 2022;16:842426. IF: 3.169

- **Baksa D**, Gonda X, Eszlari N, Petschner P, Acs V, Kalmar L, Deakin JFW, Bagdy G, Juhasz G. (2020) Financial Stress Interacts With CLOCK Gene to Affect Migraine. Front Behav Neurosci. 2020;13:284. IF: 3.558

9.2 Publications not related to the PhD thesis

- Dobos D, Szabó E, **Baksa D**, Gecse K, Kocsel N, Pap D, Zsombók T, Kozák LR, Kökönyei G, Juhász G. Regular Practice of Autogenic Training Reduces Migraine Frequency and Is Associated With Brain Activity Changes in Response to Fearful Visual Stimuli. Front Behav Neurosci. 2022;15:780081. IF: 3.558

- Gecse K, Dobos D, Aranyi CS, Galambos A, **Baksa D**, Kocsel N, Szabó E, Pap D, Virág D, Ludányi K, Kökönyei G, Emri M, Bagdy G, Juhasz G. Association of plasma tryptophan concentration with periaqueductal gray matter functional connectivity in migraine patients. Sci Rep. 2022;12:739. IF: 4.38

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