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Biochemical profiling of the brain and cerebrospinal fluid in migraine syndromes

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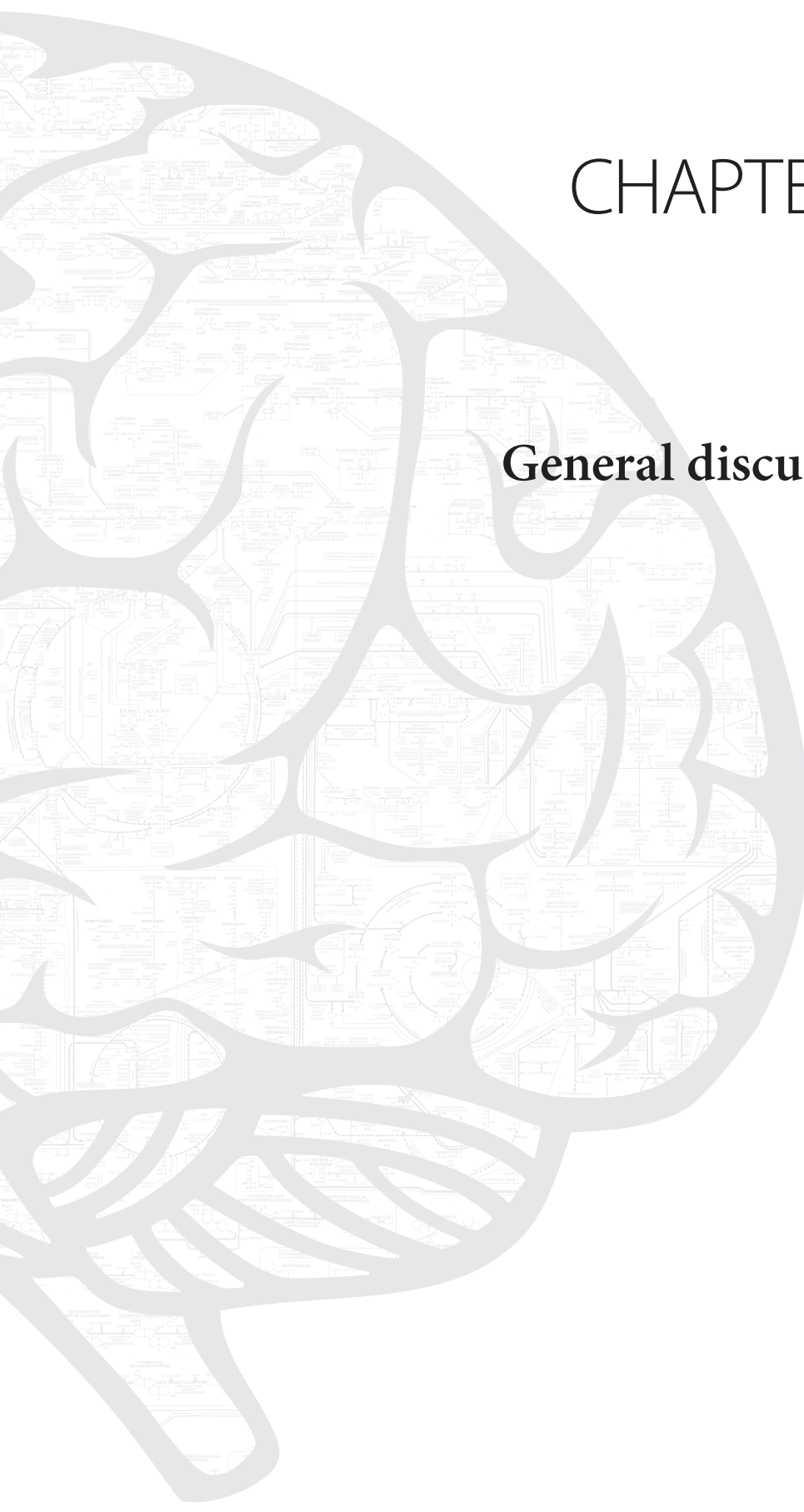


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CHAPTER 8

General discussion

General discussion

Although we have a reasonable pathophysiological explanation for most symptoms associated with migraine,¹⁻⁴ we are still missing important pieces of the puzzle. Foremost, we do not understand why and when a migraine strikes, nor do we understand why a patient can have different types of migraine attacks. There is increasing evidence that the start of a migraine attack coincides with distinct intra- and extra-cerebral biochemical changes,⁵ but our understanding of these changes is far from complete.

The aim of this thesis was to investigate biochemical mechanisms involved in migraine pathophysiology. This is challenging as the biochemistry of a living organism is a dynamic process with many regulating mechanisms that try to keep levels of biochemical compounds within functional limits.⁶ Especially when studying an episodic disorder such as migraine, it is important to realize that the biochemical constitution will likely change over the course of a migraine cycle.⁷ In addition, biochemical profiles will likely differ between anatomical regions of the brain and between different body fluids, such as blood and cerebrospinal fluid (CSF).^{8,9} By combining multiple approaches, e.g. investigating the biochemical brain composition with ¹H-magnetic resonance spectroscopy (¹H-MRS) to obtain spatially localized information of compound concentrations inside the brain and applying various types of metabolomics technologies on CSF to study various classes of compounds, we expect to obtain a better insight in biochemical processes relevant to migraine pathophysiology.

The clinical studies described in this thesis were all performed outside migraine attacks, i.e. in the interictal phase. This approach provided valuable baseline information on the biochemical constitution of the brain and CSF, and whether there are biochemical compounds with significantly different levels in migraine patients compared with healthy controls, and may give new insights in migraine pathophysiology.

Biochemical profiling of the brain in migraine patients

In **Chapters 2 and 3** we investigated the *in vivo* brain biochemistry of patients with different types of migraine. We used ¹H-MRS, a very powerful and non-invasive tool that can target specific anatomical brain regions to measure a limited number of compounds.

In vivo differences in brain biochemical profiles of hemiplegic migraine patients

In **Chapter 2** we used ^1H -MRS to investigate brain biochemical profiles in a group of eighteen hemiplegic migraine patients: two sporadic patients (SHM) and sixteen familial patients (FHM), eight of which had a known FHM mutation (five with a *CACNA1A* (FHM1) and three with a *ATP1A2* (FHM2) mutation). When compared with a group of nineteen age- and sex-matched healthy controls, the main observation was a lower concentration of total N-acetyl aspartate (tNAA) in the cerebellum of hemiplegic migraine that was most pronounced in the subgroup of FHM1 patients. The lower levels of tNAA in the cerebellum of patients with hemiplegic migraine are indicative for neuronal loss and dysfunction because tNAA is considered a marker for neuronal integrity.¹⁰ Though, only two of five FHM1 patients showed signs of cerebellar ataxia, one of which with cerebellar atrophy. A previous ^1H -MRS study in fifteen FHM1 patients also observed decreased cerebellar NAA levels but also found reduced levels of Glx (glutamate + glutamine) and elevated myo-inositol levels, which are all signs of neuronal loss and glial proliferation resulting in gliosis.¹¹ Together these observations point to neuronal loss in the cerebellum in FHM1, which can explain the ataxia and cerebellar atrophy in these patients.^{12,13} Although statistically underpowered, observations in **Chapter 2** suggest that neuronal loss in the cerebellum is not only present in FHM1 patients with cerebellar ataxia but also occurs in hemiplegic migraine patients without a *CACNA1A* mutation or with a *CACNA1A* mutation without manifested cerebellar ataxia and therefore may serve as an early biomarker for cerebellar dysfunction and/or neuronal loss in all hemiplegic migraine patients.

In **Chapter 2** we also investigated three other brain regions because of their implication in different stages of a migraine attack, namely the hypothalamus (prodrome), the occipital cortex (aura), and the pons (headache initiation), but found no biochemical difference between hemiplegic migraine patients and control subjects. A ^1H -MRS study in FHM1 patients also did not detect disease-related biochemical differences in other brain regions, i.e. the visual and parietal cortex¹¹. It should be noted however, that none of the patients was examined during a migraine attack and that the attack frequency of the patients with hemiplegic migraine was low, so biochemical changes may have been missed in the interictal phase. In this respect it is also noteworthy that in our study was the first to use high-field 7 tesla MRS for migraine research. As high-field imaging is a relatively new technique, we may not have exploited all its theoretical advantages and biochemical changes may have remained undetected. For example, we were

not able to accurately measure intracerebral glutamate concentrations which is thought to play an important role in (hemiplegic) migraine.

In vivo differences in brain biochemical profiles in migraine with and migraine without aura

For our ^1H -MRS study in migraine patients with and without aura, described in **Chapter 3**, we optimized the measurement technique to separate glutamate from glutamine that allowed accurate measurement of intracerebral glutamate concentrations. Glutamate is intimately connected to migraine pathophysiology, and despite the fact that glutamate and glutamine have distinct functions inside the brain, these molecules are metabolically closely related to each other via the glutamate-glutamine cycle. We focussed on measurements in the primary and secondary visual cortex, as these regions have been shown to be hyper-responsive in migraine patients outside a migraine attack.^{14,15} In addition, the visual cortex is the anatomical location in the brain with the highest susceptibility to develop CSD.^{14,15} We also used diffusion-weighted spectroscopy (DWS) to obtain information on the mobility of glutamate because both its concentration and subcellular compartmentalization (e.g. whether it is in synaptic vesicles or in the cytosol) is important for normal brain function.

Glutamate concentrations were higher in the visual cortex of migraine without aura patients compared with controls, but was not different between migraine with and migraine without aura nor between migraine with aura and healthy controls. Based on diffusion characteristics there was no observable difference in glutamate mobility and indirectly no differences were observed for the compartmentalization of glutamate between healthy controls and migraine patients. So we were not able to further pinpoint the intracellular location of the increased glutamate levels in migraine without aura patients. Most studies that used ^1H -MRS to investigate migraine found no biochemical difference between migraine patients and controls.¹⁶ Some studies did observe reduced NAA/tCr in the occipital lobe,^{17,18} but other studies^{19,20} including our study in **Chapter 3** could not replicate this finding. In addition, none of the previous studies was able to accurately measure glutamate; some do report on Glx (glutamate + glutamine),²¹⁻²³ but only one found elevated levels of Glx/tCr in the visual cortex of ten migraine with aura patients compared with controls.²⁴

Intracerebral glutamate concentrations in migraine with (hemiplegic) aura

The observation that we did not detect a difference, more precisely an increase, in intracerebral glutamate concentrations in patients with hemiplegic migraine and/or migraine with aura was rather unexpected. This may be due to insufficient statistical power or differences in the methodology used in **Chapters 2 and 3**. Another reason might be that our expectation is too simplistic, or more likely too optimistic to be detected with currently available technology. Cellular and animal studies of FHM mutations predict an increase in glutamate level in the synaptic cleft, perhaps already at basal level. During CSD the extracellular glutamate concentration further increases and glutamate diffuses into the extracellular space which is an important factor for propagation of the CSD wave.²⁵ Microdialysis techniques showed a 2- to 10-fold increase of extracellular glutamate during CSD compared with baseline.²⁶⁻²⁸ The highest reported extracellular glutamate concentration in humans is 2 $\mu\text{mol/L}$.²⁹ Therefore, a 10-fold increase during a CSD theoretically results in an extracellular glutamate concentration of 20 $\mu\text{mol/L}$. This translates to only 0.03% of the total amount of glutamate measured in the visual cortex as reported in **Chapter 3**. Notably, the tiny increase is much smaller than the current measurement variability of MRS. Therefore, the extracellular increase of glutamate expected during a CSD, let alone at basal levels outside an attack, is not within the detection limit of MRS imaging.^{30,31}

Biochemical profiling of cerebrospinal fluid

Because there was no clear overview of biochemical compounds measured in CSF from migraine patients, we performed a systematic review and meta-analysis described in **Chapter 4**. We aimed to identify compounds that consistently show altered concentrations in CSF from migraine patients and to assess whether these changes are also present in blood. We showed that sixty-two unique compounds have been measured in CSF from migraine patients. These compounds span a great variety of different functional classes including neurotransmitter systems, neuropeptides, endocannabinoids, neurotrophins, and cytokines. Several compounds show consistent results and have a clear implication in migraine pathophysiology as discussed below. In **Chapter 5** we performed an exploratory ¹H-NMR-based metabolomics study of CSF from a large group of patients with hemiplegic migraine, migraine with aura, migraine without aura, and healthy controls. We were able to identify nineteen different metabolites, of which only two (glutamine and glucose) were previously measured in CSF of migraine patients.

Glutamate

Our meta-analysis (**Chapter 4**) showed that glutamate concentrations are significantly increased in CSF of patients with chronic migraine. However, whether glutamate concentrations are also elevated in CSF of episodic migraine patients outside a migraine attack is unknown. Only one study showed increased CSF glutamate levels during migraine attacks in patients with episodic migraine.³² Our meta-analysis did show that glutamate levels are elevated in blood of these patients outside attacks. Elevated blood glutamate concentrations were also observed in a case-crossover study in both migraine with and migraine without aura.³³ It has not been investigated whether elevated glutamate concentrations is a specific trait of migraine or that it is also present in other primary headache disorders. Regardless, since glutamate has so many different functions in the central nervous system (CNS) it is probably not suitable to use as a diagnostic biomarker for migraine, however, it will be important to further unravel its role in the pathophysiological mechanisms of migraine.

Calcitonin gene-related peptide

Our meta-analysis (**Chapter 4**) showed that CGRP levels are significantly higher in CSF of patients with chronic migraine. CGRP levels in blood are also elevated in patients with episodic migraine, both interictal and ictal, which possibly also is the case in patients with chronic migraine.³⁴ At the moment, CGRP levels in CSF have only been measured in patients with chronic but not episodic migraine. Elevated CGRP levels have also been reported in serum and saliva both during spontaneous and nitric oxide (NO)-induced migraine attacks.^{35,36} Despite this apparent clear picture of elevated CGRP levels, there is, however, one study that did not observe an elevation of CGRP in the jugular blood during a migraine attack,^{37,38} there is debate whether the considerable delay in sample processing in the latter study could explain these negative findings. One study correlated increased CGRP levels in chronic migraine to the clinical response to onabotulinumtoxin type A (onabotA) and showed that interictal CGRP levels can predict the response.^{34,39} Responders, unlike non-responders, showed a significant decrease in blood CGRP levels. Elevation of CGRP, however, is not specific for migraine since CGRP is also released during cluster headache attacks,⁴⁰ trigeminal neuropathic pain with neurovascular features,⁴¹ and chronic paroxysmal hemicranias.⁴²

β-endorphin

Our meta-analysis (**Chapter 4**) showed that β -endorphin levels are reduced in episodic migraine outside attacks. Concentrations of β -endorphin were significantly lower in CSF and blood in both chronic and episodic migraine patients compared with controls. Individual studies regarding plasma β -endorphin level in episodic migraine, however, are conflicting;⁴³⁻⁴⁵ a case-crossover study did not show a difference between ictal and interictal β -endorphin levels.⁴⁵ So the lower β -endorphin level in patients is likely more related to migraine frequency than an indicator of migraine state (e.g. ictal versus interictal). Lower β -endorphin levels are, however, not specific for migraine as they have also been reported in other chronic pain conditions such as trigeminal neuralgia, rheumatoid arthritis,⁴⁶ neuropathic pain,⁴⁷ and, interestingly, in blood from cluster headache⁴⁸ but not chronic tension type headache⁴⁹ patients.

Nerve growth factor (NGF)

Our meta-analysis (**Chapter 4**) showed that concentrations of nerve growth factor (NGF) were increased in CSF and blood of patients with chronic migraine. Blood NGF levels were, however, not elevated in patients with episodic migraine.⁵⁰ Of the four compounds that surfaced from our meta-analysis, the evidence for NGF being relevant to migraine pathophysiology, is least convincing also because there were only five studies that measured this compound in CSF or blood of patients.⁵⁰⁻⁵⁴ Levels of NGF are elevated in patients with a variety of acute and chronic pain states including fibromyalgia,⁵¹ arthritis and spondyloarthritis,⁵⁵ endometriosis,⁵⁶ degenerative intervertebral disc disease,⁵⁷ and allergic diseases and asthma.⁵⁸ Plasma NGF levels appear not to be elevated in other primary headache disorders, such as tension type headache⁵⁹ and cluster headache.⁵⁰ Since finding an increased level of NGF in CSF or blood is not specific for chronic migraine, it is likely not a useful diagnostic biomarker. However, as is the case with CGRP, elevated levels of NGF could be used as potential biomarkers of disease evolution or prediction of treatment outcome.

Biochemical mechanisms in migraine

The role of glutamate in migraine pathophysiology

Glutamate levels in the occipital cortex are higher in patients with migraine without aura (**Chapter 3**). Our systematic literature review and meta-analysis in **Chapter 4** also showed higher glutamate levels in blood and CSF of migraine patients. Glutamate is the major excitatory neurotransmitter in the central nervous system and has since long been suggested to play an important role in migraine pathophysiology but its role in the various migraine subtypes and various phases of a migraine attacks is less clear.

Migraine aura is thought to be caused by cortical spreading depression (CSD)^{60,61} that is believed to originate in metabolically unimpaired tissue upon excessive release of potassium and glutamate into the extracellular space at a level that exceeds the buffering and removal capacity of the tissue.⁶² In experimental animals, CSD can be induced by topical application of glutamate and blocked by NMDA antagonists.^{28,63} The extracellular glutamate concentration increases during CSD, as shown by microdialysis experiments in rats.²⁸ Genetic evidence for the involvement of glutamate in migraine came from the discovery of mutations in the three genes for FHM that point towards increased glutamatergic neurotransmission and an excess of glutamate in the synaptic cleft.²⁵ The importance of neuronal glutamate release was further proven in a transgenic mouse model with a human pathogenic FHM mutation in a voltage-gated $\text{Ca}_v2.1$ calcium channel subunit⁶⁴ that showed a normalisation of the propensity for CSD when glutamatergic neurotransmission in the cortex was normalised by a $\text{Ca}_v2.1$ -specific calcium channel blocker.^{2,65}

Several other brain structures are relevant to understanding the role of glutamate (and its receptors) in migraine. For instance, pain-relay structures, such as the trigeminal ganglion, trigeminal nucleus caudalis and thalamus all contain glutamatergic neurons.⁶⁶ Moreover, glutamate receptors are present in superficial laminae of the trigeminal nucleus caudalis, neurons in the trigeminal nucleus caudalis, thalamus and other pain-related areas.⁶⁷⁻⁶⁹ Glutamate levels were shown to increase in the trigeminocervical complex after stimulation of dural structures and in the ventroposteromedial thalamic nucleus following experimentally noxious stimulation along the trigeminal nerve in rats.⁷⁰⁻⁷² Notably, glutamate receptor antagonists (especially targeting the Kainate iGluR) reduce nociceptive input in animal studies⁷³ and have proven as effective as sumatriptan in aborting migraine attacks in humans.⁷⁴

Glutamate and its relation to energy metabolism

Most of the links between migraine and glutamate focus on glutamate's role as an excitatory neurotransmitter. However, glutamate is also closely related to cellular metabolism in two important ways.

Firstly, as an alternative oxidizable substrate, glutamate serves as an important energy reserve.^{75,76} Glutamate is a central compound of cellular metabolism and its carbon skeleton is diverted to many diverse anabolic and catabolic pathways. This can only occur after glutamate is sequestered from the TCA cycle at the level of alpha-ketoglutarate.²⁹ On average a glutamate molecule is recycled one or two times in the glutamate–glutamine cycle before it is degraded in the TCA cycle.⁷⁷ *De novo* synthesis of glutamate accounts for approximately 20% of cerebral glucose metabolism, all of which initially occurs in astrocytes. Another argument that glutamate is involved in energy metabolism comes from recent functional MRS (fMRS) studies at 7 tesla that found a small, but marked, 2–3% increase of glutamate concentrations in the occipital lobe of individuals after visual stimulation.^{78–80} This glutamate increase coincided with a decrease in aspartate, which directly links to the transamination reaction between glutamate and aspartate, which is a critical step in the malate-aspartate shuttle. An increased flux in this shuttle is indicative for an increased oxidative metabolism.⁸¹ Therefore, elevated levels of glutamate observed during fMRS experiments are thought to reflect foremost an increase in oxidative metabolism.^{81,82}

Given that glutamate is a key component of the above mentioned metabolism, high levels in brain (**Chapter 3**) and CSF (**Chapter 4**) do not necessarily reflect altered glutamatergic neurotransmission but may instead reflect changes in metabolic demand. The latter explanation would be more in line with studies using ³¹P-MRS that consistently showed that different types of migraine seem to share a multi-systemic impairment of energy metabolism in both skeletal muscle⁸³ and brain.⁸⁴ The finding of an interictal deficit of brain and muscle bioenergetics has led to the hypothesis that a bioenergetics defect may be an intrinsic feature of migraine.⁸⁵ A compromised bioenergetics state may enhance the probability for developing an attack, especially when brain energy demand is increased and/or the supply of oxidizable substrates and oxygen is temporarily diminished.

Secondly, the excitatory function of glutamate as neurotransmitter is dependent on maintaining a strict compartmentalization of glutamate with high concentrations inside the cell and low concentrations outside the cell. This compartmentalization is maintained by uptake of glutamate by neurons, astrocytes,

and the blood-brain barrier (BBB), this process is highly energy-dependent.⁸⁶ So the distribution of glutamate is in dynamic equilibrium and highly sensitive to changes in the energy supply. Because of the high rates of glutamate release, inhibition or impairment of glutamate uptake can lead to high extracellular levels of glutamate within seconds.⁷⁵ High extracellular glutamate can activate glutamate receptors causing further release of glutamate, this activation increases energy consumption and Na⁺ and Ca²⁺ influx, which in turn can impair adequate glutamate uptake by glial excitatory amino acid transporters, and possibly even reverse these transporters causing further increase.⁷⁵ Such a circular process is also thought to play an important role in the initiation of CSD where high extracellular glutamate can activate glutamate receptors causing further glutamate release.³

The role of calcitonin gene-related peptide in migraine pathophysiology

CGRP is elevated in migraine patients and may serve as a biomarker to predict the treatment response to botulinum toxin (more precise onabotA) in chronic migraine (**Chapter 4**). CGRP is a neuropeptide that is widely expressed in the peripheral and central nervous system.⁸⁷⁻⁸⁹ CGRP is mainly localized in thin unmyelinated nerve fibres (C-fibres) in the cerebral vasculature, middle meningeal artery, and dura matter, which originate from the trigeminal ganglion.^{90,91} Its vascular and neuronal effects have been implicated in different aspects of migraine pathophysiology.³⁵ Activation of the trigeminovascular system (TGVS) releases several vasoactive neuropeptides, especially CGRP, from presynaptic nerve terminals.⁹² The trigeminal CGRP nerves trigger a protective, reflex vasodilation that is thought to counteract prolonged vasoconstriction of cerebral arteries supplying blood to the brain.⁹³ In addition, given the hypothesis that repeated TGVS activation sensitizes peripheral and central pain pathways which might lead to migraine chronification,⁹² CGRP is a very important compound in migraine pathophysiology. Notably, this has resulted in the creation of CGRP antagonists which are very effective in aborting migraine attacks.⁹⁴

The role of beta-endorphin in migraine pathophysiology

Levels of β -endorphin are lower in CSF and blood from both chronic and episodic migraine patients compared with controls (**Chapter 4**). The neuropeptide β -endorphin has morphine-like effects.^{95,96} Plasma and CSF levels of β -endorphin reflect activity of two functionally separate opioid systems.⁹⁷ Peripheral β -endorphin is produced in the pituitary and is circulated via the blood stream

to interact with specific opioid receptors located throughout the body where it produces analgesia by inhibiting the firing of peripheral somatosensory fibres.⁹⁵ Central β -endorphin is produced by hypothalamic pro-opio-melanocortin (POMC) neurons for release inside the CNS and the CSF. β -endorphin in CSF uses synaptic (and non-synaptic paracrine) communication via volume transport mechanisms provided by the flowing CSF⁹⁸ and plays a complicated role in controlling pain and other body functions. It is puzzling how lower β -endorphin levels may link to migraine aetiology, since β -endorphin levels do not seem to decrease during a migraine attack.⁴⁵ Although the endogenous opioid system has downstream effects on pain modulation, there is no evidence to suggest that it has an important role in the pathogenesis of the attack itself. The intriguing finding that the evolution of episodic to chronic migraine is concomitant with progressively reduced levels of β -endorphin CSF led to the hypothesis that chronification of the disease is linked to a deterioration of the anti-nociceptive system, biochemically sustained by a progressive reduction of CSF β -endorphin levels.⁹⁹

The role of neurotrophic factors in migraine pathophysiology

NGF is increased in CSF from chronic but not episodic migraine patients (**Chapter 4**). NGF is a member of the neurotrophin (NT) superfamily that also includes brain-derived neurotrophic factor (BDNF), NT-3, and NT-4/5.¹⁰⁰ NGF levels are elevated in a variety of acute and especially chronic pain states and is thought to modulate pain and hypersensitivity due to its effects on nociceptive neuron function, either directly or indirectly via an action on mast cells.¹⁰⁰ NGF receptor TrkA is expressed on nociceptive neurons¹⁰¹ that also express TRPV1.¹⁰² TRPV1 is a non-selective ligand-gated cation channel that reacts to mechanical, thermal and chemical stimuli derived from extra- and intracellular sources. An increase in NGF expression affects the function of TRPV1 in two ways, first via NGF binding to the TrkA receptor on nociceptive neurons, which activates phospholipase C that in turn leads to TRPV1 sensitization,¹⁰³ and second by increasing TRPV1 expression.¹⁰⁴ In addition it was also shown that the P2X purinergic receptor sensitivity may be affected by mediators such as CGRP, NGF, and BDNF.¹⁰⁵ More specifically, the sensitization of pain-transducing P2X3 receptors is modulated by the migraine mediators CGRP and NGF.¹⁰⁶ So increased levels of NGF tend to decrease the threshold for action potentials in nociceptive neurons, which may be a relevant factor in pain chronification.

How can we biochemically understand a complex disease like migraine?

From a clinical perspective, migraine is a very complex syndrome because of its episodic nature and the many symptoms that can be present during different phases of the migraine cycle. Currently, there are several well-established pathophysiological mechanisms that are able to explain most of the migraine symptoms. However, how these different mechanisms are connected is still largely unknown. From a biochemical perspective, migraine is also very complex because there are numerous compounds that may interact to produce the different migraine symptoms and phases of the migraine cycle. Our current understanding of the underlying biochemistry is very incomplete. The systematic review in **Chapter 4** clearly showed that biochemical research in migraine until now has been unstructured and scattered over different chemical classes, anatomical locations and body fluids, subtypes of migraine, and phases of the migraine cycle. Only few of the findings have been replicated and information on the sensitivity and specificity of potential diagnostic biomarkers is scarce.

To better understand the biochemistry underlying migraine pathophysiology and to identify useful biochemical biomarkers, there are two general approaches that can be used. The first approach is to perform hypothesis-driven biochemical research, which requires a pathophysiological framework consisting of compounds known to be involved in migraine pathophysiology. Ideally, compounds are ordered in a pathophysiological relevant way related to their specific place in the migraine cycle. The second approach is to perform non-hypothesis-driven biochemical research, in a structured way, exploring different compound classes in different phases of the migraine cycle. A more structured approach is necessary in order to identify relevant molecules and to be able to attribute these to (a) specific phase(s) of the migraine cycle and the underlying pathophysiological mechanism(s). Regardless of the approach taken, a theoretical framework of migraine pathophysiology is needed in order to make sense of identified biochemical changes.

One such framework for migraine pathogenesis is based on accumulating evidence that shows that the interplay between brain parenchyma and the trigeminovascular system (TGVS) being important for the initiation of migraine attacks.¹⁰⁷ From experimental animal research on CSD we obtained detailed information on biochemical changes that occur during a CSD.³ For instance, various molecules are released into the interstitial space (e.g. K^+ , protons, arachnidonic acid, nitric oxide, adenosine, ATP and glutamic acid), many of them

have noxious potential.^{3,107} There is compelling evidence that if these noxious molecules reach sufficiently high levels, they diffuse to pial vessels where they are able to activate a network of trigeminal axons (the visceral afferent system of the brain), which leads to lateralized pain.¹⁰⁸⁻¹¹¹ Subsequent discharge of second-order neurons causes local neurogenic inflammation and mast cell degranulation within the dura mater layer of the meninges.¹¹⁰ A recent study in wildtype mice revealed that CSD causes opening of neuronal Pannexin1 (Panx1) mega channels that lead to neuroinflammatory responses that involve neuronal caspase 1 activation and neuronal release of high-mobility group box 1 (HMGB1) into the extracellular space, nuclear factor κ B activation in astrocytes, and ultimately trigeminovascular activation.¹¹² Studies like this one emphasize that migraine is likely to be caused by interactions of cells in the brain parenchyma and the meninges, which are more intertwined and chemically connected than previously thought. It seems that a 'brain in stress' is able to communicate with the meningeal vasculature and that this is important for the pathogenesis of migraine attacks. Notably, these biochemical changes in the brain parenchyma can (theoretically) be measured using different biochemical imaging modalities such as ^1H -MRS (glutamine and glutamate; applied in **Chapters 2 and 3**), ^{31}P -MRS (energy metabolism), ^{13}C -labelled ^1H -MRS (metabolic flux) and PET. And since a lot of biochemical compounds in the brain parenchyma are able to diffuse from the interstitial space into the CSF compartment,¹¹³ it would also be very interesting to identify changes in biochemical profiles of CSF over the course of a migraine cycle.

Practical considerations for biofluid sampling in migraine

Cerebrospinal fluid to study the biochemistry of migraine pathophysiology

The studies described in **Part 2 and 3** of this thesis were all aimed at investigating the composition of CSF. From a bio-analytical point of view, CSF is an ideal body fluid because it is less complex in its biochemical constitution than blood, has a smaller biological variability⁸, and is metabolically stable (as shown in **Chapter 6**) due to the fact that there are fewer enzymes presents and hardly any cells that might have unwanted biochemical interactions.¹¹⁴ Still CSF contains hundreds of molecules.¹¹⁵ There are several important arguments why CSF also is an ideal body fluid to study migraine and CNS disorders in general.

The CSF compartment lies between the endothelial blood-brain barrier (BBB) and the epithelial blood-cerebrospinal fluid barrier (BCSFB); the CNS parenchyma is only separated from the CSF by the glia limitans, which is composed of astrocytic foot processes and a parenchymal basement membrane.¹¹⁶ Because astrocytes of the glia limitans are connected via gap junctions it is not considered part of the BBB, so material can readily pass between the foot processes from the brain parenchyma into CSF.¹¹³ The traditional view is that CSF is produced by the choroid plexus, circulates through the ventricles, the cisterns, and the subarachnoid space to be absorbed into the blood by the arachnoid villi.^{117,118} However, it was recently discovered that a pulsatile to and from movement throughout the entire brain is present with local fluid exchange between blood, interstitial fluid, and CSF.¹¹⁹ This additional CSF circulation around blood vessels that penetrates from the subarachnoid space into the Virchow Robin spaces seems to provide both a drainage pathway for waste molecules from the brain and a site for interaction of the systemic immune system with the brain, and as such seems to function as a cerebral lymphatic system.^{118,120} These new insights in CSF physiology and function are of major importance to migraine pathophysiology because if the CSF functions as vehicle for chemical messaging, this is where biochemical changes relevant to migraine pathophysiology may more readily be detected.

Practical considerations for CSF collection

A technical limitation to the use of CSF as a bio-fluid for biochemical research is the open sampling system that is normally used, which has the risk of unwanted contamination of CSF samples (as described in **Chapter 5**). It is, therefore, advised not to use volatile substances such as ethanol in the surroundings of CSF sampling, which is challenging in a hospital setting where ethanol-containing substances are widely used as disinfectant. Another practical limitation of collecting CSF via lumbar puncture is that there are misconceptions among the general lay public and even (non-neurological) physicians, leading to fear for the procedure. This makes the recruitment of study participants a challenging task. However, if executed by an experienced clinician, the burden of a lumbar puncture to patients is minimal and the risk of (serious) complications is almost negligible when patients were appropriately pre-screened for contra-indications.¹²¹ Admittedly, there remains the risk for post-dural puncture headache (PDPH) which is alike for migraine patients and healthy controls; the risk is markedly reduced with the use of a-traumatic needles.¹²²⁻¹²⁴ Fortunately, there is a noticeable change in the way researchers and

ethics committees judge the collection of CSF which will make the procedure more feasible. Since the importance of CSF to study the pathophysiology of CNS disorders has become more evident, there is less reticence, and several large CSF studies in for example Alzheimer's and Parkinson's disease are ongoing, some collecting multiple CSF samples over several years from the same subject aiming to find biochemical markers for disease progression.^{125,126}

Biomarkers in blood: advantages and disadvantages

Although CSF may represent a better source for biomarker discovery for diseases of the CNS, as it is in closer proximity to the tissue of disease origin and may better represent its neurochemical state than other body fluids (e.g. saliva, blood, urine)⁸, its collection is still cumbersome and more invasive than the collection of for instance blood, especially from control individuals.

A major advantage of blood samples is that patients can be followed up and screened over multiple time points, for example at different phases of a migraine cycle. Blood is the most commonly used bio-fluid in clinical chemistry due to the minimally invasive nature of collection, its rich metabolome¹²⁷ and proteome¹²⁸, and its reflection of the metabolic state of the entire organism. However, intracerebral compounds, in brain disorders like migraine, that are released in blood are diluted in 4 L as compared to 150 mL when entering CSF. Consequently, biochemical changes need to be profound to allow detection in blood. All in all, biomarker data from sampling blood will represent diluted steady state concentrations of molecules that are affected by complex production and numerous matrix effects, transport and clearance kinetics. Regardless, it is of importance to know how metabolic profiles of CSF and blood correlate, which is challenging in itself given the various brain-fluid barriers (blood-brain, blood-CSF, brain-CSF) that have a profound impact on identifying differences in metabolic profiles between CSF and blood. Whether choosing CSF or blood is better as bio-fluid for biochemical research of migraine remains unclear. After all, a disease biomarker initially discovered in CSF will probably cause difficulties to get translated in a broadly used blood-based test, and a disease biomarker initially discovered in blood may be a weak reflection of the disease process in the brain.

Future perspectives

Future research efforts based on spectroscopy

Because high-field MRS is a relative new technique, it is subject to rapid development and ongoing technical improvements. Potential future research may comprise of further robust, quantitative and multinuclear brain MRS studies. Multinuclear brain MRS studies, combining ^1H -MRS and ^{31}P -MRS, could address the question whether there is a relation between the previously found energy metabolism and the higher glutamate concentrations reported in **Chapter 3**. Performing such studies before and during the onset of for instance an NTG-provoked migraine attack will give valuable information on biochemical changes in migraine during different stages of the migraine cycle. This could specifically test the hypothesis whether changes in glutamate concentration are related to the onset of migraine attacks. To investigate the link between CSD and *in vivo* glutamate it would be interesting to investigate whether glutamate levels are changing during the course of an aura. This is practically challenging, but the scientific gain is high because that would provide direct evidence in humans whether and to what extent the glutamatergic system is actually involved in the aura phase in humans. To further investigate the link between elevated glutamate concentrations and altered cortical excitability in migraine patients a combination of visual stimulation, visual-evoked potentials and functional MRS would also be interesting. Recent advances in fMRS make this kind of research feasible and hopefully will provide direct insight into brain metabolism by investigating the metabolic responses of the brain to physiological stimuli. This could provide objective proof that an altered cortical excitability in migraine is also reflected at the biochemical level.

Future research efforts based on body fluid biomarker discovery

Biomarker discovery studies have an exploratory setup with numerous compounds measured in a single run. Although several candidate neurochemical biomarkers have been identified for different neurological diseases, including migraine, translation into clinical application is hindered by the small sample size of current studies, multiplicity issues, and the lack of independent verification of promising findings. In order to push knowledge of migraine biochemistry forward, it will be extremely important for future biochemical studies to combine hypothesis-driven and non-hypothesis-driven studies. Hypothesis-driven studies in larger study populations can be aimed at replicating previous findings from

smaller studies, which is desperately needed. Non-hypothesis-driven studies can basically screen as many biochemical compounds as possible to provide valuable novel clues for migraine disease mechanisms that may have escaped our attention. There is increasing evidence that noxious compounds originating from the brain are causing activation of the trigeminovascular system via activation of primary afferent nociceptive neurons that innervate cephalic tissues, primarily the cranial meninges and their related blood vessels.^{3,107,129} Despite this compelling evidence from animal studies, direct evidence in humans is still lacking.^{130,131} However, it might be possible to test the hypothesis of noxious stimuli directly in humans. The recent discovery, that the CSF also functions as the brain's lymphatic system and flows freely from the arachnoid space along blood vessels into brain tissue, makes the CSF a very important body fluid to study in the context migraine. Because CSF reflects, in part, the biochemical constitution of the ECF of the brain, it might be possible to detect the noxious compounds from the brain in CSF. It will therefore be important to study different phases of the migraine attack, especially during a migraine attack because that is where the biggest changes are expected. To know whether potential neurochemical biomarkers are specific for migraine, or whether they are generic for headache disorders, it will also be important to collect CSF and blood from patients suffering from other primary headaches. These new research approaches are likely to substantially enhance our knowledge of the pathogenesis of migraine which should result in the identification of dearly needed novel prophylactic treatment targets, predictive biomarkers for treatment response, and objective diagnostic biomarkers to facilitate the reliable differentiation of migraine from other primary headache syndromes that require different treatment approaches.

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