The evolving genetic and pathophysiological spectrum of migraine

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8.0

General Discussion

Migraine is an episodic neurovascular disorder that is characterized by severe headache, autonomic, and other neurological symptoms.[1] The identification and characterization of migraine genes and molecular pathways will help increase our knowledge of migraine pathophysiology. As the identification of genetic susceptibility factors for complex disorders is particularly challenging, this thesis also focused on alternative approaches to improve our insight in the molecular mechanisms of migraine. These approaches range from genetic and functional studies of gene mutations in rare monogenic migraine subtypes (e.g., hemiplegic migraine and other disorders with a high migraine prevalence) to genetic studies in a Dutch genetically isolated population, and gene expression studies in transgenic migraine mouse models.

8.1 Hemiplegic migraine: a monogenic form of migraine with aura

Genetic studies in FHM: genes encode ion transporters

A successful approach to identify genes and unravel pathways for migraine has been the investigation of monogenic subtypes of the disease. The best example is Familial Hemiplegic Migraine (FHM), a rare form of migraine with aura. FHM can be considered a model for the common forms of migraine because the headache and aura features, apart from the hemiparesis, are identical[2] and two-thirds of FHM patients have, in addition to attacks of FHM, also attacks of common non-hemiplegic migraine.[3] Linkage studies in FHM families resulted in the identification of three FHM genes; *CACNA1A* (FHM1), *ATP1A2* (FHM2) and *SCN1A* (FHM3). The fact that not all FHM families are linked to one of these three known FHM loci implies that there are additional FHM genes.

The *CACNA1A* FHM1 gene encodes the α1 subunit of $Ca_V2.1$ channels.[4] Until now, 28 different FHM1 missense mutations have been described (figure 1). FHM1 mutations are associated with a broad spectrum of clinical features. Besides hemiplegic migraine, FHM1 patients can have cerebellar ataxia[5-9] and/or epilepsy.[10,11] FHM1 patients carrying the S218L mutation can have a particularly severe phenotype with attacks that can be triggered by mild head trauma and that in some cases may lead to fatal, cerebral edema and coma.[12-15] In **Chapter 2.1**, we report on a monozygotic twin pair with a novel *de novo CACNA1A* V1696L mutation. This mutation causes an overlap syndrome between FHM and alternating hemiplegic of childhood (AHC), a severe neurological childhood disorder that shares several clinical features with FHM. This study provided the first evidence that a mutation in the *CACNA1A* gene can cause an AHC/FHM overlap syndrome.

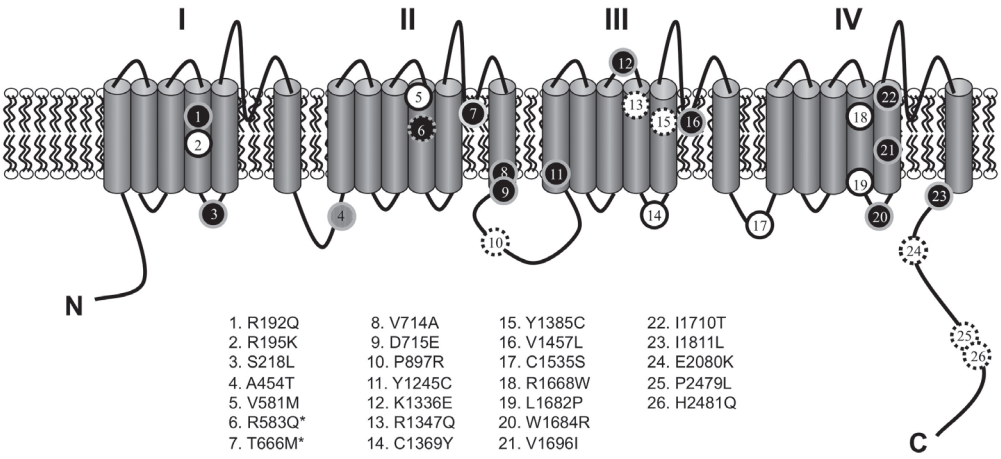
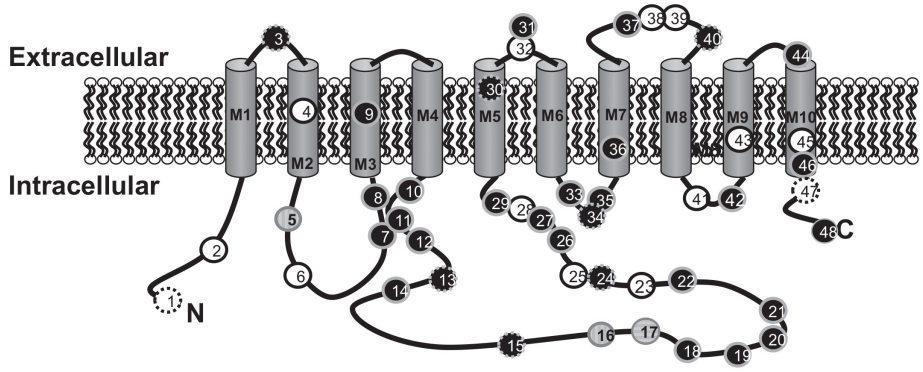


Figure 1. *Mutations in the α1 subunit of the voltage-gated $Ca_V2.1$ Ca^{2+} channel encoded by the FHM1 CACNA1A gene (Genbank Ac. nr. X99897). The protein is located in the plasma membrane and contains four repeated domains, each encompassing six transmembrane segments. Symbols: Circle with solid line = FHM, circle with dotted line = SHM. Asterisk = Mutation for which also SHM was reported, black circles mutation was tested for functional consequences, white circle mutation was not tested for functional consequences*

The second FHM gene, *ATP1A2* (FHM2) encodes the α2 subunit of sodium-potassium pumps.[16] To date many mutations in the *ATP1A2* gene have been described (Figure 2) and the vast majority is associated with pure FHM without additional clinical symptoms.[16-20] However, some are associated with FHM and cerebellar problems[21], benign familial neonatal convulsions (BFIC)[22], epilepsy[18,23], or permanent mental retardation.[18,24] In **Chapter 2.2** we present a novel *ATP1A2* G855R mutation with functional consequences. Besides hemiplegic migraine, mutation carriers can also have febrile seizures. With this study, we further expanded the clinical spectrum associated with *ATP1A2* mutations. Also non-hemiplegic migraine phenotypes were found to be associated to some *ATP1A2* mutations, including basilar migraine[25] and even common migraine[26], although causality has not been established for all mutations by testing their functional consequences.



1. Y9N	11. V362E	21. V628M	31. P796S	41. 935K-940Sdelinsl
2. R65W	12. T376M	22. R689Q	32. P796R	42. R937P
3. E120A	13. R383H	23. E700K	33. M829R	43. S966fs
4. V138A	14. T415M	24. N717K	34. R834X	44. P979L
5. E174K	15. E492K	25. D718N	35. R834Q	45. D999H
6. R202Q	16. C515Y	26. M731T	36. G855R	46. R1002Q
7. T263M	17. R548H	27. R763H	37. W887R	47. Y1009X
8. I286T	18. R593W	28. R763C	38. G900R	48. X1021R
9. G301R	19. A606T	29. L764P	39. E902K	
10. T345A	20. G615R	30. P786L	40. R908Q	

Figure 2. *Mutations in the α2 subunit of the Na^+,K^+ ATPase encoded by the FHM2 ATP1A2 gene (Genbank Ac. nr. NM_000702). The protein is located in the plasma membrane and contains ten transmembrane segments. Symbols: Circle with solid line = FHM, circle with dotted line = SHM, circle with horizontal striped pattern = basilar-type migraine, circle with vertical striped pattern = common migraine. Asterisk = mutation for which also SHM was reported, black circles = mutation was tested for functional consequences, white circle = mutation was not tested for functional consequences.*

Finally, the *SCN1A* (FHM3) gene encodes the α1 subunit of neuronal $Na_v1.1$ voltage-gated sodium channels.[27] The *SCN1A* gene is a well-known epilepsy gene with over 100 mutations that are associated with childhood epilepsy, i.e., severe myoclonic epilepsy of infancy (SMEI) or generalized epilepsy with febrile seizures (GEFS+).[28,29] Only five FHM3 mutations (Figure 3) have been identified. First confirmation of *SCN1A* as a migraine gene is described in **Chapter 2.3**. The *SCN1A* L1649Q mutation was identified in a Caucasian North American FHM family with 'pure' FHM, without cerebellar signs or epilepsy symptoms. The third FHM3 mutation was identified in a FHM family in which three out of five carriers of the L263V mutation had generalized tonic-clonic epileptic attacks, occurring independently from their hemiplegic migraine attacks.[30]

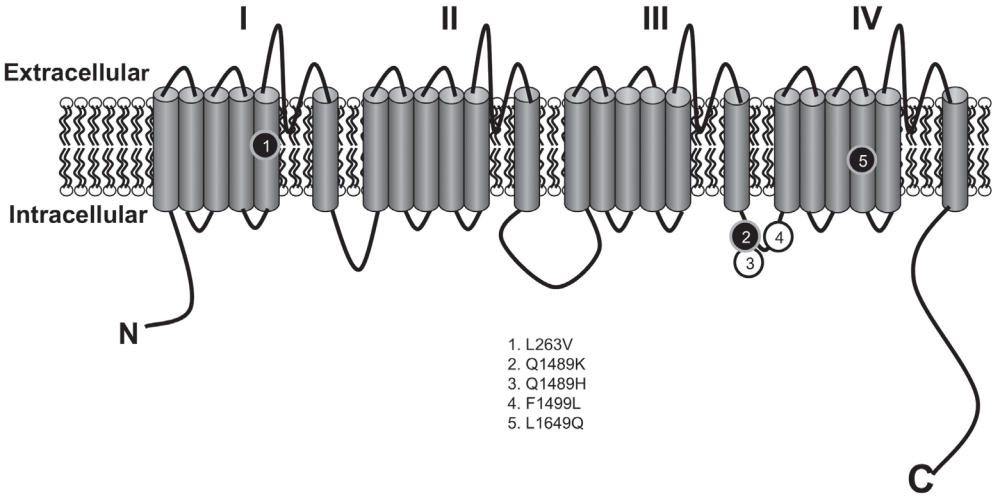


Figure 3. *Mutations in the α1 subunit of the voltage-gated $Na_V1.1$ Na^+ channels encoded by the FHM3 SCN1A gene (Genbank Ac. nr. NM_006920). The protein is located in the plasma membrane and contains four repeated domains. Symbols: Black circles = mutation was tested for functional consequences, white circle = mutation was not tested for functional consequences.*

Functional studies of FHM mutations

Functional consequences of FHM gene mutations have been studied in cellular and animal models. At the single channel level, FHM1 mutations were shown to cause opening of $Ca_V2.1$ channels at more negative voltages and have an enhanced channel open probability, compared to normal channels.[31-33] These *gain-of-function* consequences predict an increased calcium influx and increased neurotransmission. The generation and analysis of transgenic knock-in migraine mice with human pathogenic FHM1 mutations (i.e., FHM1 R192Q and FHM1 S218L mutant mice) revealed *gain-of-function* consequences of these mutations[34,35]; not only with respect to calcium influx, but also for spontaneous and evoked neurotransmission at the neuromuscular junction, a synapse in the peripheral nervous system where transmitter release is predominantly determined by $Ca_V2.1$ calcium channels. Most relevant for migraine pathophysiology, in both strains of mutant mice, the threshold for inducing a CSD was lowered and the propagation velocity of the CSD wave was increased. Whereas FHM1 R192Q migraine mice have no overt phenotype, FHM1 S218L mice exhibit cerebellar ataxia, seizure susceptibility, and head trauma induced brain edema as also seen in FHM1 S218L patients. These observations indicate that the FHM1 mutant mice are useful models to study the pathophysiology of migraine in vivo. Future studies have to reveal exactly how a lower activation threshold of mutated $Ca_V2.1$ calcium channels can lead to an episodic disease. One may envisage that only when the stimulus is strong (for instance with repetitive neuronal firing) and the threshold is temporarily lowered (for instance by hormonal changes), hyperexcitability of neurons in a susceptible brain leads to a cascade ending with a full-blown migraine attack.

The functional consequences of a large number of *ATP1A2* mutations causing either FHM or SHM have been investigated in various in vitro assays. Many were shown to be dysfunctional as they – unlike wildtype - were not (or only partially) able to rescue cell survival in assays in which endogenous sodium potassium pumps were inactivated by the drug ouabain.[36] In these assays, wildtype or mutant α2 Na^+,K^+- ATPase cDNAs were made insensitive to the ouabain challenge. More detailed functional studies revealed that FHM2 mutations G301R, T376M, L764P, W887R, R855R lead to non-functional proteins[37,36,38,39] or sodium potassium pumps with partial activity with decreased (in the case of T345A and A606T) or increased (in the case of R689Q, M731T, R763H, and X1021R) affinity for potassium.[40,41,38] For five FHM2 mutations (i.e., R383H, R689Q M731T, R763H, and R834Q) a reduced turn-over rate was shown. Since FHM2 mutations compromise pump function, *Atp1a2* knockout mice may serve as a good model for FHM. However, *Atp1a2* knockout mice that lack the α2 subunit have a very severe phenotype and die immediately after birth because of their inability to take a first breath.[42,43] Heterozygous mice are viable and exhibit enhanced fear and anxiety following conditioned fear stimuli.[42] These mice have not been evaluated as potential migraine mouse models.

The functional consequences of three FHM3 mutations have been investigated.[27,44] Whereas early functional studies of FHM3 mutations Q1489K and L1649Q revealed various *gain-of-function* effects when using a cardiac $Na_V1.5$ cDNA as backbone for making the constructs[27,44], more recent studies that investigated the consequences of these FHM3 mutations in the more appropriate $Na_V1.1$ protein revealed clear *loss-of-function* effects.[45] The third FHM3 mutation L263V that in patients causes FHM and in the majority of carriers also generalized tonic-clonic epilepsy, essentially had *gain-of-function* effects.[45] It was hypothesized that loss of sodium channel activity primarily disturbs the functioning of inhibitory neurons, where the $Na_V1.1$ normally are expressed[46,47], whereas gain of activity has a predominant effect on excitatory neurons. Interestingly, when overexpressed in neurons, depending on the test paradigm, the Q1489K mutation seemed to have functional consequences fitting either with hyperexcitability or hypoexcitability (i.e., self-limiting hyperexcitability capacity)[48], but this has not been tested in knock-in mice.

8.2 How do FHM mutations cause disease?

Can the molecular genetic findings of the three FHM genes (*CACNA1A*, *ATP1A2* and *SCN1A*) be integrated into a common pathway? More specifically, can we link the functional consequences of the three genes to for instance an increased propensity for CSD? Mutant $Ca_V2.1$ calcium channels from FHM1 R192Q and S218L knock-in mice predict increased glutamate release in the cerebellar cortex and thereby can easier induce, maintain, and propagate CSD; this is in line with the observed decreased threshold for CSD in knock-in mice.[34,35] FHM2 mutations in

the sodium-potassium pump predict in vivo reduced glial uptake of K^+ and glutamate from the synaptic cleft. FHM3 mutations in the $Na_v1.1$ sodium channel predict in vivo hyperexcitability of excitatory neurons. Therefore, the consequence of FHM1, FHM2, and FHM3 mutations all seem to cause increased levels of glutamate and K^+ in the synaptic cleft and thereby facilitate CSD (Figure 4). The increased propensity for CSD could well explain the aura, but, it remains to be established whether this also would result in a more readily activated trigeminovascular system with structures in the brainstem from which the headache originates.

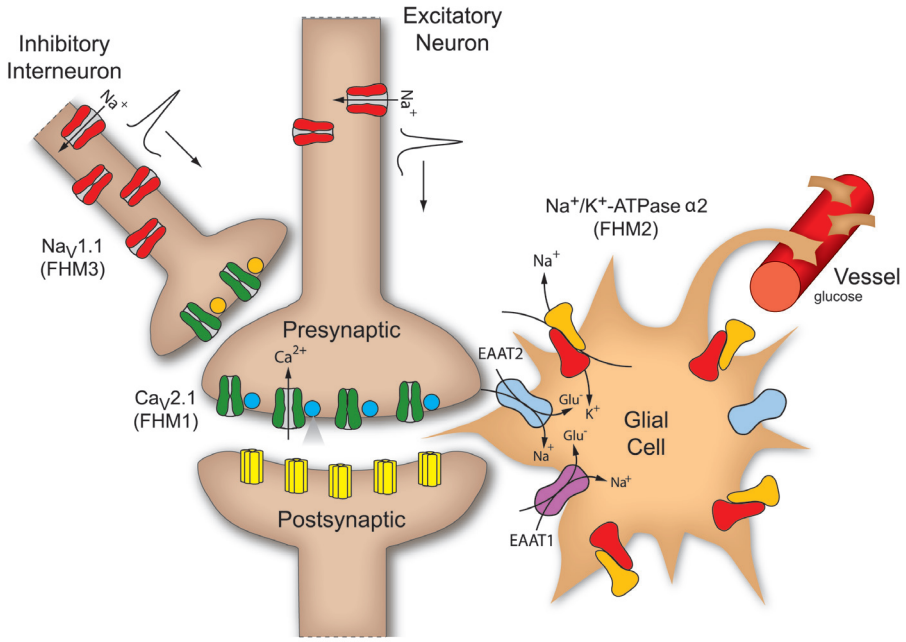


Figure 4. *Schematic representation of a glutamatergic sysnapse and the proteins encoded by the three FHM genes and the SLC1A3 gene (adapted from Barret et al. 2008). $Na_v1.1$ channels (encoded by SCN1A) are essential for the generation and propagation of action potentials. In response to an action potential, calcium enters the cell via $Ca_v2.1$ channels (encoded by CACNA1A) and glutamate will be released by vesicles into the synaptic cleft. Potassium in the synaptic cleft is removed in part by the action of the Na^+/K^+-ATPase (encoded by ATP1A2) which is located at the surface of glial cells (astrocytes). Removing extracellular K^+ generates a Na^+ gradient, which drives uptake of glutamate from the cleft by transporters, for example, EAAT1 (encoded by SLC1A3). Energy is required and achieved by glucose utilization after uptake from blood vessels. Gain-of-function mutations in $Ca_v2.1$ and loss-of-function mutations in the ATPase, $Na_v1.1$ and EAAT1 will each lead to of increased general excitability.*

8.3 Do FHM genes play a role in SHM?

Not all hemiplegic migraine patients are part of FHM families. So-called sporadic hemiplegic migraine (SHM) patients do exist, and exhibit clinical symptoms that are very similar to those of familial cases.[49] For instance, SHM - like FHM - patients can have attacks of common migraine that are not associated with hemiparesis. Also the prevalence of familial and sporadic hemiplegic migraine in the population is similar; both are rare with a prevalence of approximately 0.01%.[50]

Therefore, an interesting question is whether FHM genes also play a role in SHM? Previously, only one study addressed that question, and investigated the involvement of the *CACNA1A* gene in 27 SHM patients; two *CACNA1A* mutations were identified.[51] **Chapter 3** of this thesis describes a study that reports a systematic mutation screen of all three FHM genes in 39 clinic-based 'pure' SHM patients without cerebellar signs or epilepsy. About 15% of our SHM patients had mutations in FHM genes; predominantly in the *ATP1A2* gene. SHM could not be explained by mutations in known FHM genes in the majority of patients. The frequency of mutations was even lower in a Danish population-based study.[52] In one hundred SHM patients only 8 sequence variants in *CACNA1A* and *ATP1A2*, of which only 2 were considered pathogenic, were identified; no functional studies were performed to proof causality. This indicates a difference between Dutch and Danish patients, diagnosis, and/or mutation detection methodology. Regardless, these genetic studies indicate that (i) SHM belongs to the genetic migraine spectrum, and that (ii) other genetic factors likely play a role in SHM. Future research must show whether these patients have a mutation in yet undiscovered hemiplegic migraine genes, or whether they have an unfavorable combination of low-risk gene variants present in a single patient.

8.4 Is it possible to translate genetic results from HM to common migraine?

As the main clinical symptoms of headache and aura are similar in FHM and common migraine, it is thought that they may share a common pathophysiology.[3] Several studies have investigated the role of FHM1 and FHM2 loci in the common forms of migraine. These studies led to conflicting results with some evidence in favor of the hypothesis[53-55,26], while others find no evidence for their involvement in common migraine.[56-58] Some of the studies hypothesized that mutations found in FHM may cause common migraine, while it is more likely that 'milder', less penetrant, DNA variants are involved. A recent comprehensive study, including some 2800 migraine patients from various European countries, tested whether common DNA variants in ion transport genes are involved in common migraine.[59] Over 5,000 SNPs in 155 ion transport genes (including the three FHM genes) were studied, but none of the original significant SNPs (66 SNPs in 12 genes) was significant across all four replication cohorts. From this study it seems that common variants in ion transport genes do not play a major role in susceptibility for common migraine. Rare variants or variants with a very small effect size would not have been detected with this study design.

8.5 Genetic studies in other disorders in which migraine is prevalent

Another approach to identify genes and pathways for complex disorders is to study disorders that are comorbid with that particular genetically complex disease. Migraine can be part of the clinical spectrum of certain monogenic disorders. A good example is Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) (for recent review see Stam et al)[60]. CADASIL is caused by mutations in the *NOTCH3* gene, which encodes the

Notch3 receptor that plays a key role in vascular smooth muscle cell function in small arteries and arterioles of the brain.[61] Up to one-third of CADASIL patients suffers from migraine with aura, where migraine often is the presenting clinical symptom.[62] The link with MA and not the more frequent MO, suggests that increased susceptibility for CSD is somehow caused by *NOTCH3* mutations. This hypothesis is strengthened by the fact that transgenic *Notch3* mice have a decreased threshold for CSD.[63] A second example is Retinal Vasculopathy with Cerebral Leukodystrophy (RVCL) that is caused by mutations in the *TREX1* gene that encodes the major mammalian 3'-5' DNA exonuclease (**Chapter 5.1**). RVCL originally was described in three families under different disease names; cerebroretinal vasculopathy (CRV), hereditary vascular retinopathy (HVR), and hereditary endotheliopathy, retinopathy, nephropathy and stroke (HERNS).[64-67] RVCL is primarily characterized by progressive blindness due to vascular retinopathy and can be associated with a wide range of systemic and cerebral symptoms, including cerebral infarcts and white matter hyperintensities, vascular dementia, liver and kidney dysfunction, Raynaud's phenomenon, and migraine. Particularly in a Dutch RVCL family, migraine and Raynaud's phenomenon are prominent.[66] Comorbidity of migraine with CADASIL and RVCL indicates that cerebral or meningeal vasculopathy and vascular dysfunction may play a role in migraine.[68] *TREX1* mutations were also identified in other vascular and immune-related disorders, such as Systemic Lupus Erythomatosus (SLE) and Familial Chilblain Lupus (FCL).[69,70] **Chapter 5.2** describes a *TREX1* mutation screen in 60 patients with neuropsychiatric SLE (NPSLE), with and without white matter hyperintensities (WMH). We identified the first gene mutation in a NPSLE patient with WHM. Interestingly, this patient also has severe migrainous headache, which is known to be common in SLE patients.[71]

Another pathway that is important in migraine pathophysiology is the 'neuronal hyperexcitability pathway'. Depression and epilepsy are two genetic disorders in which neuronal hyperexcitability is thought to play a role. It is consistently found that migraine patients have an increased risk for migraine and epilepsy. This relation is bi-directional, meaning that patients with depression or epilepsy also have an increased risk for migraine.[72-77] This reinforces the hypothesis that overlapping pathways play a role in migraine and epilepsy and in migraine and depression. For instance co-occurrence of epilepsy and migraine was reported for several FHM mutation carriers.[22,11,78,21,79] Recent genetic research points to yet another gene that seems to fit perfectly into the FHM pathway of cortical hyperexcitabiltiy: the *SLC1A3* gene that encodes EAAT1, a glutamate transporter that is located on astrocytes (Figure 4). A P290R mutation in this gene was shown to cause severe episodic ataxia and progressive ataxia, seizures, alternating hemiplegia, and migraine headache.[80] Mutant EAAT1 showed severely reduced glutamate uptake. In **Chapter 4**, we describe a novel C186S EAAT1 mutation in a patient with episodic ataxia. Functional studies showed a modest but significant reduction of glutamate uptake, which is in line with the milder

phenotype. Because of the known relation between ataxia and hemiplegia with FHM1 mutations, the finding of *SLC1A3* mutations causing both ataxia and hemiplegia has potential relevance for FHM as well.

8.6 Linkage studies in common migraine

A major genetic strategy to identify migraine susceptibility genes has been classical linkage analysis that aims to find linked chromosomal loci using a family-based approach. Over the years, a number of chromosomal loci (Table 1) have been identified using either migraine without or with aura patients diagnosed according to the International Headache Criteria (i.e., IHS diagnosis).[1] However, with a few exceptions, replication of initial findings was unsuccessful. One of the most promising migraine susceptibility loci resides on chromosome 4; initial linkage to chromosome 4q24 in Finnish MA families[81] was replicated in an Icelandic sample of MO patients.[82] Although the Finnish and Icelandic migraine loci are not identical, but seem to overlap, it is yet unclear whether they harbor different migraine susceptibility genes. Lack of success with the linkage approach probably is due to the high prevalence of migraine making it difficult to ascertain "clean" pedigrees for linkage where migraine genes from spouses do not interfere with the analysis.

Table 1 *Summary of relevant linkage results performed for migraine using the International Headache Classification (IHC) classification guidelines.*

Chromosomal locus	Phenotype	Genotyping method	Reference
1q31	MA, MA/MO*	Regional microsatellite markers	Lea et al. 2002
4q21	MO	Genome-wide scan	Bjornsson et al. 2003
4q24	MA	Genome-wide scan	Wessman et al. 2002
6p12.2-p21.1	MA/MO	Genome-wide scan	Carlsson et al. 2002
10q22-q23	MA	Genome-wide scan	Anttila et al. 2008
11q24	MA	Genome-wide scan	Cader et al. 2003
14q21.2-q22.3	MO	Genome-wide scan	Soragna et al. 2003
15q11-q13	MA	Regional microsatellite markers	Russo et al. 2005
19p13	MA	Regional microsatellite markers	Jones et al. 2001
Xq25-q28	MA/MO	Regional microsatellite markers	Nyholt et al. 1998; 2000

*MO = migraine without aura; MA = migraine without aura; *Only suggestive linkage for MA/MO combined.*

In the last few years, linkage studies made use of alternative phenotyping approaches that are based either on individual migraine traits in "trait component analysis (TCA)" or that use a combination of clinical migraine features in "latent class analysis (LCA)". Whereas TCA is rather straightforward, LCA involves a complex statistical empirical clustering approach based on factor analysis that combines the information of several migraine symptoms.[83,84] The classification reflects disease severity and does not specifically separate MO from MA. In principle, TCA and to a certain degree LCA, reflects the underlying processes in migraine pathophysiology as they utilize the questionnaire-based information in a more optimal manner, compared to the dichotomous IHS end diagnosis.[85] It can be expected that by using TCA, the clinical heterogeneity will be reduced, since traits better reflect the biological pathways that are influenced by specific genetic variations. Several migraine loci were identified using this alternative phenotyping strategy (see Table 2).

Table 2 *Summary of linkage results performed for migraine grouped for phenotyping methods Latent Class Analysis (LCA) and Trait Components Analysis (TCA).*

Chromosomal locus	Phenotypic trait (analysis method)*	Reference
4q24	Age at onset, photophobia, phonophobia, photo- and phonophobia, pain intensity, unilaterality, pulsation, nausea and vomiting (TCA)	Anttila et al. 2006
5q21	Pulsation (LCA)	Nyholt et al. 2005
10q22-q23	Migrainous headache (LCA)	Anttila et al. 2008
10q22-q23	Unilaterality, pulsation, pain intensity, nausea/vomiting, photophobia, phonophobia (TCA)	Anttila et al. 2008
17p13	Pulsation (TCA)	Anttila et al. 2006

*IHS = International Headache Society; LCA = Latent Class Analysis; TCA = Trait Component Analysis. *Order based on level of significance (most significant trait mentioned first).*

8.7 Candidate gene association studies

In common disorders, such as migraine, many common genetic factors (present in more than 1–5% of the population) are thought to play a role in disease susceptibility. This phenomenon is known as the 'common disease, common variant' hypothesis. A frequently used strategy to identify these common gene variants for common disorders are case-control association studies.[86-89] These

studies test for significant differences in allele frequencies between cases and controls. Many candidate gene association studies have been performed in migraine research; mainly of genes involved in the serotonin and dopamine pathways - but also in other genes with an already suspected function in migraine pathophysiology. Unfortunately, the majority of the associations could not be replicated, suggesting that many of the original findings may in fact represent false positive findings (for review see De Vries et al)[90]. Studies often contained rather low numbers of cases and controls; the ones that used 275 cases and controls or more are summarized in Table 3. A positive exception is a recent study that analyzed several SNPs in ten different genes from the dopamine system in over 600 MA cases and controls using a two-step design and showed a positive association with single SNPs in *DBH*, *DRD2* and the *SLC6A3* genes, also after multiple testing.[91] Functional consequences of any of these associated SNPs are still unknown.

Another promising association finding seems to be with the 5',10'-methylenetetrahydrofolate reductase (*MTHFR*) gene. MTHFR is a key enzyme in folate and homocysteine metabolism.[92] Most studies found an association of the T-allele of the *MTHFR* C677T polymorphism with migraine [93-98], although negative findings have been reported as well.[99,100] The T-allele results in moderately increased levels of homocysteine that may cause migraine through a vascular endothelium dysfunction effect, but evidence for this hypothesis is still lacking. Recently, also two meta-analyses were reported for *MTHFR* and migraine.[101,102] Both studies revealed that the T-allele is associated with MA, but not with migraine without aura or migraine overall. However when the results in one of the meta-analyse were stratified for ethnicity, the results were driven by studies in non-Caucasian populations.[102]

8.8 Genome wide association studies

In recent years, high-throughput genotyping techniques developed rapidly allowing extensive genotyping in large cohorts for Genome Wide Association Studies (GWAS) became feasible. In contrast to hypothesis-driven candidate gene-based association studies, GWAS do not require prior knowledge about the disease mechanism. In **Chapter 6**, GWA studies in migraine are described. The first GWAS was conducted for the migraine subtype MA by the International Headache Consortium (**Chapter 6.1**). The initial study cohort contained a total of 2,748 clinic-based European MA cases, of which 655 cases were of Dutch origin and were collected via the LUMINA (Leiden University Migraine Neuro Analysis) initiative.[103] The most significantly associated SNP (*P*-value 5.1×10^{-9}) is located on chromosome 8q22.1 and could be replicated in several independent migraine cohorts.[104] Interestingly, the associated allele of this SNP was significantly correlated with expression levels of the adjacent *AEG-1* gene. *AEG-1* is expressed on astrocytes and

downregulates EAAT2, which is an important glutamate transporter in the brain that is important for glutamate from synaptic cleft.[105] The *AEG-1* gene seems to perfectly fit into the 'glutamate pathway' that is seen in FHM and common migraine.

For **Chapter 6.2** we initially performed a GWAS for migraine in the Erasmus Rucphen Family (ERF) population, and subsequently this data was included in the first meta-analysis for migraine. The ERF population is a well-studied Dutch genetically isolated population, which resides in the Southwest of the Netherlands and consists of roughly 3,000 descendants of a relatively small numbers of founders in the mid 18th century. The main advantage of genetic studies in genetically isolated populations is that these populations are more homogeneous due to their relatively small number of founders that underwent rapid population expansion (i.e., genetic drift).[106] In our GWA study none of the in total approximately 2.5 million SNPs reached genome-wide significance (i.e., a *P*-value < 5.0×10^{-8}). However, several SNPs in genes that seem relevant to migraine pathophysiology showed nearly significant association with migraine. Subsequently a meta-analysis was performed using the GWAS data of six population-based cohorts from the Dutch Icelandic (DICE) consortium, including the ERF cohort. For only one SNP a *P*-value below 10^{-4} was obtained in both the GWA in ERF and the meta-analysis study. The best SNP in the meta-analysis was located in the neuronal growth factor receptor gene (NGFR), which is a good candidate gene for migraine, due to is relevance to pain perception. Future replication and/or functional studies must reveal their true relevance to migraine pathophysiology.

The most significant SNP from the clinic-based GWAS did not show a signal in the ERF GWAS and in the meta-analysis of the population-based migraine cohorts. This might be due to the difference between population-based and clinic-based migraine patients. Perhaps this specific SNP has an effect on disease severity. Furthermore, previous studies for other disorders also showed that often different GWAS for the same disease do not yield the same peaks[107], which could be due to clinical heterogeneity between the samples.

Hormone receptor system; estrogen receptor 1 and 2 (ESR1 and ESR2), follicle stimulating hormone receptor (FSHR), androgen receptor (AR), CYP19 aromatase polypeptide A1 (CYP19A1), nuclear receptor-interacting protein 1 (NRIP1), progesterone receptor (PGR)						
ESR1	c.594G>A (G594A)	484 (360/124)	484	594A: p=0.003 (p=0.01/p=0.02)	Two cohorts combined; *P*-values based on initial cohort of 224 migraine cases and 224 controls	Colson et al. 2004
ESR1	c.2014G>A (G2014A)	898 (898/-)	900	- (NS/-)		Kaunisto et al. 2006
ESR1	c.325G>C (G325C)	356 (198/158)	374	325C: p=0.03 (p=0.045/NS)		Oterino et al. 2008
ESR2	c.2100A>G (A2100G)				2100A: NS (p=0.03/NS)	
FSHR	c.2039G>A (Ser680Asn)				2039G: NS (p=0.01/NS)	
CYP19A1	c.1672C>T (C1672T)				NS (NS/NS)	
NRIP1	c.225G>A (Gly75Gly)				NS (NS/NS)	
AR	CAG repeat in exon 1	509 (371/138)	454	NS (NS /NS)	*P*-values based on initial cohort of 275 migraine cases and 275 controls; PROGINS was replicated	Colson et al. 2005
PGR	PROGINS ins in intron 7			PROGINS ins: p=0.02 (NS/p=0.008)		
Inflammation related genes; tumor necrosis factor-α and –β (TNFA and TNFB) and lymphotoxin α (LTA)						
TNFA	c.308G>A (G308A)	299 (38/261)	306	308G: p<0.001 (NS/p<0.001)		Rainero et al. 2004
TNFA	Multiple variants tested	439 (65/327)	382	NS	15 SNPs were tested	Lee et al. 2007
LTA	-294T>C (rs2844482; promoter)			-294C: p=0.0002 (p=0.006/p=0.0008)		
TNFA	c.308G>A (G308A)	299 (-/299)	278	- (-/NS)		Asuni et al. 2009
TNFB	c.252G>A (G252A)			252A: - (-/p=0.018)		
Insulin receptor gene (INSR)						
INSR	c.2946-713C>A (SNP84; intron 15)	827 (377/450)	765	c.2946-713A: NS (p=0.002/NS)	48 SNPs tested in region 19p13; SNP84: rs2860172; SNP90: rs2860174; SNP274: rs1799817/His1085His; *P*-values based on 331 migraine cases and 466 controls	McCarthy et al. 2001
	c.2842+1451T>A (SNP90; intron 14)			c.2842+1451A: NS (p=0.007/NS)		
	c.3255C>T (SNP274)			c.3255T: NS (p=0.008/NS)		
INSR	c.2842+1451T>A (rs2860174; intron 14)	1278 (1278/-)	1337	c.2842+1451T: - (p=0.005/-)	Two-step design (haplotype-tagging); 35 SNPs tested in region 19p13; *P*-values based on total cohort	Netzer et al. 2008
Angiotensin converting enzyme (ACE), angiotensin receptor 1 (AGTR1) and angiotensin (AGT)						
ACE	Ins/del (rs1799752; intron 15)	342 (187/155)	403	NS (NS/NS)		Tronvik et al. 2008
ACE	Ins/del (rs1799752; intron 15)	3226 (1275/1951)	20423	NS (NS/NS)		Schurks et al. 2009a
AGTR1	c.1166A>C (A166C)	3226 (1275/1951)	20423	NS (NS/NS)		Schurks et al. 2009b
AGT	c.803T>C (Met235Thr)			NS (NS/NS)		
Association studies with other genes						
NOS3	c.-51-898G>A (rs1800779; intron 1)	337 (188/149)	341	NS (NS/NS)	NOS3 encodes for endothelial nitric	Toriello et al. 2008
	c.894T>G (rs1799983)			NS (NS/NS)	oxide synthase	
Ion transporter genes	Multiple variants tested	3676	3624	NS (NS/NS)	>5000 SNPs (haplotype-tagging) in 155 ion transporter genes tested in initial cohort; replication cohorts included	Nyholt et al. 2008

*MA = migraine with aura; MA = migraine without migraine; NS = not significant; - = not tested/not available; SNP = single nucleotide polymorphism; Ins = insertion; Del = deletion; VNTR = variable number of tandem repeats. *Nomenclature of DNA variant in the original study; for intronic DNA variants, the respective intron number is indicated; **number of cases and ***p-values are given for all migraine cases combined or, when specified between brackets, for migraine with aura cases only and/or migraine without aura cases only.*

Gene	DNA variant*	Cases (n)** Migaine (MA/MO)	Controls (n)	Associated allele with phenotype (*P*-value)***	Remarks	Reference
5',10'-Methylenetetrahydrofolate reductase (*MTHFR*)						
MTHFR	c.677C>T (C677T)	652 (465/187)	320	677T: NS (p=0.017/-)	Combined single cases and families; *P*-values based on initial cohort of 270 migraine cases and 270 controls; replication provided	Lea et al. 2004
MTHFR	c.677C>T (C677T)	413 (187/226)	1212	677T: - (p<0.006/NS)		Scher et al. 2006
MTHFR	c.677C>T (C677T)	898 (898/-)	900	677T: - (NS/-)		Kaunisto et al. 2006
MTHFR	c.677C>T (C677T)	2961 (2170/791)	3844	677T: NS (p=0.0005/NS)	Meta-analysis	Rubino et al. 2007
MTHFR	c.677C>T (C677T)	4577 (1275/1951)	20424	677T: p=0.03 (p=0.02/NS)	Protective effect of 677T	Schurks et al. 2008
Dopaminergic system; catechol-O-methyltransferase (*COMT*), dopamine β-hydroxylase (*DBH*), dopamine transporter (*DAT1*)						
COMT	c.472A>G (Val158Met)	305	1468	NS		Hagen et al. 2006
DBH	-1021C>T +1603C>T	830 (588/242)	500	-1021T: p=0.004 (p=0.011/NS) NS (NS/NS)	*P*-values based on initial cohort of 275 migraine cases and 275 controls; replication provided; protective effect of T-allele	Fernandez et al. 2009
DBH	rs2097629 (A>G intr 9)	650 (650/-)	650	c.1434+1579G: - (p=0.01/-)	Two-step design (haplotype-tagging); 35	Todt et al. 2009
DRD2	rs7131056 (T>G intr 1)			c.-32+16024T - (p=0.006/-)	SNPs tested in dopamine pathway;	
SLC6A3	rs40184 (G>A intr 14)			c.1840-204A: - (p=0.03/-) corrected	*P*-values based on total cohort	
DRD1	rs251937 (T>C)	543 (318/225)	561	0.026 (-/-)	Two-step design (haplotype-tagging);	Corominas et al. 2009
DRD2	rs2283265 (G>T)			0.0030 (p=0.037/p=0.0081)	Non of the SNPs were significant in	
DRD3	multiple SNPs tested			0.0033 (-/-)	both samples	
DRD5	multiple SNPs tested			NS (-/-)		
DBH	multiple SNPs tested			NS (-/-)		
COMT	multiple SNPs tested			NS (-/-)		
SLC6A3	multiple SNPs tested			NS (-/-)		
TH	rs2070762 (T>C)			0.0035 (NS/p=0.023)		
SLC6A3	VNTR in intron 8 550	(401/149)	550	NS (NS/NS)	SLC6A3 is also known as DAT1	McCallum et al. 2007
Serotonergic system						
HTR2C	c.69G>C (Cys23Ser) c.2831T>G (T2831G)	275	275	NS NS		Johnson et al. 2003
HTR2C	c.69G>C (Cys23Ser)	335 (184/151)	335	NS (NS/NS)		Oterino et al. 2007
HTR2C	c.69G>C (Cys23Ser)	561 (561/-)	1235 NS (NS/-)		Meta-analysis	Oterino et al. 2007
HTR2B	rs16827801T-rs10194776G	528 (220/308)	528	- (-/p=0.0017)	19 Serotonin-related genes covered by	Corominas et al. 2009
DDC	rs2329340A-rs11974297C-rs2044859T			- (p=0.0019/-)	122 SNPs. None of the individual	
	-rs11761683G			- (-/p=0.006)	SNPs was significant after multiple	
MAOA	rs3027400G-rs2072743C			- (-/p=0.006)	testing correction.	
Gamma-Aminobutyric acid type A (GABA-A) receptor system; GABA-A receptor α5 (GABRA5), β3(GABRB3), receptor ε (GABRE), γ3 (GABRG3), receptor θ (GABRQ) subunits						
GABRA5	Multiple variants tested	898 (898/-)	900	- (NS/-)	34 SNPs in region 15q11-q13	Oswell et al. 2007
GABRB3	Multiple variants tested			- (NS/-)	(haplotype-tagging)	
GABRG3	Multiple variants tested			- (NS/-)		
GABRA5	Multiple variants tested	649 (649/-)	652	- (NS/-)	56 SNPs tested in region 15q11-q12	Netzer et al. 2008
GABRB3	Multiple variants tested			- (NS/-)	(haplotype-tagging); p-values for	
GABRG3	Multiple variants tested			- (NS/-)	two cohorts	
GABRE	Multiple variants tested	384 (254/130)	275	NS (NS/NS)	3 SNPs tested in GABRE	Fernandez et al. 2008
GABRQ	c.1432T>A (I478F)			- (NS/-)		

8.9 Gene expression studies in knock-in migraine mouse models

The availability of transgenic FHM1 knock-in mouse models[34,35], gave opportunities to investigate gene expression profiles in brain tissues. The difference in severity of clinical features in patients with the R192Q (pure FHM) and the S218L mutation (FHM with cerebellar ataxia, epilepsy and increased susceptibility to head trauma induced brain edema) is reflected in the mouse models with the same FHM1 mutations. The fact that the more 'severe' mouse model also exhibits more profound changes in neuronal calcium influx, (cortical) neurotransmission, and susceptibility to CSD warrants an in-depth molecular analysis of the consequences of both mutations on gene expression profiles. Therefore, in **Chapter 7** of this thesis, we performed a first gene expression study in both transgenic mouse models to investigate whether the observed hyperexcitabiliy might be associated with changes in gene expression levels under basal (i.e., untriggered) conditions. To this end, the occipital cortex (i.e., the origin of the CSD) and the cerebellum (i.e., the origin of the ataxia) were investigated. Gene expression levels in the occipital cortex were notably similar in cortices of mutant and wildtype mice. However, gene expression in the cerebellum of S218L mice was somewhat different from that in wildtype and R192Q mice. Several of the differentially expressed genes in the cerebellum of S218L mice could be related to neurotransmission and more specifically to ataxia, which is a prominent feature in patients and mice with this mutation. As an example, the gene with the highest fold change, tyrosine hydroxylase (*Th*), which could be confirmed at the protein level, had already been implicated in ataxia of several natural mouse *Cacna1a* models (i.e. *Rolling Nagoya*[108], *Tottering*[109], and *Leaner*.[110] Perhaps additional microarray experiments using migraine-relevant triggers are needed before gene expression profiles are more pronounced and can be combined with GWAS and/or exome-genome sequencing to prioritize findings.

8.10 Future perspectives

This thesis focused mainly on the identification and characterization of migraine gene mutations and pathways. Three FHM genes have been identified. The genetic spectrum of FHM mutations and their associated clinical features have been investigated in this thesis. Not all FHM families can be explained by mutations in known FHM genes, so additional FHM genes must exist. It is interesting to assess whether novel FHM genes will fit in the same pathway as the known FHM genes, or whether they will highlight additional pathways with relevance to migraine pathophysiology. With the availability of 'Next Generation Sequencing' technology, which allows high-throughput sequencing of either desired regions of the genome, all exons of the genome (the so-called exome), or the entire genome, these FHM genes will probably soon be identified. First successes in gene identification for monogenic disorders using this exome strategy were published.[111]

For common migraine, clinical and genetic heterogeneity make the identification of susceptibility genes even more difficult than gene discovery in FHM. The diagnosis of migraine is mainly based on questionnaires and (sometimes) interviewing the patients. Unfortunately, a more objective method of diagnosing patients, such as biochemical testing in blood (or cerebrospinal fluid or urine) is currently not available. Systematic studies to identify such reliable biomarkers are dearly needed as they will help defining more homogeneous groups of patients for genetic studies. Ideally, biomarker information should somehow be combined with other endophenotyping approaches such as previously discussed LCA and TCA. Particularly TCA seems to reduce clinical heterogeneity. Endophenotyping likely will increase the power of the genetic analyses. Also because, as was shown for several other complex disorders such as Attention-Deficit/Hyperactivity Disorder (ADHD) and schizophrenia, the heritability of the individual traits may be higher than of the syndrome as a whole (i.e., combination of traits).[112,113] One appealing strategy to decrease heterogeneity in migraine is to take co-morbidity with other diseases into account. A recent study in ERF indicated that migraine and depression may share, at least to some extent, genetic factors.[77] By stratifying for depression, gene discovery in migraine may become (a little) easier. At the moment, most investments in migraine genetics go into GWAS. For many complex disorders, GWAS already led to successes.[114-116] The coming two years will be very exciting as additional GWAS are currently being performed for migraine. Still, most gene variants identified with GWAS have a low relative risk (RR) of often 1.1 - 1.3 and seem to explain only a small proportion of disease heritability. Therefore, it is now being questioned whether GWAS will contribute much to understanding the majority of the genetic load. The question at hand is where the majority of the genetic burden is and how to increase our understanding in migraine mechanisms. Is it in epistasis or copy number variation? Can pathway analyses on GWA data increase our insight[117,118]? Or is most of the genetic load carried in large number of allelic variants that combine a very low allele frequency with a reasonably high relative risk? As this genetic variation (usually) is not captured in current GWAS, other approaches (i.e., large-scale deep sequencing) are needed. Also for this approach, the technology is available. The next few years will have to show what this new technology can bring for migraine. In conclusion, although the last decade has produced major advances in our knowledge of migraine pathophysiology, the best perhaps is yet to come. It will require a true multidisciplinary approach to harvest this knowledge and translate it to novel treatment options to help migraine patients.

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