Migraine genetics : from monogenic to complex forms

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Voor mijn ouders

Contents

Chapter 1

General Introduction

1.1 Clinical characteristics of migraine

1.1.1 Migraine with and without aura (MO and MA)

Migraine is a common episodic neurovascular headache disorder characterised by attacks of severe headache and autonomic and neurological symptoms. The one-year prevalence of migraine in the Western countries is 11% overall (6-8% in men and 15-18% in women). [1-4] Onset of migraine is nearly always below age 50 (in 90% of patients). Peak age of onset of migraine is 10-12 years old for males and 14-16 years old for females. [5, 6] Among active migraineurs, the median attack frequency is 1.5 per month; at least 10% of patients have weekly attacks. [5-7]

Migraine patients suffer from recurrent attacks. Diagnosis of migraine is based on the patient's description of the attacks and exclusion of other possible secondary causes of headache. In 1988, a set of diagnostic criteria was defined by the Headache Classification Committee of the International Headache Society (IHS). The IHS criteria were revised in 2004 (Table 1). [8, 9] Migraine can be divided into two major subtypes, migraine without aura (MO) and migraine with aura (MA), based on the presence or absence of the aura phase. Up to 33% of migraineurs experience both types of attacks during their lifetime. [10] Migraine with aura, occurring in one-third of patients, shares the same headache qualities, but the headache is usually preceded by an aura: attacks of focal neurological symptoms, which develop gradually within 5-20 minutes and last less than 60 minutes. [1, 10] Aura symptoms include most frequently visual symptoms, including the classical scintillating scotoma (99% of patients) (Figure 1), sensory disturbances (paraesthesia, 31%), speech difficulties (dysarthria, aphasia, 18%) and motor symptoms (weakness, paresis, 6%). [11]
Typical migraine headache is unilateral, throbbing, moderate to severe and aggravated by physical activity and usually lasts between 4 and 72 hours. [9] The headache is often accompanied by other symptoms, like nausea, vomiting and sensitivity to light (photophobia) and/or sound (phonophobia).

Figure 1. Drawing of development and progression of a migraine-scotoma by Hubert Airy (1870). Scintillating scotoma is the most common visual aura preceding migraine and was first described by 19th century physician Hubert Airy (1838–1903). While many variations occur, the scintillating scotoma usually begins as a "ball of light" in the center of the visual fields, which obscures vision to some degree. It then takes on the form of a shimmering arc of white or coloured lights. The arc of light gradually enlarges, becomes more obvious, and may take the form of a definite zig-zag pattern, sometimes called a fortification spectrum, because of its resemblance to the battlements of a castle or fort. It may be difficult to read or drive while the scotoma is present. Symptoms typically resolve within 15-30 minutes, leading to the headache in classic migraine, or resolving without consequence in acephalgic migraine.

1.1.2. Familial Hemiplegic Migraine (FHM)

Familial hemiplegic migraine is a rare autosomal dominantly inherited subtype of MA, in which attacks are associated with hemiparesis (Table 1). In a Danish epidemiological study, the prevalence of familial hemiplegic migraine was estimated to be 0.01%. [12] The mean age of onset is lower (about 11 years) than in other types of migraine. [12, 13] FHM attacks are characterized by motor aura symptoms consisting of typically unilateral motor weakness or paralysis that may last from minutes to weeks; in other respects they resemble typical MA attacks. Nearly always at least three aura symptoms are present in FHM (Table 1) and they typically last longer than in MA. [12] Some FHM patients can have atypical severe attacks with signs of diffuse encephalopathy, impairment of consciousness (coma), fever, prolonged hemiplegia and/or seizures. [13] Patients may have permanent neurological signs between attacks, mostly nystagmus and ataxia. [12-14] These features led to the distinction between families with pure hemiplegic migraine and those with hemiplegic migraine and cerebellar signs (~20% of families). FHM is characterized by large clinical variability in the severity and frequency of attacks among individuals of different families, but also within the same family. Emotional stress and minor head trauma are among the most common triggers of FHM attacks. [13]

Hemiplegic migraine may run in families (FHM), but may also be sporadic (SHM). Clinically, FHM and SHM attacks are indistinguishable and prevalence of SHM was estimated to be similar as FHM. [15] It is unknown whether and to what extent SHM and FHM are pathophysiologically related and whether genes for FHM are also involved in SHM.

1.1.3. Comorbidity of migraine

Migraine patients, especially those with migraine with aura, have an increased risk (comorbidity) of a number of other episodic brain disorders. [4] The highest and most consistently found increased risks are for epilepsy (2-4 fold), [16, 17] depression and anxiety disorders (2-10 fold), [18-20] Patent Foramen Ovale (PFO) (3 fold), [21] and stroke (3-14 fold) increased risk depending on age and cofactors such as smoking and use of oral contraceptives. [21] Migraine patients with a high attack frequency have a 16 fold increased risk of white matter and cerebellar lesions visible on MRI. [22] The increased risk for all these diseases is bi-directional, suggesting common underlying mechanisms.

The association between migraine and epilepsy is especially evident for FHM. Both diseases are considered to be disorders of neuronal hyperexcitability and share common pathways of ion transport dysfunction. FHM patients are frequently initially misdiagnosed with epilepsy. Genes involved in FHM are associated with different types of epilepsy (this thesis).

Table 1. International Headache Society Criteria for Migraine With and Without Aura and Familial Hemiplegic migraine

Migraine without aura

A. At least five attacks fulfilling criteria B-D
B. Headache attacks lasting 4 to 72 hours (untreated or unsuccessfully treated)
C. Headache has at least two of the following characteristics:
    1. Unilateral location
    2. Pulsating quality
    3. Moderate or severe pain intensity
    4. Aggravation by or causing avoidance of routine physical activity (e.g., walking or climbing stairs)
D. During headache al least one of the following:
    1. Nausea and/or vomiting
    2. Photophobia and phonophobia
E. Not attributed to another disorder

Migraine with aura

A. At least two attacks fulfilling criteria B-D
B. Aura consisting of at least one of the following, but no motor weakness:
    1. Fully reversible visual symptoms including positive features (e.g., flickering lights, spots, or lines) and/or negative features (i.e., loss of vision)
    2. Fully reversible sensory symptoms including positive features (i.e., pins and needles) and/or negative features (i.e., numbness)
    3. Fully reversible dysphasic speech disturbance
C. At least two of the following:
    1. Homonymous visual symptoms and/or unilateral sensory symptoms
    2. At least one aura symptom develops gradually over ≥ 5 minutes, and/or different aura symptoms occur in succession over ≥ 5 minutes
    3. Each symptom lasts ≥ 5 and ≤ 60 minutes
D. Headache fulfilling criteria B-D for migraine without aura begins during the aura or follows aura within 60 minutes
E. Not attributed to another disorder

Familial Hemiplegic Migraine

A. At least two attacks fulfilling criteria B and C
B. Aura consisting of fully reversible motor weakness and at least one of the following:
    1. Fully reversible visual symptoms including positive features (e.g., flickering lights, spots, or lines) and/or negative features (i.e., loss of vision)
    2. Fully reversible sensory symptoms including positive features (i.e., pins and needles) and/or negative features (i.e., numbness)
    3. Fully reversible dysphasic speech disturbance
C. At least two of the following:
    1. At least one aura symptom develops gradually over ≥ 5 minutes, and/or different aura symptoms occur in succession over ≥ 5 minutes
    2. Each symptom lasts ≥ 5 and ≤ 24 hours
    3. Headache fulfilling criteria B-D for migraine without aura begins during the aura or follows aura within 60 minutes
D. At least one first- or second-degree relative has had attacks fulfilling these criteria A-E
E. Not attributed to another disorder

1. 2 Towards identification of genes underlying migraine

1.2.1 Genetic mechanisms are involved in migraine

Migraine shows a high familial aggregation, which could be due, purely by chance, to the high prevalence of the disorder. Genetic epidemiological studies are necessary to prove the involvement of genetic factors. Twin studies have been used to assess the respective roles of genetic and environmental factors in migraine. [23-31] These studies are based on the comparison of concordance rates between monozygotic (MZ) and dizygotic (DZ) twins. Several twin studies support a strong genetic component for migraine, showing consistently elevated migraine concordance rates among monozygotic versus dizygotic twins. For example, in a large Danish twin study, the pairwise concordance rate was significantly higher among MZ than DZ twin pairs for migraine without aura (28 *vs* 18 %, $p<0.05$) and for migraine with aura (34 *vs* 12%, $p<0.001$). [24, 25] The contribution of genetic factors to disease susceptibility, termed heritability (h^2), can be estimated from twin studies and varied between 34% and 65% in several studies. [23-31]

In addition, population-based studies provide information about the effect of genetic factors and the potential mode of inheritance. These studies investigate whether first- or second-degree relatives of a subject with a certain disease have a higher frequency to suffer from the disease than expected by chance alone. Studies have shown that the risk of migraine in first-degree relatives is 1.5–4 fold increased. The familial risk appeared greatest for patients with migraine with aura, with a young age at onset and a high attack severity and disease disability. [32-34]

Although family and twin studies indicate involvement of genetic factors in the etiology of migraine, the exact contribution of genes and the mode of inheritance of such factors remain unknown. Most studies favoured a multifactorial inheritance, combining genetic and environmental factors. For example, a large Danish population-based segregation analysis including 126 MO families and 127 MA families indicated that a multifactorial inheritance is most likely for MO and MA. [35]

1.2.2 FHM as a model for migraine

A successful approach for understanding complex diseases is to study closely related rare Mendelian subtypes of these diseases. New hypothesis can be created and novel pathway components explored in complex diseases based on the findings in Mendelian diseases. Less severe variants in these Mendelian disease genes might act as susceptibility factors for the more common forms of the disease. FHM is considered as a rare monogenic subtype that is part of a migraine spectrum. Apart from the hemiparesis, the other headache and aura features of the FHM attack are identical to those of attacks of the common types of migraine. Only, FHM patients have a significantly longer duration of the visual and sensory aura symptoms and the headache compared with migraine with aura. [12] In addition to attacks with hemiparesis, the majority of FHM patients also experience attacks of “normal” migraine with or without aura. [13, 36] As in the common forms of migraine, attacks of FHM may be triggered by mild head trauma. Thus, from a clinical point of view, FHM seems a valid model for the common forms of migraine. [37] Therefore, genes and their pathways involved in FHM may also be promising candidates for the much more frequent migraine types. Major clinical differences, apart from the hemiparesis, include that FHM in 20% of the cases may

also be associated with cerebellar ataxia and other neurological symptoms such as epilepsy, mental retardation, brain oedema, and (fatal) coma.

1.2.3 Approaches to gene mapping

Human genetic diseases can be roughly divided in Mendelian disorders and complex disorders. Mendelian disorders have a monogenic inheritance, caused by changes or mutations that occur in the DNA sequence of a single gene. Mendelian disorders are inherited in recognizable patterns: autosomal dominant, autosomal recessive, and dominant or recessive X-linked. Mendelian diseases can show reduced penetrance: individuals carrying the disease mutation do not develop the disease. This indicates that environmental and/or genetic factors (i.e. modifier loci) influence the severity of the phenotype. Complex disorders have a multifactorial inheritance caused by a combination of environmental factors and mutations in multiple genes. The exact strategy to map genes for complex diseases in general differs from that for monogenic disorders. For Mendelian disorders the most successful approach to map genes has been genome-wide linkage analysis in large multi-generational families. For mapping genes for complex disorders several study designs are possible, from large pedigrees to groups of cases and controls, using linkage as well as association analysis approaches (Figure 2)

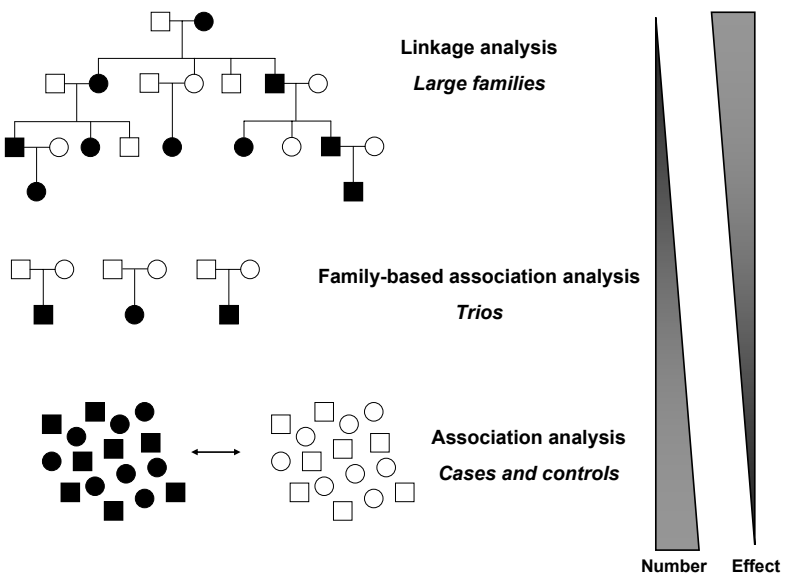

Figure 2. Mapping of disease genes using genetic markers.

Different study designs (large families, trios and case-control) and their respective statistical analyses are shown. In addition, triangles indicate: (left) the ratio in the number of families or cases needed for gene mapping and (right) the ratio in the expected effect size of disease alleles feasible to detect using these strategies.

Linkage analysis

In a genome scan a narrow grid of polymorphic markers evenly spaced over the genome is tested and marker alleles are subsequently correlated with the segregation of the disease by linkage analysis. When a chromosomal region (locus) is transmitted with the disease

phenotype within families, this region is likely to contain the gene of interest. Linkage analysis is based on the fact that loci located close to each other on the same chromosome are usually not separated by recombination but inherited together during meiosis. The recombination fraction (θ) is a measure of the dependence in inheritance between two loci and approximates 0 if these loci are close to each other and 0.5 if the loci are inherited independently. For calculating linkage, the LOD (logarithm of odds) score is calculated, which is the logarithm of a ratio of the likelihood of two loci being linked at a given θ and the likelihood that they are unlinked (θ=0.5). [38] Statistical analysis for linkage can be done with parametric (model-based) or non-parametric (model-free) methods. Parametric methods require specification of several parameters that define the model and mode of inheritance of the disorder, for example gene frequency, disease probabilities among those who carry no mutation (the phenocopy rate) and probabilities of being affected while carrying one or two copies of the allele (penetrance). If good estimates for these parameters are known, parametric linkage analysis is very powerful. [39] Since non-parametric methods do not require specification of the inheritance model, these methods are often regarded as more suitable for complex diseases, although they have lower power.

Association analysis

Whereas linkage analysis in general is family-based, association studies can be performed in homogenous case-control cohorts. Genetic association studies aim to detect association between one or more genetic polymorphisms and a trait, which may be a quantitative (e.g. blood pressure) or binary (affected or not affected) trait. Association differs from linkage in that the same allele (or alleles) is associated with the trait in a similar manner across the population, while linkage allows different alleles to be associated with the trait in different families. Association studies compare the frequency of alleles of a genetic marker between patients and healthy controls. If an allele increases susceptibility to a disease, it should be at a higher frequency among affected individuals than among controls. Significant association means (1) the polymorphism has a causal role; (2) the polymorphism has no causal role, but is associated with a nearby causal variant; or (3) the association is due to some underlying stratification or admixture (substructure) of the population.

Genetic isolates

Geneticists have targeted genetically isolated populations for mapping genes for Mendelian as well as complex diseases. Each genetically isolated population has its own demographic history, and each might have its own advantages and disadvantages for gene mapping. For studies of complex traits, preferably younger population isolates (10-20 generations old) are studied that originated from a relatively small number of founders and that underwent rapid population expansion. Genetically isolated populations have a reduced genetic variability compared to an outbred population, because of a limited number of founders (and consequently a limited gene pool), the absence of migration and genetic drift and inbreeding effects. These evolutionary forces have a great influence on young founder populations, especially compared to large stable outbred populations. This is explained by the Hardy-Weinberg equilibrium principle that describes the unchanging frequency of alleles and genotypes in a stable, idealized population. In this population there is random mating and sexual reproduction without normal evolutionary forces such as mutation, natural selection, or genetic drift. In the absence of these evolutionary forces, the population would reach equilibrium in one generation and maintain that equilibrium over successive generations.

It is also shown that in young isolates, linkage disequilibrium (LD) extends over much larger chromosomal regions than in outbred populations, a feature that can facilitate gene mapping. [40] LD is the non-random association of alleles at two or more loci on a chromosome that is gradually lost over generations by recombination. Another advantage of genetically isolated populations is that they generally have a more uniform environment and culture and, often extensive, genealogical records. Isolated populations, such as Finns, Icelandic's, Bedouin-Arabs, and Amish, have proven to be ideal for the identification of genes causing rare monogenic diseases. [41] Several genome-wide scans for complex diseases in genetically isolated populations have yielded numerous loci, but for most studies the positional cloning has remained a problem. [42]

Human Genome project

The ability to discover genes underlying human diseases has been tremendously facilitated by the genetic and physical maps, and technologies for gene identification that emerged from the Human Genome Project (HGP). The goal of their project was to complete the sequencing of the total human genome, develop genetic maps to assign genes to specific regions on chromosomes, to identify genes associated with disease, and to develop new technologies for furthering genetic research and clinical testing. The project also intended to investigate ethical, social, and legal issues as well as to provide education about genetics to professionals and to the public. [43] Before the HGP was underway, scientists had estimated that human complexity would require a genome in excess of 100,000 genes. The completion of the human genome sequence surprisingly showed that the human genome encodes only 20,000-25,000 protein-coding genes. The next major goals are: (1) systematic identification of all genetic polymorphisms carried in the human population to facilitate the study of their association with disease; (2) systematic identification of all functional elements in the human genome, including genes, proteins, regulatory controls and structure elements; (3) systematic identification of all the 'modules' in which genes and proteins function together. [43] Already millions of single nucleotide polymorphisms (SNPs) have been deposited in public databases, like dbSNP (www.ncbi.nlm.nih.gov). The International HapMap Project (www.hapmap.org), aimed to develop a freely-available haplotype map of the human genome (the HapMap) which describes the common patterns of human DNA sequence variation, has contributed significantly to this knowledge.

From disease locus to mutation

After mapping a disease locus to a certain genomic region, the causal gene can be identified by sequencing candidate genes for mutations. Selection of the most promising candidate genes is done by compiling all knowledge about expression, functional pathways, animal models, association with disease etc, from public databases, like NCBI (www.ncbi.nlm.nih.gov). Three classes of small-scale mutations can be distinguished: base substitutions (involve replacement of usually a single base), deletions (one or more nucleotides are eliminated from a sequence) and insertions (one or more nucleotides are inserted into a sequence). It is important to distinguish a causal mutation from a non-causal DNA variation or polymorphism. First the presence of the identified DNA variation needs to be tested in a group of control persons to exclude the possibility that it represents a common polymorphism (frequency >0.01). In addition, segregation with the disease phenotype and evolutionary conservation of the mutated amino acid are good indicators for a causal mutation. Essential proof is provided by functional studies characterising the effect of specific mutations. These

studies can be divided in cellular and animal model studies. Functional consequences of ion channel mutations are often studied with electrophysiological experiments in transfected cells. Electrophysiology involves measurement of voltage change or electrical current flow either on single channel or whole cell level. Generating transgenic knockin mouse models, carrying a human pathogenic mutation, can further knowledge of functional consequences of mutations and may reveal important dysfunctional pathways.

1.2.4 Challenges of gene mapping in complex diseases like migraine

Identification of genes for complex diseases is challenging. The contribution of genetic factors to susceptibility of complex diseases, termed heritability, is estimated from family or twin studies and is typically between 30 and 50%. [44] Thus environmental factors have a significant role. Genetic heterogeneity categorised in allelic heterogeneity (where the disease is caused by different variants within the same gene) and locus heterogeneity (disease caused by variants in genes at different chromosomal loci) complicates the analysis of complex diseases. In addition, multiple genetic factors may contribute to the phenotype (i.e. polygenic inheritance), and these genetic factors will most likely only have a small effect on disease risk and are therefore difficult to detect. Different points of view on the allelic structure of common diseases exist. The *common disease-common variant* (CD-CV) hypothesis predicts that the genetic risk of common diseases will often be due to disease-predisposing alleles with weak effect sizes and relatively high frequencies – that is, there will be one or a few predominating disease alleles at each of the major underlying disease loci. [43-48] Another hypothesis for allelic structure of common disease is the *common disease-rare variant* (CD-RV) hypothesis, predicting the presence of multiple rare alleles with moderate to large effect size. [49, 50] Most of the confirmed alleles associated with common diseases tend to support the CD-CV hypothesis, although this conclusion might be biased as these frequent alleles are easier to identify. [51] A few prototypical examples of such common variants are the *APOE* ε4 allele in Alzheimer's disease, [52] Factor V Leiden in deep venous thrombosis, [53] and PPARg Pro12Ala in type II diabetes. [54]

A complicating factor for the identification of genetic factors in migraine is the absence of any biological or radiological marker to establish the diagnosis, which is based on self reporting by the patient. Although international diagnostic criteria have been established, [8, 9] problems remain with their implementation in genetic studies. Migraine is a disease with high phenotypic variability within patients (attacks varying in frequency and clinical symptoms during lifetime) and within families (family members suffering from different types of migraine). This variability can cause problems in defining the affected status of persons in a genetic study. Also, in 10% of migraine patients, the age of onset is after 50 years, so that the diagnostic status of a young person who does not suffer from migraine remains uncertain. Because of the high prevalence of migraine, pure migraine families are hard to find because often other migraine patients are 'married in' into the pedigrees.

For migraine gene mapping different approaches are used to deal with the high genetic and clinical heterogeneity. The contribution of genetic factors among cases can possibly be increased by selecting patients with a strong family history and/or earlier onset of a disease. Another approach to decrease genetic complexity is to make use of intermediate or endophenotypes, because they may be controlled by fewer susceptibility factors than the disease state itself. An Australian study, in over 6,000 twin pairs, identified disease subtypes ("latent classes") by using so-called *latent class analysis* on the basis of the patterns and

severity of the symptoms. [55, 56] A Finnish study used the individual clinical symptoms of migraine - *trait component analysis* - to determine affection status in genome-wide linkage analyses of 50 migraine families.[57] The use of symptom components of migraine rather than the full end diagnosis is a promising novel approach to stratify samples for genetic studies.

1.3 Pathophysiology of migraine

Migraine is a disorder of the brain, with vascular changes being secondary to brain deregulation. The pathophysiology once a migraine attack has started is now beginning to be well understood. However why and how migraine attacks are triggered is essentially unknown. Therefore, identification of susceptibility genes will help to increase our knowledge about pathways that are involved in the migraine attack, and especially why they are triggered. Activation of the trigeminal vascular system (TGVS) appears essential in the development of the headache phase. [58, 59] The TGVS consists of the cranial blood vessels, innervated by sensory afferent fibers of the ophthalmic division of the trigeminal nerve. Activation of these fibers leads to activation of second-order neurons in the trigeminal nucleus caudalis (TNC) and the two uppermost levels of the spinal cord dorsal horn, together termed the trigeminocervical complex. Impulses are then carried to brain regions involved in the modulation and perception of pain, including the thalamus and the periaqueductal gray region (PAG). Activation of the TGVS also leads to the release of vasoactive neuropeptides contained in the peripheral nerve endings, including calcitonin gene-related peptide (CGRP) and substance P. [60] In experimental animal models, release of these of these inflammatory mediators from activated trigeminal nerve endings initiates an inflammatory reaction characterized by vasodilatation of the meningeal vessels, plasma protein leakage and mast cell degranulation with secretion of proinflammatory substances in the dura mater. [61-63] The headache in migraine is believed to result from similar activation of trigeminal neurons and subsequent inflammatory reaction in the meninges. [64]

It is now well accepted that the migraine aura is most likely caused by the human equivalent of the cortical spreading depression (CSD) of Leao. [65, 66] CSD is a slowly propagating (3-5 mm/min) wave of sustained strong neuronal depolarization that spreads across the cortex and is followed by prolonged nerve cell depression synchronously with a dramatic failure of brain ion homeostasis, efflux of excitatory amino acids from nerve cells and enhanced energy metabolism. [65] Several studies with neuroimaging recordings during aura in humans support the conclusion that visual aura arises from CSD. [67-70] While the evidence that CSD causes the migraine aura is mounting, there is much debate as to whether CSD may trigger the rest of the migraine attack as well through activation of the trigeminovascular system. Animal studies have identified a potential link between CSD and headache by showing that CSD, induced by either pinprick or electrical stimulation, can activate the meningeal trigeminovascular afferents and evoke a series of alterations in the meninges and brainstem consistent with the activation of trigeminal nociceptive pathways and the development of head pain. [71, 72] However direct human evidence for this hypothesis is still lacking.

1.4 Aim and outline of thesis

Migraine is a very common and disabling episodic disorder of largely unknown etiology. Identification and characterisation of susceptibility genes for migraine is an important step

in understanding the pathophysiological mechanisms underlying the disease and might give novel leads for drug development. The studies in this thesis have focussed on the genetic mechanisms underlying familial hemiplegic migraine, sporadic hemiplegic migraine and migraine with and without aura. The main aim of this work was to search for genetic loci and genes involved in these types of migraine, using different research strategies.

FHM is considered a good model to study the genetics of migraine. At the start of the thesis, the first gene for FHM was already identified on chromosome 19p13: *CACNA1A*, encoding the $Ca_v2.1$ α1-subunit of voltage-gated neuronal P/Q-type calcium channels. [73] In **chapter 2**, the link between FHM and epilepsy was investigated by testing the involvement of the FHM1 *CACNA1A* gene in a family with FHM, childhood epilepsy and cerebellar ataxia. In **chapter 3**, the FHM2 *ATP1A2* gene mutation spectrum and the clinical phenotypes associated with it were studied. To investigate functional consequences of several *ATP1A2* mutations, cellular survival assays were performed. In **chapter 4**, the role of the voltage-gated sodium channel epilepsy gene *SCN1A* in FHM families was studied. To learn the functional consequences of a novel *SCN1A* mutation, electrophysiological characterisation of mutant channels was performed. In **chapter 5,** the involvement of the three FHM genes in sporadic hemiplegic migraine (SHM) was studied. Although clinically indistinguishable, it was unknown whether and to what extent SHM and FHM are pathophysiologically related and whether and to what extent known genes for FHM are also involved in SHM. Thirty-nine well-characterized SHM patients were systematically scanned for mutations in these FHM genes. For all novel sequence variants functional assays were performed. In **chapter 6** the genetics of common migraine was investigated. Two genome-wide scans were performed to map susceptibility loci for migraine, the first with an outbred linkage approach with Dutch MO families and the second with a family-based association approach with severe MA patients from a genetic isolate. **Chapter 7** provides a general discussion. Results from this thesis are reviewed and future possibilities for genetic research of migraine are discussed.

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Chapter 2

Childhood epilepsy, familial hemiplegic migraine, cerebellar ataxia, and a new *CACNA1A* mutation

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Introduction

The *CACNA1A* gene encodes the pore-forming subunit of neuronal P/Q type Ca^{2+} channels. Mutations in this gene cause a spectrum of neurological diseases, including familial hemiplegic migraine (FHM). [1,2] We report a novel *de novo CACNA1A* mutation in a Swedish family. Three mutation carriers had FHM and early onset ataxia; additional childhood epilepsy occurred in two.

Family Description

The proband, II-3, is a 54-year-old woman with slowly progressive cerebellar ataxia since childhood and cerebellar atrophy on CT. She was hospitalized twice at ages 7 and 8 because of decreased consciousness and vomiting for 1 day, starting with a lucid interval after a fall. She experienced four hemiplegic migraine attacks between ages 14 and 30 years and weekly at age 47. Seizures were never observed.
Her 32-year-old son (III-5) and 30-year-old daughter (III-6), who have different fathers, showed cerebellar ataxia at age 4. Ataxia is now prominent in both, and brain imaging shows cerebellar atrophy. Both have had attacks of migraine without aura since age 8 and so far two episodes of hemiplegic migraine.
Both III-5 and III-6 had seizures during childhood. III-5 had at age 5 several episodes of complex partial seizures. Carbamazepine treatment was instituted. After a generalized tonic-clonic seizure, the treatment was changed to phenytoin. He remained seizure-free under treatment until the age of 11, and afterward without medication. At ages 5 and 8 he had periods of decreased consciousness for several hours after a fall with a lucid interval. III-6 had a complex partial seizure at the age of 1.5, following an upper respiratory tract infection. At age five she had a generalized tonic-clonic seizure not associated with infection or fever. Phenytoin treatment was instituted. Four months later, while having an upper respiratory tract infection and fever (39° C), she had generalized (tonic-clonic) seizures during 2 days, lasting a few minutes each. Phenytoin treatment was continued to age 9. Repeated interictal EEGs in both III-5 and III-6 did not demonstrate epileptiform activity. Subjects I-I, I-2, and II-4 had no history of seizures or ataxia, I-1 and II-4 had migraine without aura.

Mutation analysis *CACNA1A*

Direct sequencing of all exons of the *CACNA1A* gene revealed a heterozygous nt 5404 T>C substitution in exon 33 of genomic DNA of II-3, III-5, and III-6, which was not found in DNA of subjects I-1, I-2, and II-4, or 50 control subjects (*CACNA1A* reference sequence: Genbank Ac. nr. X99897) (figure). This point mutation changes an Isoleucine to a Threonine residue, at position 1710, located within the transmembrane segment 5 of the fourth domain. Haplotyping analysis of genetic markers D19S221, D19S1150 and D19S226 excluded nonpaternity of I-1, indicating that the mutation arose de novo in II-3 (figure, for primer sequences and PCR conditions, see http://gdbwww.gdb.org/). Sequence alignments showed strong evolutionary conservation in the protein sequence both of the Isoleucine that is mutated as well as the neighboring amino acids (data not shown).

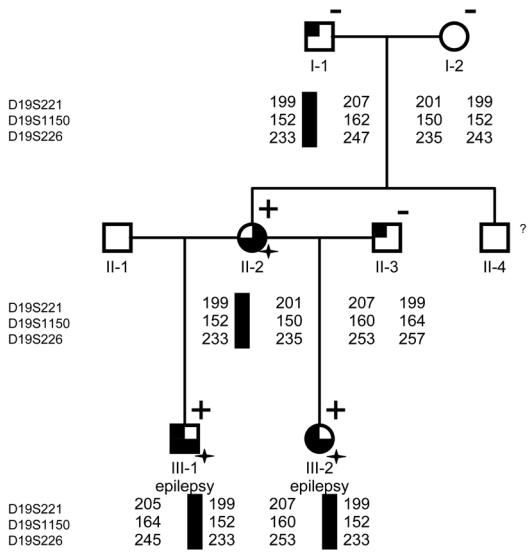

Figure. Pedigree of the family.

Hemiplegic migraine: black lower half box (males)/circle (females); migraine with aura: black upper right quadrant; migraine without aura: black upper left quadrant; cerebellar ataxia: black star; question mark: information was provided by other family members; +: mutation carriers; -: no mutation detected. Alleles of D19S221, D19S1150, and D19S226 are shown. Black bar: haplotype cosegregating with hemiplegic migraine.

Discussion

In this family, epileptic seizures occurred independently from FHM attacks in two of three mutation carriers with FHM and cerebellar ataxia. Seizures separated from FHM attacks have not been described before in FHM families with *CACNA1A* mutations. In contrast, epilepsy is not uncommon in FHM associated with *ATP1A2* mutations. [2,3] The proband, with the de novo I1710T mutation, had no epilepsy, indicating reduced penetrance. Her two children had complex partial seizures. Patient III-6 had additional generalized tonic-clonic seizures (febrile and afebrile), where a focal start could not be inferred. A shared genetic etiology of the seizures is likely: they had different fathers, both had inherited the I1710T mutation from their mother, and both had cerebellar ataxia and FHM. Seizures isolated from FHM attacks were restricted to childhood and did not reappear after discontinuation of medication. Epilepsy has been associated with the calcium channel subunit gene *CACNA1A* before: natural mutant mice have absence epilepsy and ataxia, [4] and polymorphisms within *CACNA1A* are associated with idiopathic generalized epilepsy.[5] Furthermore, a boy with the Y1820X mutation has absence seizures, generalized tonic-clonic seizures, and ataxia (but no hemiplegic migraine attacks). [6] Finally, seizures may also occur during severe hemiplegic migraine attacks. [7] This report confirms *CACNA1A*-associated epilepsy in human and extends the *CACNA1A* phenotype by the co-occurrence of FHM and childhood epilepsy.

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Chapter 3

Mutations in the FHM2 Na,K-ATPase gene *ATP1A2*

3.1

Novel mutations in the Na^+,K^+-ATPase pump gene *ATP1A2* associated with Familial Hemiplegic Migraine and Benign Familial Infantile Convulsions

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Novel Mutations in the Na^+,K^+-ATPase Pump Gene *ATP1A2* Associated with Familial Hemiplegic Migraine and Benign Familial Infantile Convulsions

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Familial hemiplegic migraine (FHM) is a rare, severe, autosomal dominant subtype of migraine with aura. Up to 75% of FHM families have a mutation in the P/Q-type calcium channel $Ca_v2.1$ subunit *CACNA1A* gene on chromosome 19p13. Some CACNA1A mutations also may cause epilepsy. Here, we describe novel missense mutations in the ATP1A2 Na^+,K^+-ATPase pump gene on chromosome 1q23 in two families with FHM. The M731T mutation was found in a family with pure FHM. The R689Q mutation was identified in a family in which FHM and benign familial infantile convulsions partially cosegregate. In this family, all available affected family members with FHM, benign familial infantile convulsions, or both, carry the ATP1A2 mutation. Like FHM linked to 19p13, FHM linked to 1q23 also involves dysfunction of ion transportation and epilepsy is part of its phenotypic spectrum.

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Familial hemiplegic migraine (FHM) is a rare, severe autosomal dominant subtype of migraine with aura associated with hemiparesis.[1] Up to 75% of the reported FHM families are linked to chromosome 19p13 and have missense mutations in the *CACNA1A* gene encoding the $Ca_v2.1$ subunit of neuronal voltage-gated P/Q-type calcium channels.[2,3] Approximately 10% of the reported FHM families are linked to chromosome 1q21-23.[4–8] In the remaining families, linkage to chromosomes 19 and 1 was excluded, suggesting at least a third gene for FHM.

Recently, two missense mutations have been identified in the *ATP1A2* gene, coding for the $\alpha 2$ subunit of the Na^+,K^+-ATPase, in two families with FHM linked to 1q23.[8,9] Both mutations lead to a *loss-of-function* of the Na^+,K^+ pump.[9] Here, we describe two novel mutations in the *ATP1A2* gene, one in a Dutch family with pure FHM, and the other in a Dutch-Canadian family in which FHM and benign familial infantile convulsions (BFIC) partially cosegregate.[10] BFIC is a rare autosomal dominant benign familial childhood epilepsy, with strictly partial, nonfebrile, convulsions that begin at age 3 to 12 months and disappear after the first year.[11] These findings confirm that migraine and epilepsy, at least in part, have overlapping mechanisms involving dysfunction of ion transportation.

Subjects and Methods

All subjects gave written informed consent. Their neurological examinations were unremarkable. Diagnoses of migraine[1] and BFIC[12] were made according to standardized criteria.

Family 1

This Dutch family is depicted in Figure 1. The proband (III-2) was a 33-year-old female who, since the age of 3 years, had attacks of headache and vomiting accompanied by one-sided sensory symptoms, weakness in one arm, and

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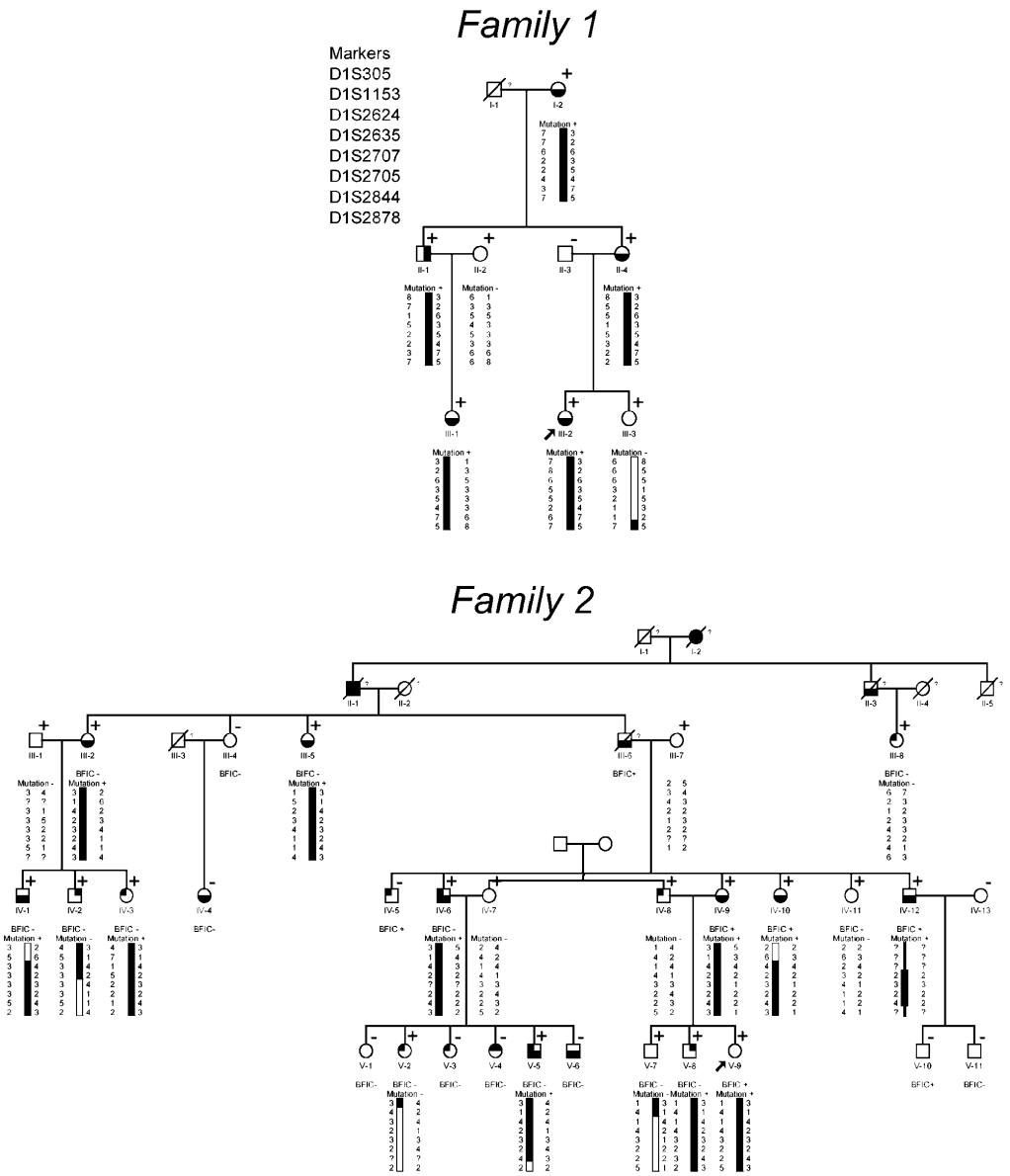

Fig 1. Pedigree of Families 1 (FHM) and 2 (FHM and BFIC). Pedigrees of Families 1 and 2 are depicted. The arrows indicate the probands. The following symbols are used to indicate the diagnosis: FHM: black lower half*; MA:* right upper square*; MO:* left upper square*; MsM:* black right half*; ?: heteroanamnestic information. BFIC + and BFIC − indicate a positive and negative BFIC diagnosis, respectively. Mutation + and Mutation − indicate carriers and noncarriers of the pathogenic mutations, respectively. Black bars show the haplotypes segregating with FHM and/or BFIC. Additional markers indicated that the recombination in Patient V-7 was located between D1S1153 and D1S2624 (data not shown). (+) Genomic DNA is available; (−) no genomic DNA available.*

speech disturbances for 15 to 30 minutes. Total attack duration was 1 to 2 days, and attack frequency ranges from twice a year to bimonthly. There was no history of epilepsy.

The mother of the proband (II-4, 54 years) had, from age 7 years until menarche at age 16 years, attacks, twice a year, of headache and aura (black spots, sensory symptoms, and weakness in both hands and right side of the face) for 1 to 2 days.

The grandmother of the proband (I-2, 89 years) had from age 9 years until menarche at age 16 years, attacks of headache, vomiting, and aura symptoms including dysarthria, visual problems (not been able to read, seeing spots), and weakness in one arm, lasting for 1 day.

An uncle of the proband (II-1, 57 years) has, since the age of 11 years, two to four times a year attacks of aura without headache lasting for 15 minutes. During these attacks, he saw black spots and had speech difficulties and sensory symptoms in the tongue and one hand with loss of control without clear weakness. Before the age of 11 years, he had had four similar episodes after mild head trauma's. These episodes were at that time diagnosed as cerebral concussions but in retrospect might well have been episodes of trauma-triggered attacks of migraine aura.[13]

A cousin of the proband (III-1, 32 years) had had, from age 14 years, three attacks with headache, vomiting, visual symptoms (black spots), speech difficulties, confusion, sensory symptoms in one arm, and weakness in arm and face. These attacks lasted 1 to 2 days.

Family 2

This Dutch-Canadian family has been described in detail elsewhere.[10,14] The pedigree is depicted in Figure 1. Since the previous publication, the diagnosis of one subject (V-8) has changed. This previously healthy boy now had a diagnosis of migraine with aura.

In summary, 11 subjects were diagnosed with FHM, and 7 subjects had infantile seizures. The infantile seizures were all generalized and started at a median age of 3 months (range, 1.5–5 months); all patients had normal psychomotor development. An exact diagnosis of the epilepsy syndrome in this family is difficult because ictal electroencephalogram information was unavailable. However, because the onset of epilepsy is clearly infantile and not neonatal we are confident that the diagnosis in our family is indeed BIFC. Four subjects had both FHM and benign infantile convulsions. Two FHM patients also had migraine without aura. An additional seven subjects had migraine with or without aura but no hemiplegic migraine attacks. One spouse had migraine without aura. Two subjects (IV-3 and V-9) once had a febrile convulsion, and one subject (IV-9) had a generalized convulsion at age 8 years.

Genomic DNA Samples

Blood samples were collected from Subjects I-2, II-1, II-2, II-4, III-1, III-2, and III-3 of Family 1 and III-1, III-2, III-5, III-7, III-8, IV-1, IV-2, IV-3, IV-6, IV-7, IV-8, IV-9, IV-10, IV-11, IV-12, V-2, V-5, V-7, V-8, and V-9 of Family 2. Genomic DNA was isolated from leukocytes.[15]

Haplotype Analysis

Markers D1S305, D1S1153, D1S2624, D1S2635, D1S2707, D1S2705, D1S2844, and D1S2878 were tested for Families 1 and 2. All primer sequences and polymerase chain reaction (PCR) conditions are available through the Human Genome Database (http://gdbwww.gdb.org/). After PCR, amplified products were separated using an ABI 3700 DNA sequencer. All genotypes were analyzed and independently double scored by E.E. Kors and K.R.J. Vanmolkot using Genescan and Genotyper 2.1 software (Applied Biosystems, Foster City CA). Haplotypes were constructed by inspection of segregation and assuming a minimal number of recombinations.

Mutation Screening

All exons of ATP1A2 were amplified by PCR from genomic DNA of one (II-4) or two (III-2, V-9) affected subjects from Families 1 and 2, respectively. The genomic structure was available from the Human Genome Project (NCBI database http://www.ncbi.nlm.nih.gov/). Details of intron–exon structure and primer sequences are available from us upon request. Double-strand sequencing was performed using the dideoxy termination method and an ABI3700 sequencer (Prism Big Dye Terminators Cycle Sequencing kit; ABI Applied Biosystems).

Carrier Detection

To identify carriers of the T-to-C substitution in Family 1 resulting in a M731T missense mutation, we amplified exon 16 by PCR using primers exon 16F (5′-CCAAGACAAGCATGGAGTGA-3′) and exon 16R (5′-AAGGGACAGGGAACAGAGGT-3′) and genomic DNA as a template. PCR products were digested with restriction enzyme *Nco*I using standard protocols and electrophoresed on a 3% agarose gel.

Similarly, for the G-to-A substitution in Family 2 resulting in an R689Q missense mutation, PCR products of exon 15 which was amplified by PCR using primers exon 15F (5′-AGGAGGGGCTGGTACAGGT-3′) and exon 15R (5′-GAAGACGGCCACACTTGAG-3′) and genomic DNA as a template were digested with restriction enzyme *Taq*I using standard protocols and electrophoresed on a 3% agarose gel.

As controls, genomic DNA of 50 subjects from the general Dutch population with no history of migraine, epilepsy, or cerebellar ataxia were used.

Results

Haplotype Analysis

Family 1 was too small for linkage analysis, but haplotype analysis suggested involvement of 1q21-23 (see Fig 1). Previous mutation analysis of the *CACNA1A* gene located on chromosome 19p13 failed to detect pathogenic mutations.

Family 2 was not linked to 19p13 but showed suggestive linkage to 1q21-23 (data not shown, for methodology see Terwindt and colleagues[10]). A single haplotype was found to consistently cosegregate with the disease phenotype in all tested patients with FHM, BFICs, or a combination of both disorders (see Fig 1).

Mutation Analysis

Mutation analysis of the *ATP1A2* gene was performed by direct sequencing of all exons. Two point mutations were identified. In Family 1, the M731T mutation causes a change of a threonine for a methionine residue in exon 16 (nucleotide 2296 T→C) (Figs 2 and 3). The R689Q mutation in Family 2 changes a glutamine into an arginine residue in exon 15 (nucleotide 2170 G→A) (see Figs 2 and 3). Sequence alignments indicate a strong conservation of amino acids M731 and R689 among several α units of Na^+,K^+-ATPases, H^+,K^+-ATPases and sarco/endoplasmic reticulum Ca^{2+} (SERCA) ATPases from several species (Fig 4). Neither of the mutations was observed in a panel of 100 control chromosomes.

Discussion

We describe two novel missense mutations in the Na^+,K^+-ATPase gene on chromosome 1q23: the M731T mutation in a new family with pure FHM and the R689Q in a previously described family with co-segregating FHM and BFIC.

For the FHM/BFIC family, we initially argued that two genes could be involved.[10] The current mutation analysis, however, shows that all available patients with FHM, BFIC, or both carry the R689Q mutation. This suggests that this ATP1A2 mutation may cause FHM, BFIC, or both, possibly depending on other genetic or nongenetic cofactors. The *ATP1A2* gene therefore may be the first gene for BFIC. Other loci for BFIC have been identified on chromosome 19q,[16] 16p,[17] and 2q24,[18] but, as yet, no responsible genes. The *ATP1A2* gene is likely to be involved in other types of epilepsy because non-BFIC Epilepsy has been reported in other families with FHM linked to 1q21-q23.[4,6,8]

The Na^+,K^+-ATPase is an integral plasma membrane enzyme that couples the hydrolysis of ATP to the countertransport of Na^+ and K^+ across the membrane.[19] As a key regulator of cellular ion homeostasis, it is important to several cellular functions, including control of cell volume and pH, and the generation of action potentials. The Na^+,K^+-ATPase protein complex is composed of three heteromeric subunits, the α or catalytic subunit, the β or regulatory subunit, and the γ subunit, whose function is unknown.

There are four α subunit genes of which α1-3 are expressed in the central nervous system. The α1 isoform is ubiquitously expressed and serves as the "housekeeping" form. The α2 isoform is also expressed in muscle and adipose tissue (for review, Mobasheri and colleagues[19]); in mouse central nervous system, expression of the α2 isoform is primarily

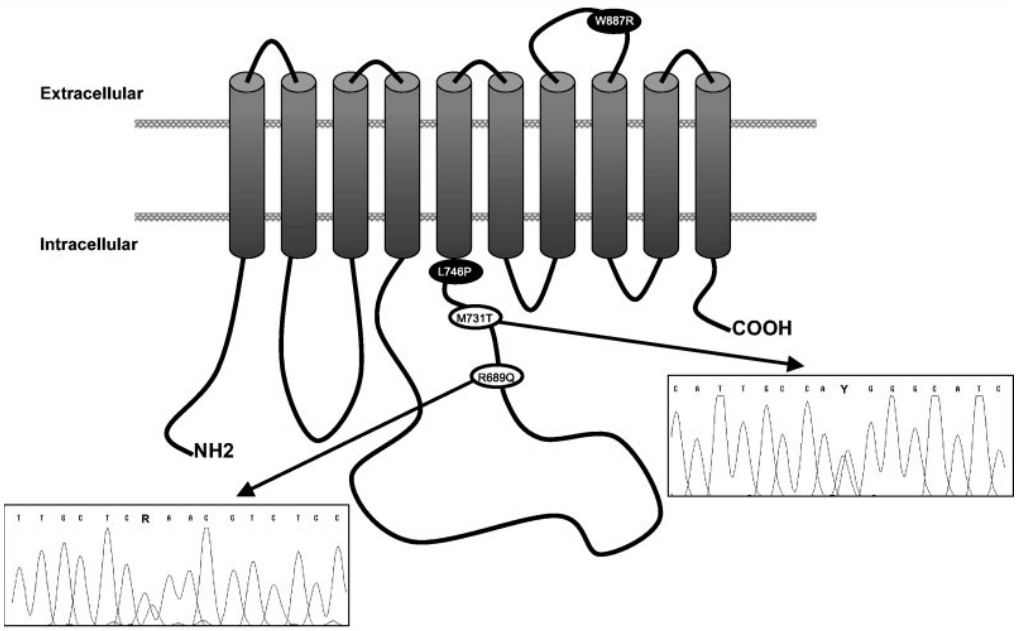

Fig 2. Predicted transmembrane topology model of the Na^+,K^+-ATPase α2 subunit and location of mutations. The location of the amino acid substitutions of the two new mutations (M731T and R689Q) and those previously published (L746P and W887R)[9] are depicted. Arrows point to the boxes with electropherograms showing the respective heterozygous mutations.

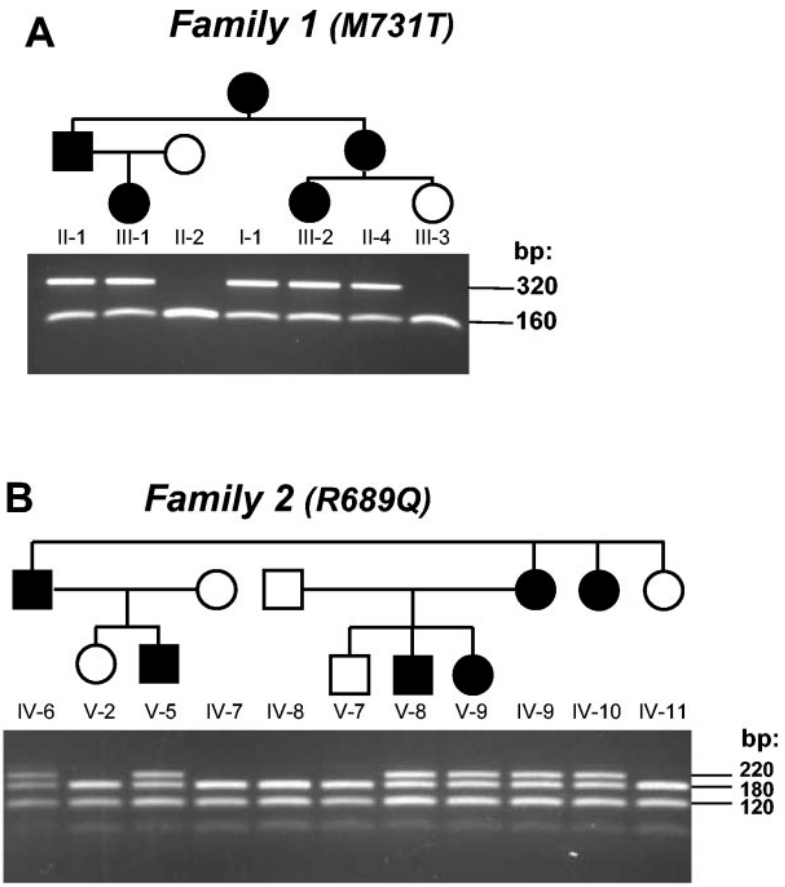

Fig 3. Carrier detection by restriction enzyme analysis in the two families. (A) M731T mutation of Family 1 is detected by a loss of a Nco*I site, which results in an extra 320bp band for the mutant allele. All the haplotype carriers are depicted in black. (B) R689Q mutation of Family 2 is detected by a loss of a* Taq*I site, which results in an extra 220bp band for the mutant allele.*

within the neurons during late gestation and the early neonatal stage but primarily in astrocytes in adult tissue.[20]

Only a small part of the α subunit is exposed on the extracellular side of the membrane (see Fig 2). Both the amino and carboxyl termini are located intracellularly. The M731T and R689Q mutations are present in the large intracellular loop between transmembrane domains M4 and M5. This loop harbors the ATP-binding and hydrolase domains and has been implicated in several functions.[21] The M731 is located in the highly conserved "hinge" sequence in the junction region.[21] This domain and the phosphorylation motif are the most highly conserved regions amongst all prokaryotic and eukaryotic ATPases. It is essential for the interactions between the catalytic and membranous cation binding sites during cation translocation.[19,21,22] The R689 residue is located in the same important functional hydrolase domain but outside the "hinge" sequence.

It is likely that the M731T and R689Q mutations have a *haploinsufficiency* effect similar to that reported for the L764P mutation, which is located in the same intracellular loop.[9] Homozygous ATP1A2 knockout animals are severely affected, have reduced contractility of cardiac and skeletal muscles, and die at birth because of failure to breath.[20,23] Heterozygous animals, however, grow normally and show increased muscle contractility.[20]

The Na^+,K^+-ATPase pump exchanges intracellular Na^+ for extracellular K^+. Loss of Na^+,K^+-ATPase function therefore may depolarize neurons and may

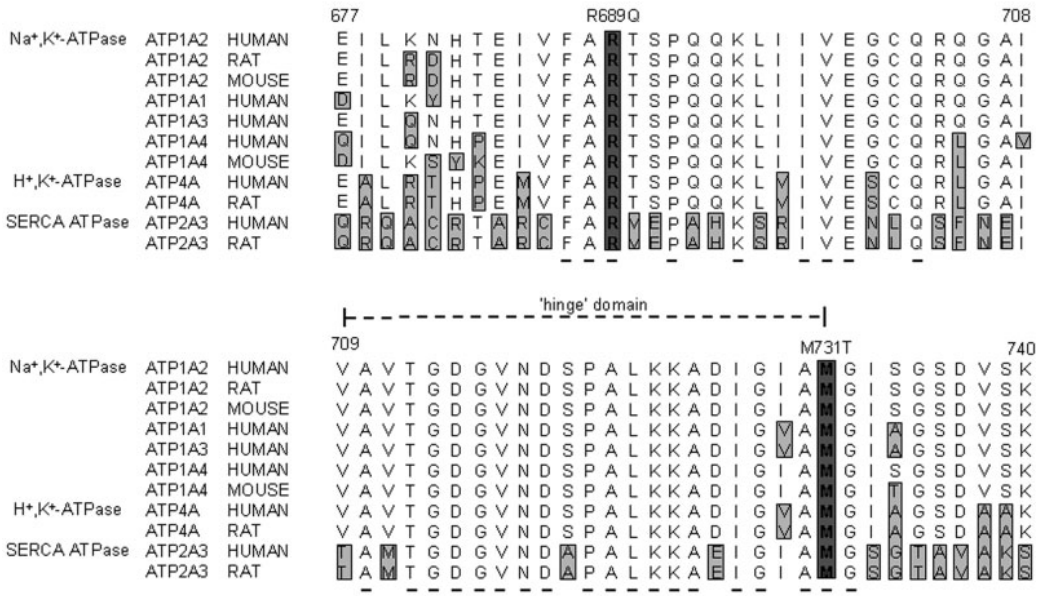

Fig 4. Alignments of amino acid sequences of relevant parts of α subunits of ATPases. Conservation of the mutated amino acids R689 and M731 residues are depicted as dark boxes in alignments of various α subunits of different species (accession numbers are indicated). Variations from human ATP1A2 are indicated by light gray boxes. Amino acid residues that are not variable are indicated by horizontal lines at the bottom. Na^+,K^+-ATPases; ATP1A2 of human (P50993), rat (P06686), and mouse (AAH25807), human ATP1A1 (P05023), human ATP1A3 (P13637), human ATP1A4 (Q13733), mouse ATP1A4 (Q9WV27); H^+,K^+,ATPase; ATP4A of human (NP_000695) and rat (P09626); SERCA ATPase ATP2A3 of human (Q93084) and rat (NP_037046). The strongly conserved "hinge" domain,[21] is indicated by a dotted line. The amino acid positions in the human ATP1A2 are shown.

render them hyperexcitable. Furthermore, increase of extracellular K^+ (Kraig and colleagues[24]) will facilitate cortical spreading depression (CSD), the likely mechanism for migraine aura,[25] and increase of intracellular Na^+ will enhance intracellular Ca^{2+} through decreased Na^+-Ca^+-exchanger function, which will also facilitate CSD.[26] Interestingly, ouabain, which blocks ATPase function, changes extracellular pH, causes cation shifts, and also induces CSD.[27] Finally, studies on the functional consequences of CACNA1A FHM mutations also suggest increase of intracellular Ca^{2+} as a major effect.[28] In summary, these studies support the concept that dysfunction of ion transportation are important in the pathogenesis of FHM, presumably through facilitation of CSD.

The differential expression of the Na^+,K^+-ATPase α2 isoform over life may provide an attractive explanation for the different clinical effects of ATP1A2 mutations in the very young (infantile epilepsy caused by neuronal hyperexcitability) versus in adulthood (FHM caused by a reduced threshold for CSD caused by low reuptake of extracellular K^+ and neurotransmitters by glial cells). This needs to be confirmed in functional studies.

In conclusion, we confirm the role of ATP1A2 in FHM and expand the clinical spectrum to BFIC, a specific form of epilepsy. Migraine and epilepsy seem to have partially overlapping and partially opposite mechanisms related to dysfunction of ion transportation.

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3.2

Severe episodic neurological deficits and permanent mental retardation in a child with a novel FHM2 *ATP1A2* mutation

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Severe Episodic Neurological Deficits and Permanent Mental Retardation in a Child with a Novel FHM2 *ATP1A2* Mutation

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Objective: **Attacks of familial hemiplegic migraine (FHM) are usually associated with transient, completely reversible symptoms. Here, we studied the *ATP1A2* FHM2 gene in a young girl with episodes of both very severe and transient neurological symptoms that were triggered by mild head trauma as well as permanent mental retardation. Her family members suffered from hemiplegic and confusional migraine attacks.** ***Methods:*** **Mutation analysis of the *ATP1A2* gene was performed by direct sequencing of all exons and flanking intronic regions, using genomic DNA of the proband. Functional consequences of the mutation were analyzed by cellular survival assays.** ***Results:*** **We identified a novel G615R *ATP1A2* mutation in the proband and several of her family members. Functional analysis of mutant Na,K-ATPase in cellular survival assays showed a complete loss-of-function effect.** ***Interpretation:*** **Permanent mental retardation in children may be caused by *ATP1A2* mutations.**

Ann Neurol 2006;59:310–314

Familial hemiplegic migraine (FHM) is a rare autosomal dominant subtype of migraine, in which attacks are associated by hemiparesis in addition to other neurological aura symptoms.[1,2] Typically, these symptoms are fully reversible. The majority of patients have mutations in the neuronal calcium channel gene *CACNA1A* (FHM1).[3,4] Approximately 20% of FHM in families is linked to chromosome 1q23 (FHM2),[5] with mutations in the *ATP1A2* gene,[6] encoding the α2-subunit of a Na,K-ATPase. Recently a mutation in the sodium channel gene *SCN1A* has been associated with FHM (FHM3).[7]

The clinical spectrum associated with *ATP1A2* mutations is expanding as childhood epilepsy, alternating hemiplegia of childhood, coma, sensory deficits, as well as transient and permanent cerebellar signs have been reported.[8,9–13] Apart from cerebellar ataxia, permanent neurological symptoms have rarely been described in FHM. Here, we describe a novel *ATP1A2* loss-of-function mutation in a young girl with permanent mental retardation after a few episodes of severe neurological deficits that were triggered by mild trauma. Several family members with the mutation suffered from hemiplegic and confusional migraine attacks, which were initially misdiagnosed as epilepsy.

Patients and Methods

Patient and Family Description

The proband, III-10, was a 9-year-old girl who initially showed a normal psychomotor development. For pedigree, see Figure 1. At age 2 years, she had two episodes of transient blindness, restlessness, and fever that were triggered by mild head trauma. The first episode lasted a few hours, the second several days. Cerebral magnetic resonance imaging (MRI) was unremarkable; the electroencephalogram (EEG) showed diffuse θ and δ activity. After the second episode, there was a severe regression in development and she showed autistic behavior that recovered slowly and incompletely over the next 2 years. She remained mildly retarded (IQ [Wechsler Intelligence Scale for Children–Revised] of 70 at age 8

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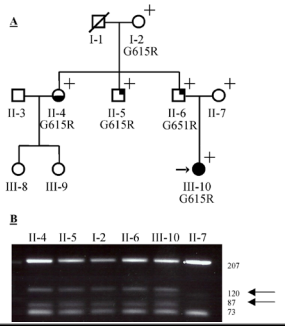

Fig 1. Pedigree of the family and carrier detection. (A) Hemiplegic migraine is indicated as a black lower half box (males)/ circle (females), trauma-triggered neurological episodes in the proband (arrow) *as a full black circle, confusional migraine as a black upper right quadrant. A plus symbol indicates that DNA was available for genetic testing; G615R indicates heterozygosity for the G615R mutation. (B) MvaI-digested mutant polymerase chain reaction product results in two additional bands of 120 and 87bp* (arrows)*, in addition to wild-type bands of 207, 73, and 11 (not visible) bp.*

years). At ages 4 and 7 years, she was admitted to the hospital after a fall on her head, with fever, left-sided hemiparalysis, conjugated right-sided deviation of the eyes, somnolence, generalized seizures, and during the last episode status epilepticus. Cerebral MRI at the second day of the third episode was unremarkable. Fluid-attenuated inversion recovery (FLAIR) MRI imaging 2 weeks later showed a discrete swelling and hyperintensity of the cortex of the right hemisphere without enhancement after gadolinium (Fig 2A). The EEG displayed diffuse very slow, polymorph, δ activity over the right hemisphere with a maximum in the frontal regions, and some slow activity over the left hemisphere. Focal epileptiform activity was seen in the left temporal region (see Fig 2B). The girl recovered completely from these episodes. After the last episode acetazolamide 250mg twice daily was started and she has remained attack-free since, for now more than 2 years. A recent MRI did not show any abnormalities.

Her father (II-6) reported that he, his brother (II-5), and his sister (II-4) were diagnosed with childhood epilepsy. Medical records of his sister (II-4) showed attacks of hemiplegia, aphasia, altered consciousness, amnesia, headache, and/or aggressive behavior since the age of 3 years. These episodes were sometimes provoked by physical exercise or a fall on the head. Her last episode occurred at age 33 years. Medical information of father (II-6) and uncle (II-5) was not available. According to their 68-year-old mother (I-2), they each had had one or two episodes at school age, involving strange behavior and headache for a few hours. These episodes resembled the episodes of their sister when she was confused. II-5 also had a learning disability and received special education. Retrospectively, II-4, II-5, and II-6 most likely had hemiplegic migraine with a few episodes of confusional migraine.[14]

Genetic Analysis

Genomic DNA was isolated according to standard protocols. Direct sequencing was performed for all 23 exons of the *ATP1A2* gene. For detection of the heterozygous substitution (nucleotide 1947 G→A; accession no. NM_000702), exon 14 was amplified by polymerase chain reaction (291bp product) and products were digested with restriction enzyme *Mva*I and electrophoresed on a 3% agarose gel. The wild-type allele results in bands of 207, 73, and 11bp, whereas the mutant allele produces two extra bands of 120 and 87bp.

Functional Analysis

Human Na,K-ATPase α2-subunit cDNA was subcloned into a modified pCDNA3.1 vector.[15] To distinguish endogenous Na,K-ATPase activity from that of transfected Na,K-ATPase, we used a cDNA encoding ouabain-resistant wild-type (α2-WT)[16] to introduce the G615R mutation with site-directed mutagenesis (Quikchange; Stratagene, La Jolla, CA) (mutant α2-G615R). HeLa cells (5×10^5) were transfected with plasmid DNA of either α2-WT or α2-G615R, using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA). Two days after transfection, two thirds of the cells was harvested for immunoblotting and the α2-subunit was detected using specific polyclonal antibody HERED.[17] The remaining one third of the cells was seeded on 10cm petri dishes and subsequently 1μM ouabain was added to the culture medium. After 5 days of ouabain challenge, colonies were stained with 1% methylene blue in 70% methanol, scanned, and analyzed with Image Pro Plus (MediaCybernetics, Silver Spring, MD). Each transfection was performed eight times.

Results

A novel G615R mutation was identified in the *ATP1A2* FHM2 gene in the proband and family members I-2, II-4, II-5, II-6, and III-10, but was absent in spouse II-7 (see Fig 1). The mutation was absent in 100 Dutch control individuals with no history of migraine or epilepsy. Mutation G615R is located in the highly conserved "TGD" motif of the ATP-binding domain in the large intracellular loop.[18,19]

In survival assays, the endogenous Na,K-ATPase activity was completely inhibited by ouabain challenge in HeLa cells. In such an assay, we assessed whether trans-

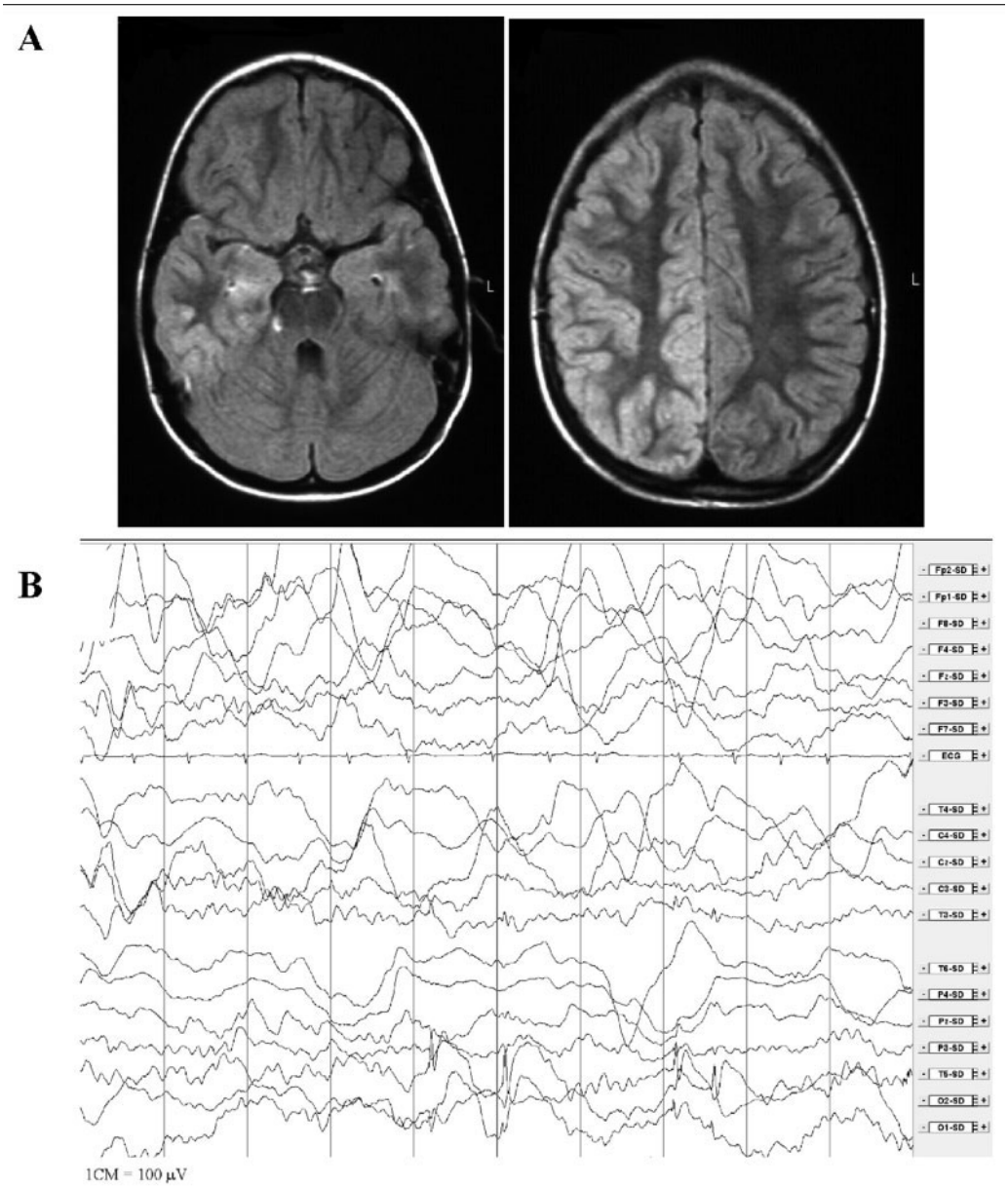

Fig 2. Magnetic resonance imaging (MRI) and electroencephalogram (EEG) at third episode (at age 4 years). (A) MRI 2 weeks after the start of the third episode. Discrete swelling and a hyperintense signal of the cortex of the right hemisphere especially parietooccipital, in the uncus and the parahipppocampal gyrus (fluid-attenuated inversion recovery imaging). (B) EEG on day 3 of the third episode showing diffuse very slow polymorph δ activity over the right hemisphere with a maximum in the frontal region and some slow activity over the left hemisphere. Focal epileptiform activity is seen over the left temporal region.

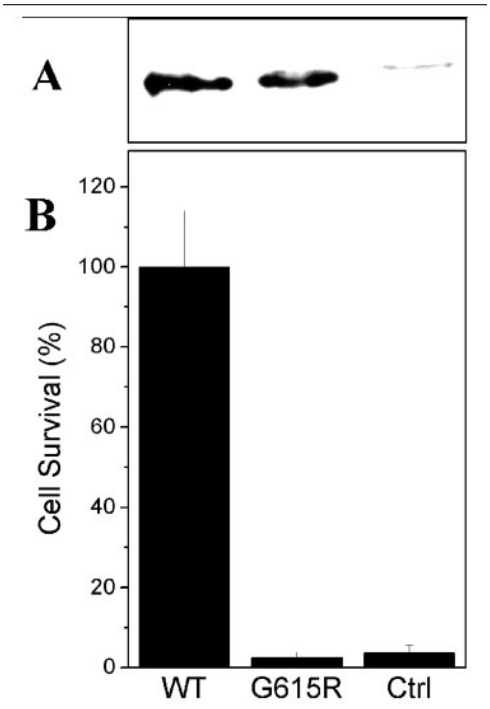

Fig 3. Ouabain survival assay. (A) Western blot analysis of transfected HeLa cells. Detection was performed using a polyclonal anti–Na,K-ATPase α2-subunit antibody. (B) Ouabain sensitivity of cells transfected with either wild-type or mutant ATP1A2 *cDNA constructs. Graphic representation of cell survival after 5 days of ouabain treatment (n = 8). (lane 1) WT: Na,K-ATPase α2-WT construct; (lane 2) G615R: Na,K-ATPase α2-G615R construct; (lane 3) Ctrl: mock-transfected.*

fected mutant Na,K-ATPase is able to compensate for the loss of endogenous Na,K-ATPase activity. No compensation will lead to cell death, revealing clear functionality of the mutation. In the assay, we expressed special Na,K-ATPase α2-subunits (either wild-type or mutant G615R) that are not sensitive to ouabain. Importantly, Western blot analysis showed that our constructs were expressed at comparable, sufficient levels (Fig 3A). In the survival assay (see Fig 3B), only cells expressing the wild-type construct were able to survive ouabain treatment. Cells expressing mutant construct G615R (and untransfected cells) did not survive, clearly indicating that mutant G615R is unable to compensate for loss of endogenous Na,K-ATPase activity.

Discussion

We describe a Dutch FHM2 family with a novel G615R missense mutation in the Na,K-ATPase *ATP1A2* gene associated with loss of function of the Na^+, K^+ pump. The G615R mutation replaces glycine[615] for arginine in the highly conserved segment "TGD" that surrounds the phosphorylation loop "DKTGTLT." This is one of the most critical residues involved in ATP binding.[20,21] The introduction of a bulky positively charged arginine residue likely causes space and electrostatic problems. Therefore, the reduced survival of mutant G615R cells is most likely caused by a lack of ATP binding causing complete inactivation of the pump.

In the proband, this mutation is associated with a drastic clinical phenotype consisting of both episodic and permanent severe neurological features, starting at a young age. The episodic symptoms were precipitated by mild head trauma and included hemiplegia, epileptic seizures, and cortical blindness. Transient blindness after mild head trauma has been reported, sometimes in combination with headache, confusion, irritability, anxiety, nausea, and vomiting.[22–24] Elicited repetitive daily blindness (ERDB) is a recently recognized autosomal dominant condition associated with childhood epilepsy and familial hemiplegic migraine.[25] Genetic linkage to the *CACNA1A* FHM1 gene was excluded. The *ATP1A2* FHM2 gene was not scanned but seems a good candidate gene for this syndrome. We previously described delayed cerebral edema and fatal coma after minor head trauma in carriers of the S218L mutation in the FHM1 *CACNA1A* gene.[26] Here, we describe an ATP1A2 mutation that also seems to cause delayed swelling after minor head trauma. FLAIR MRI imaging 2 days after onset of the patients' third episode was unremarkable, but repeated images 2 weeks later showed discrete swelling and an hyperintense signal of the right hemispheral cortex. The most striking feature in this girl, however, is the incomplete recovery after her second episode resulting in mental retardation. Mental retardation has been reported previously in four cases of FHM1 associated with the Y1385C or T666M *CACNA1A* mutations[27,28] and in six cases of FHM2 associated with the D718N, P979L, or L746P *ATP1A2* mutations.[6,10,29]

Mutations in FHM genes cause a high variability in clinical symptoms, even within families.[2] In our family, the same mutation causes a clinical spectrum ranging from clinically unaffected (incomplete penetrance), confusional, and hemiplegic migraine to mental retardation. Our findings indicate that FHM should be considered as a cause for mental retardation in children, even in cases with a personal and family history that is atypical for FHM. Mild head trauma-

triggered transient neurological symptoms should alert the physician to consider this diagnosis.

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3.3

Two *de novo* mutations in the Na,K-ATPase gene *ATP1A2* associated with pure Familial Hemiplegic Migraine

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ARTICLE

Two *de novo* mutations in the Na,K-ATPase gene *ATP1A2* associated with pure familial hemiplegic migraine

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Familial hemiplegic migraine (FHM) is a rare autosomal dominantly inherited subtype of migraine, in which hemiparesis occurs during the aura. The majority of the families carry mutations in the *CACNA1A* gene on chromosome 19p13 (FHM1). About 20% of FHM families is linked to chromosome 1q23 (FHM2), and has mutations in the *ATP1A2* gene, encoding the α2-subunit of the Na,K-ATPase. Mutation analysis in a Dutch and a Turkish family with pure FHM revealed two novel *de novo* missense mutations, R593W and V628M, respectively. Cellular survival assays support the hypothesis that both mutations are disease-causative. The identification of the first *de novo* mutations underscores beyond any doubt the involvement of the *ATP1A2* gene in FHM2.
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Keywords: familial hemiplegic migraine (FHM); *ATP1A2*; Na, K-ATPase

Introduction

Familial hemiplegic migraine (FHM) is a rare autosomal dominant subtype of migraine characterized by hemiplegia as part of the aura.[1,2] The majority of FHM families is linked to chromosome 19p13 (FHM1) and carry a mutation in the *CACNA1A* gene encoding the $Ca_v2.1$ subunit of neuronal voltage-gated P/Q-type calcium channels.[3,4] A smaller proportion of FHM families is linked to chromosome 1q23 (FHM2)[5–9] with mutations in the *ATP1A2* gene, encoding the Na,K-ATPase α2-subunit.[10–15] Recently, a third FHM gene, *SCN1A*, located on chromosome 2q24, encoding a neuronal voltage-gated sodium channel, was identified.[16] *ATP1A2* mutations have been associated with FHM, benign familial infantile convulsions (BFIC),[11] alternating hemiplegia of childhood (AHC)[17,18] and additional features like seizures, prolonged coma and cerebellar signs.[10–15]

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Functional studies of mutant Na,K-ATPases revealed a broad spectrum of abnormalities, including a complete loss of Na,K-pump function effect described for mutations T378N,[18] L764P and W887R.[10,19,20] Mutations R689Q and M731T resulted in a decreased catalytic turnover[21] whereas the T345A mutation showed a reduced affinity for potassium.[22]

Here we describe the first *de novo ATP1A2* mutations segregating with pure FHM. Moreover, in cellular survival assays where the endogenous Na,K-pump was blocked by ouabain, we could demonstrate that these mutations are disease-causative, since both mutant Na,K-ATPases are associated with reduced cellular survival.

Material and methods

Clinical data

Family 1 The proband of this Turkish family (III-6) (Figure 1) is a 47-year-old woman who has had, since the age of 13, attacks of headache and vomiting accompanied by homonymous hemianopsia and paresthesia of the ipsilateral upper and lower limbs, progressing to hemiplegia lasting up to 30 min. The attacks occurred twice a month but the last 8 years the frequency dropped to 4 attacks a year. Two of her children (IV-7 and IV-9) were also diagnosed with hemiplegic migraine. Her other two children (IV-6 and IV-8) were not diagnosed with migraine.

The elder sister (III-4) of the proband has hemiplegic migraine attacks, as well as all her five children. For the monozygotic twin brothers, a clinical description of attack characteristics is given in detail. Twin brother IV-1 (26 years of age) has 4–6 migraine with aura attacks per year. In addition, from age 18 until 20, he has had two migraine attacks with hemiplegia, but none since then. His twin brother (IV-2) has had 2–6 migraine attacks with hemiplegia every year from age 13 until 20, but none since then. Attacks in both brothers lasted 24–36 h and were associated with dysphasia, hemianopsia, motor weakness and ipsilateral sensory disturbances. The parents of the proband (II-1 and II-2), the grandparents (I-1 and I-2), the maternal aunt (II-4) and her offspring (III-8, IV-10, IV-11, IV-12), as well as her two brothers (III-1 and III-2) were not diagnosed with migraine. Of importance, all clinical diagnoses were made before genetic testing.

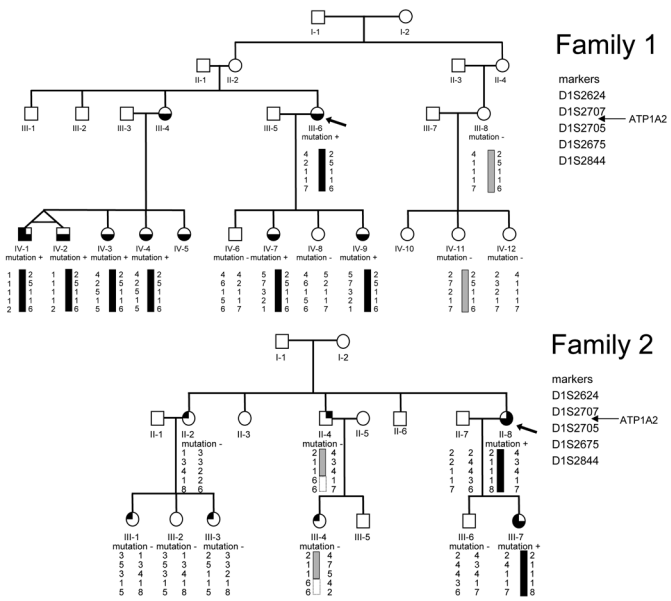

Figure 1 Pedigrees of *families 1* and *2*. The arrows indicate the probands. The following symbols are used to indicate the diagnosis: FHM: black lower half; MA: right upper square; MO: left upper square. Mutation + and Mutation− indicate carriers and noncarriers of the pathogenic mutations, respectively. Black bars show the haplotypes segregating with FHM; gray bars indicate the identical haplotype without the mutation; a dotted bar indicates a recombination. Genetic markers and the position of the *ATP1A2* gene are indicated on the right.

Family 2 The proband (II-8) of this Dutch family (Figure 1) is a 48-year-old woman who has monthly attacks of hemiplegic migraine from the age of 4. These attacks are accompanied by visual aura, with contralateral paresthesias around the mouth and paresthesias and motor weakness in one arm and leg for half an hour. She once had an atypical attack, for which she was admitted to a hospital. Prophylactic treatment using valproic acid was associated with an attack-free period of 2 years. Her daughter III-7 (23 years of age) has attacks of migraine without aura approximately once a week. In addition, during the last 6 years, she has had five attacks of hemiplegic migraine, starting with decreased hearing, followed by one-sided motor weakness and visual symptoms. She has had two episodes of unilateral motor weakness and temporary unconsciousness after a fall, not followed by headache. A sister of the proband (II-2) has migraine without aura, a brother (II-4) has migraine with visual aura, and three cousins have migraine without aura (III-1, III-3 and III-4). The parents of the proband (I-1 and I-2) never reported any headache attacks.

Genetic analysis

Blood samples were collected from 10 members of *family 1* and *family 2* each. Genomic DNA was isolated from peripheral blood using a standard salting out extraction method.[23] Microsatellite markers (D19S221, D19S1150, D19S226 and D1S2624, D1S2707, D1S2705, D1S2675, D1S2844) were selected to test the involvement of the FHM1 and FHM2 loci, respectively. Oligonucleotide primer sequences were obtained from the Human Genome Database (GDB) (http://www.gdb.org/). After amplification, PCR products were detected on automated sequencer (ABI 3700 DNA sequencer, Applied Biosystems, Foster City, CA, USA). All genotypes were analyzed and independently scored by KRJV and SKhK using Genescan and Genotyper 2.1 software (Applied Biosystems, Foster City, CA, USA). Haplotypes were constructed by inspection of segregation and assuming a minimal number of recombinations.

Proof of monozygosity of the twin brothers in *family 1* was established by monozygotic-probability calculation (MZ Probability > 99.99%) using 17 autosomal markers (results not shown).[24]

Mutation analysis of the *ATP1A2* gene was performed by direct sequencing of all exons and flanking intronic regions using genomic DNA of probands of both families as described in Vanmolkot *et al.*[11] Genomic DNA of 100 population-matched subjects was used as a control group to test each mutation. For detection of the V628M mutation (G > A, nt position 1987; Ac no. NM_000702) amplification refractory mutation system PCR (ARMS-PCR) was performed.[25] In brief, the wild-type allele was detected using forward primer 'Universal' (5′-GGGCTGAGGAAC CAGTCACAA-3′) and reverse primer 'G' (5′-AGGCCATTG CCAAAGGCG-3′), whereas the mutant allele was detected with reverse primer 'A' that differed at the ultimate 3′-base position (5′-AGGCCATTGCCAAAGGCA-3′). Both PCRs gave a product of 172 bps. The GG (wild type) genotype showed a PCR product only in case reverse primer 'G' was used; the AA (mutant) genotype gave only a PCR product with reverse primer 'A'. In case of a heterozygote (eg GA genotype') PCRs with either reverse primer gave a product. As an internal control for PCR, in every reaction, forward primer 'Control forward' (5′-TGTCATCTTGGATGGCA CTG-3′) and reverse primer 'Control reverse' (5′-TGCGTT GATCTGCATCTTCT-3′) were included resulting in a 500-bp PCR product.

For detection of the R593W mutation (C > T, nt position 1881, Ac no. NM_000702), exon 13 was amplified by PCR using primers 'exon13F' (5′-GGGATTCCCAAGCCTCTG-3′) and 'exon13R' (5′-TCTCTGAGTCAGTGGGAAGGA-3′), resulting in a 398-bp product. Subsequently, PCR products were digested with restriction enzyme *Sma*I using standard protocols, and electrophoresed on a 3% agarose gel. The R593W mutation causes a loss of a *Sma*I site, which results in an uncut band of 398 bps for the mutant allele, besides the wild-type bands of 172 and 226 bps.

Functional analysis

cDNA constructs Human Na,K-ATPase α2-subunit cDNA was subcloned into a modified pCDNA3.1 vector (originally from Invitrogen, Carlsbad, CA, USA), which additionally contained the 5′- and 3′-untranslated regions of the *Xenopus* β-globin gene flanking the multiple cloning site.[19] To distinguish endogenous Na,K-ATPase activity from that of transfected Na,K-ATPase, mutations Q116R and N127D were introduced in the original α2-subunit cDNA to express an ouabain-resistant isoform (α2-WT).[26] Next, mutations R953W and V628M were introduced into the ouabain-resistant wild-type α2-subunit construct by site-directed mutagenesis (Quikchange, Stratagene, La Jolla, CA, USA) to obtain mutants α2-R593W and α2-V628M, respectively. All constructs were sequence-verified.

Transfection and ouabain treatment HeLa cells (5×10^5) were transfected with 1.6 μg plasmid DNA of either α2-WT, α2-R593W or α2-V628M, using Lipofectamine 2000 Transfection Reagent in Opti-Mem medium (Invitrogen, Carlsbad, CA, USA) and cultured in DMEM-containing Glutamax and 10% FCS (Invitrogen, Carlsbad, CA, USA). At 2 days after transfection, one-third of the cells (1.7×10^5) were seeded on 10 cm Petridishes and subsequently 1 μM ouabain was added to the culture medium. After 5 days of ouabain challenge, colonies were stained with 1% methylene blue in 70% methanol, scanned and analyzed with Image Pro Plus (MediaCybernetics, Silverspring, MD, USA). Each transfection was performed nine times.

Electrophoresis and Western blot analysis At 2 days after transfection, two-third of the cells (3.3×10^5) were harvested for Western blot analysis.[19] In brief, proteins were resuspended in a mix of protease-inhibitor (Complete Mini, Roche, Basel, CH, USA) and DNaseI. Subsequently, Laemmli-loading buffer and 0.1 M DTT were added and the samples were heated for 10 min at 65°C. Next, equal amounts of protein as measured by Bio-Rad protein assay (Bio-Rad Laboratories, Munchen, Germany) were separated on 7.5% SDS-polyacrylamide gels for 40 min at 200 V. Proteins were electroblotted onto nitrocellulose membranes (Hybond, Amersham, Buckinghamshire, UK) and incubated overnight with the α2-subunit-specific polyclonal antibody HERED.[27] The primary antibody was detected with goat-anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma, St Louis, MO, USA). Protein bands were visualized with Super Signal Substrate (Pierce Biotechnology, Rockford, IL, USA).

Results

Our two pure FHM families were compatible with involvement of the 1q23 FHM2 locus, because single haplotypes consistently co-segregated in all tested patients with FHM (Figure 1). No indications for involvement of the 19p13 FHM1 locus were found either by haplotype analysis (in case of *family 1*) or sequencing of the *CACNA1A* gene (in case of *family 2*). However, sequence analysis of the *ATP1A2* gene identified two missense mutations, V628M and R593W, in *families 1* and *2*, respectively. The V628M mutation in exon 14 (nt 1987 G>A) causes a Valine to Methionine substitution, whereas the R593W mutation in exon 13 (nt 1881, C>T) causes an Arginine to Tryptophan substitution (Figure 2). Both mutations were not observed in a panel of 100 population-matched control individuals. Taxonomy analysis indicates a strong conservation of amino acids Arg[593] and Val[628] among several alpha subunits of the P_2-type ATPase subfamily (Figure 3). Haplotype analysis showed that both mutations occurred *de novo*. Haplotypes identical by descent, but without the mutations, were not associated with FHM (Figure 1). Interestingly, in *family 1*, we identified monozygotic twin brothers that carry the V628M mutation and have similar hemiplegic migraine attack characteristics and age of onset, although the attack frequency seems to vary between them.

To evaluate the functional consequences of both mutations, survival assays were performed. In these assays, the endogenous Na,K-ATPase activity was completely inhibited by ouabain challenge in HeLa cells. In such an assay, we assessed whether transfected Na,K-ATPase is able to compensate for this loss. For transfections we used either

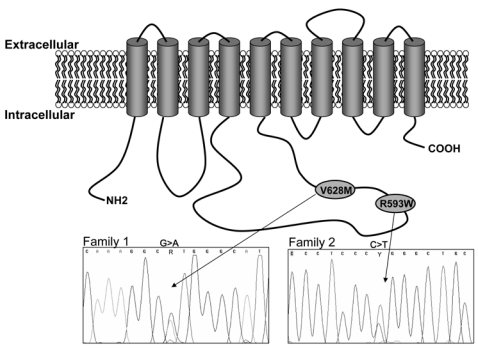

Figure 2 Transmembrane topology model of the Na,K-ATPase α2-subunit and location of novel mutations. The location of the amino-acid substitutions V628M and R593W are shown. Arrows point to electropherograms with the respective heterozygous mutations.

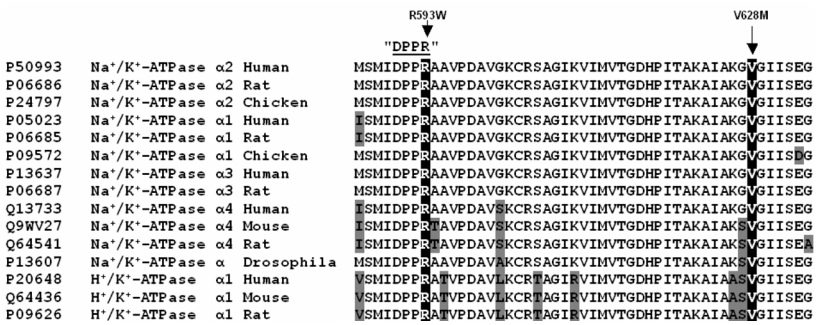

Figure 3 Amino-acid sequence alignments. Conservation of the Arg[593] and Val[628] residues (in black) in several subunits of various Na,K- ATPase, H,K-ATPase and SERCA ATPases. The 'DPPR' motif is indicated. Accession numbers are indicated on the left. Variations from the human *ATP1A2* sequence are given in gray.

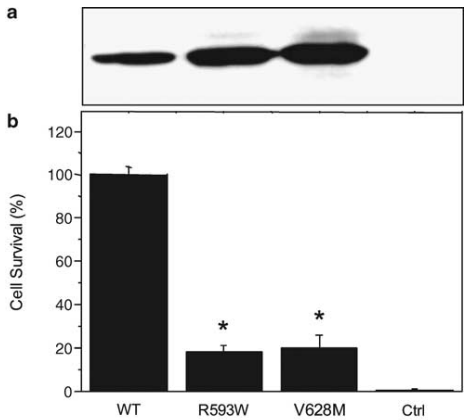

Figure 4 Ouabain survival assay. (**a**) Western blot analysis of transfected HeLa cells. Detection was carried out using a polyclonal antibody specifically directed against the Na,K-ATPase α2-subunit (anti-HERED). (**b**) Ouabain sensitivity of cells transfected with either wild type or mutant *ATP1A2* cDNA constructs. Graphic representation of cell survival after 5 days of ouabain treatment ($n = 9$). Lane 1: WT: α2-WT construct; lane 2: α2-R593W; lane 3: α2-V628M; lane 4: Control: mock-transfected cells.

wild-type or mutant (α2-R593W or α2-V628M) Na,K-ATPase α2-subunits that were made insensitive to ouabain by mutagenesis.[26] Compromised rescue will lead to cell death, revealing clear functionality of the mutation. Importantly, Western blot analysis showed that our constructs were expressed at comparable, sufficient, levels (Figure 4a). In the survival assay (Figure 4b), cells expressing the wild-type construct were able to survive ouabain treatment. Cells expressing mutant constructs α2-R593W or α2-V628M showed a significantly reduced rate of survival, clearly indicating that both mutants are unable to compensate sufficiently for loss of endogenous Na,K-ATPase activity.

Discussion

We have identified two novel missense mutations, R593W and V628M, in the FHM2 *ATP1A2* gene in two families with pure FHM. Both mutations co-segregate consistently with the FHM phenotype. Haplotype analysis of each family revealed that both mutations had occurred *de novo*, as identical haplotypes without the mutation were present in several unaffected family members. *Family 1* is the first Turkish family reported with FHM. These are the first *de novo* mutations reported for *ATP1A2*, underscoring that mutations in the *ATP1A2* gene are indeed causing FHM.

Transfection studies with the *ATP1A2* cDNA constructs showed sufficient residual function of both mutants R593W and V628M to allow cell growth, although at a significantly reduced rate compared to the ouabain-resistant wild-type isoform. In this respect, they differ from the previously published T378N, L764P, W887R mutations that all produce pumps completely incapable of a functional rescue.[10,18–20] On the other hand, FHM2 mutants R689Q and M731T located in the same cytoplasmatic loop as R593W and V628M also allow partial or complete cell growth in survival assays, but show decreased catalytic turnover in additional functional studies.[21]

Both mutated amino acids are well conserved within the P_2-type subfamily. The Na,K-ATPase α2-subunit has a structure similar to Ca^{2+}-ATPases, which comprises 10 transmembrane helices and three cytoplasmic domains: the A-domain (actuator), the P-domain, which contains the residue of phosphorylation, and the N-domain that binds ATP.[28] The Na,K-ATPase Val^{628} is the equivalent amino acid of Ca^{2+}-ATPase Ile^{639} that resides in the P-domain. Ile^{639} is located at the border of one of the short conserved helices that together with the seven-stranded parallel β-sheet form a typical Rossman fold.[28] This residue is rather well conserved in all P-type ATPases.[29] We showed that the substitution of Valine with a Methionine decreases the cell survival. The Na,K-ATPase Arg^{593} that is equivalent to Ca^{2+}-ATPase Arg^{604} is located at the border of the P-domain, directly after the two prolines that form the hinge between the P- and N-domains. Large domain motions require the presence of a flexible hinge region, with an invariant DPPR motif. It has been reported that the hydrogen-bond network involving the conserved Gly^{354}, Arg^{604}, and Asp^{737} seems to link the movement of this hinge region to that of M5, thereby effectively transmitting the phosphorylation signal to the Ca^{2+} binding sites.[28] Abnormal Na,K-ATPase functioning observed with our R593W mutant is in line with abnormal functioning in Ca^{2+}-ATPase mutant R604M that revealed a 35% reduced Ca^{2+} transport.[30]

Until now, 12 variations in the intracellular domain of the Na,K-ATPase α2-subunit have been reported in FHM patients.[10–15] ATP1A2 mutations have been shown to result in a broad spectrum of functional abnormalities. Two mutations associated with pure FHM (eg L764P and W887R), and mutation T378N that causes AHC are incapable of a functional rescue in survival assays, implicating complete loss-of-function.[10,18–20] The T345A mutation does not lead to reduced rescue in survival assays, but additional experiments showed a functionally altered pump with reduced affinity for K^+.[22] Mutants R689Q and M731T, also allow partial or complete cell growth in survival assays, but show decreased catalytic turnover.[21] Therefore, the partial survival of our novel mutations (R593W and V628M) supports the hypothesis that they are disease-causative.

Both the R593W and the V628M mutation occurred *de novo*, underscoring that mutations in the *ATP1A2* gene are indeed causing FHM in these families. In addition, the

occurrence of *de novo* mutations indicates that such mutations may also be found in sporadic hemiplegic migraine (SHM), that is, in hemiplegic migraine patients with no other family members suffering from this disease. The genetic etiology of SHM is not well established and only a few causative mutations in FHM1 and FHM2 genes have been identified in SHM patients.[14,31] Therefore, our findings suggest that the *ATP1A2* gene is a good candidate for genetic testing in SHM cases.

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3.4

First case of compound heterozygosity in Na,K-ATPase gene *ATP1A2* in Familial Hemiplegic Migraine

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ARTICLE

First case of compound heterozygosity in Na,K-ATPase gene *ATP1A2* in familial hemiplegic migraine

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Familial hemiplegic migraine (FHM) is a rare autosomal-dominant subtype of migraine with aura, associated with hemiparesis during the aura. Here we describe a unique FHM family in which two novel allelic missense mutations in the Na,K-ATPase gene *ATP1A2* segregate in the proband with hemiplegic migraine. Both mutations show reduced penetrance in family members of the proband. Cellular survival assays revealed Na,K-ATPase dysfunction for both *ATP1A2* mutants, indicating that both mutations are disease causative. This is the first case of compound heterozygosity for any of the known FHM genes.
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Keywords: familial hemiplegic migraine (FHM); *ATP1A2*; Na,K-ATPase

Introduction

Familial hemiplegic migraine (FHM), a rare and severe form of migraine with aura, has an autosomal-dominant inheritance pattern and is characterized by transient motor weakness in addition to a wide range of other neurological aura symptoms.[1] Mutations for FHM have been identified in three genes; the *CACNA1A* calcium channel FHM1 gene, the *ATP1A2* Na,K-ATPase FHM2 gene and the *SCN1A* sodium channel FHM3 gene.[2–5] Here, we report compound heterozygosity for two novel allelic missense *ATP1A2* mutations in the proband of a FHM family. Functional consequences were shown for both mutations in cellular survival assays. This is the first case of compound heterozygosity for any of the FHM genes.

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Patients and methods

Subjects

This family is of British Caucasian origin. The proband and available family members were diagnosed according to the International Classification of Headache disorders, second edition.[1] All family members were seen by a neurologist, who interviewed them directly and where relevant, examined the patients.

The proband (III-2, Figure 1) (aged 29 years) has, since the age of 8 years, typical hemiplegic migraine with an attack every 4–6 weeks that can last between 2 h and 9 days with an average duration of 2 days. Attacks are sometimes triggered by mild head injury. A typical attack starts with a visual aura of zigzag lines, which on occasion can progress to complete hemianopia. Subsequently, she develops

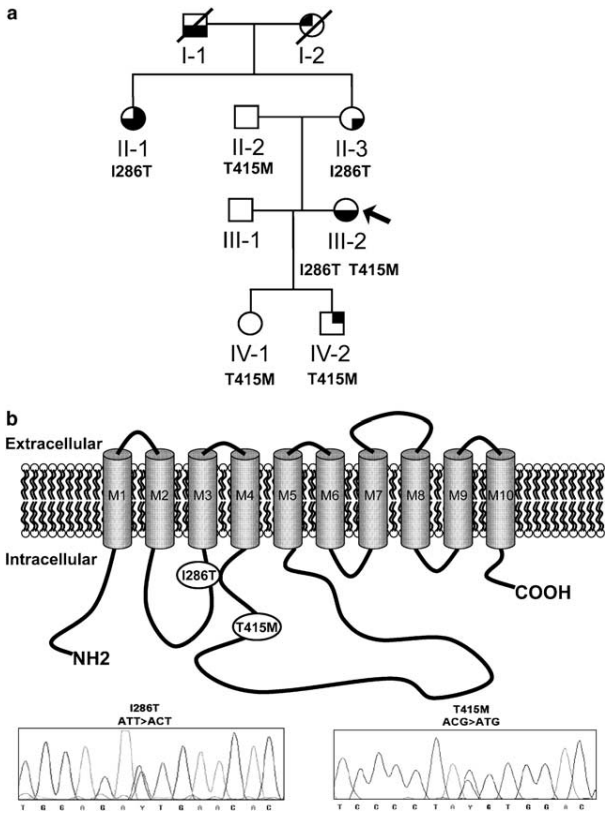

Figure 1 (**a**) Pedigree of the family. Symbols represent the following; lower black half: hemiplegic migraine; lower right quadrant: aura without headache; right upper quadrant: migraine with aura; left upper quadrant: migraine without aura; square: male; circle: female. The arrow indicates the proband. I286T and T415M indicate the presence of the *ATP1A2* allele harbouring the respective mutation. (**b**) Schematic representation of the ATP1A2 protein. The location of mutations I286T and T415M is shown. Boxes with electropherograms show the respective heterozygous mutations.

dysphasia and within 10 min complete right- or left-sided hemiplegia, followed by severe unilateral throbbing headache, associated with vomiting and photophobia. The nature of the headache can vary form a sharp, stabbing pain to an explosive thunderclap headache. Severe attacks are accompanied by disorientation, drowsiness and confusion. At the age of 20 (at 12 weeks pregnancy), she had a particularly severe attack associated with drowsiness, dysphasia and right hemiparesis lasting 9 days. During this attack, at the peak of her symptoms, MRI brain imaging including diffusion-weighted imaging showed no abnormalities, but perfusion imaging showed hyperaemia of the left hemisphere (data not shown). EEG showed high voltage slow activity over the left hemisphere (data not shown).

The maternal aunt (II-1) of the proband also suffers from hemiplegic migraine. Her attacks started at the age of 13 and are characterized by mild weakness in hand and arm with an average duration of 30 min to 5 h. The headache is severe, unilateral, throbbing and aggravates by physical activity. The attack frequency ranges from once a week to twice a year. Her attacks are not provoked by head trauma. The proband's maternal grandfather (I-1) heteroanamnestically had severe migraine with possible hemiplegic

attacks and her maternal grandmother (I-2) suffered from migraine without aura. Other family members suffer from migraine with visual and sensory aura (IV-2, aged 8 years), a few isolated visual auras without headache (II-3), or do not suffer from migraine until now (IV-1, aged 6 years and II-2).

Mutation detection

Direct sequencing was performed for all 23 exons of the *ATP1A2* gene in the proband. For detection of the first heterozygous substitution (c.961T>C; I286T, Ac no. NM_000702), exon 8 was amplified by PCR using specific primers. Subsequently, PCR products were digested with restriction enzyme *Alw*261 using standard protocols and electrophoresed on a 3% agarose gel. Detection of the second heterozygous substitution (c.1348C>T; T415M, Ac nr NM_000702) was performed by amplification of exon 10, and subsequently, digestion of PCR products with restriction enzyme *Tai*I. Family members and 180 control individuals were tested for the presence of both mutations.

Functional analysis

Human Na,K-ATPase $\alpha 2$ subunit cDNA was subcloned into a modified pCDNA3.1 vector.[6] To distinguish endogenous Na,K-ATPase activity from that of transfected Na,K-ATPase, a cDNA encoding ouabain-resistant wild-type ($\alpha 2$-WT)[7] was used to introduce the I286T and T415M mutations with site-directed mutagenesis (Quikchange, Stratagene, La Jolla, CA, USA). For the assay, HeLa cells (5×10^5) were transfected with 1.6 μg plasmid DNA of either $\alpha 2$-WT, $\alpha 2$-I286T or $\alpha 2$-T415M, using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). Two days after transfection, two-third of the cells were harvested for immunoblotting and $\alpha 2$ subunit was detected using specific polyclonal antibody HERED.[8] The remaining one-third of the cells were seeded on 10 cm Petri dishes, and subsequently, 1 μM ouabain was added to the culture medium. After 5 days of ouabain challenge, colonies were stained with 1% methylene blue in 70% methanol, scanned and analyzed with Image Pro Plus (Media Cybernetics, Silverspring, MD, USA). Each transfection was performed 15 times and the average values were calculated for each construct, respectively. The average number of colonies obtained with the WT construct was used as a reference (100% cell survival). Next, relative percentages of cell survival were calculated for both mutant constructs.

Results

In the proband with hemiplegic migraine, two heterozygous sequence variants I286T (exon 8; c.961T>C) and T415M (exon 10; c.1348C>T) were identified in the *ATP1A2* gene (Figure 1). The sequence variants are located on different alleles because I286T was inherited from the mother (II-3) and T415M from the father (II-2). Both variants were absent in 180 Dutch control individuals. Sequence alignments indicate a strong evolutionary conservation of both amino acids Ile^{286} and Thr^{415} among several α subunits of the P-type ATPase family (Figure 3).

Functional consequences of both variations were investigated in cellular survival assays in HeLa cells. In these assays, the endogenous Na,K-ATPase activity is completely inhibited by ouabain challenge. Transfected WT Na,K-ATPase $\alpha 2$ cDNA, that was made insensitive to ouabain by mutagenesis, is able to rescue cell survival (Figure 2b). We assessed whether mutant ouabain-insensitive Na,K-ATPase $\alpha 2$ cDNA ($\alpha 2$-I286T or $\alpha 2$-T415M) can do the same. Compromised rescue will lead to cell death, thus demonstrating that the mutations are functional-defective and can be considered pathogenic. Mutant T415M did not show cell survival at all. Cells expressing mutant I286T resulted in significantly reduced survival (Figure 2b). Western blot analysis showed that the constructs were expressed at levels comparable to the WT, indicating that

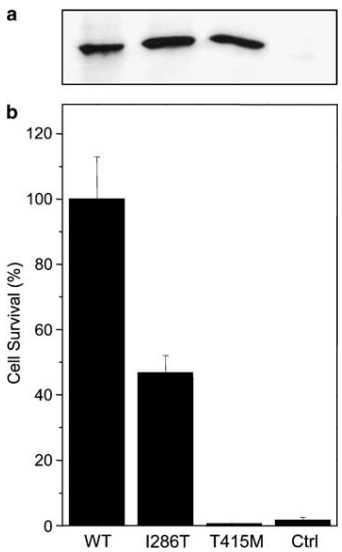

Figure 2 Ouabain survival assay. (**a**) Western blot analysis of transfected HeLa cells. Detection was performed using a polyclonal anti-Na,K-ATPase $\alpha 2$ subunit antibody. (**b**) Ouabain sensitivity of cells transfected with either wild-type (WT) or mutant *ATP1A2* cDNA constructs. Graphic representation of cell survival after 5 days of ouabain treatment ($n=15$). Survival obtained with the WT construct was set to 100% and compared to the relative survival for both mutants constructs. Lane 1: WT: Na,K-ATPase $\alpha 2$-WT construct; Lane 2: I286T: Na,K-ATPase $\alpha 2$-I286T construct; Lane 3: T415M: Na,K-ATPase $\alpha 2$-T415M construct; Lane 4: Control: mock-transfected. Error bars indicate SEM.

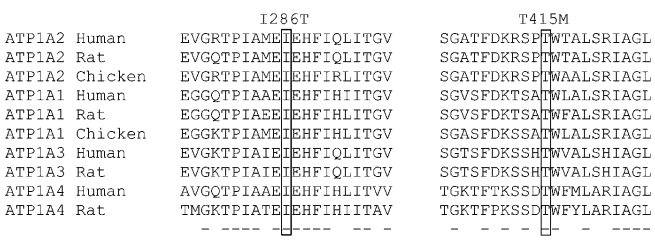

Figure 3 Evolutionary conservation of the mutated amino acids Ile^{286} and Thr^{415}. Alignment of amino-acid sequence from several vertebrate Na,K-ATPase α subunits, showing evolutionary conservation of the amino acids Ile^{286} and Thr^{415} (in boxes). Dashed lines indicate fully conserved amino acids. Protein sequences were obtained from Genbank. Human: P50993 (ATP1A2), P05023 (ATP1A1), P13637 (ATP1A3), Q13733 (ATP1A4); rat: P06686 (ATP1A2), P06685 (ATP1A1), P06687 (ATP1A3), Q64541 (ATP1A4); chicken: P24797 (ATP1A2), P09572 (ATP1A1).

compromised rescue was not due to a reduction in protein expression level (Figure 2a). These results clearly indicate that I286T and T415M mutant Na,K-ATPase pumps are unable to (fully) compensate for the loss of endogenous Na,K-ATPase activity, thereby indicating that both variations are disease causative.

Discussion

Here we describe a case of two allelic, novel, *ATP1A2* missense mutations in a patient with hemiplegic migraine. In fact, this is the first report of a hemiplegic migraine patient with compound heterozygosity, that is the presence of two different mutant alleles at a hemiplegic migraine gene locus. Several lines of evidence suggest that these two mutations are disease causative. First, both DNA variations were not present in 180 control individuals. Second, both affect amino acids that are highly conserved among α subunits of Na,K-ATPases from different species. Third, cell survival assays revealed that both mutations result in dysfunctional sodium–potassium pumps. The I286T mutation is located in the intracellular loop between M2 and M3 and showed a partial rescue of cell survival. Mutation T415M, resulting in complete *loss-of-function* on cell survival, is located in the large intracellular loop between M4 and M5, in which the majority of FHM2 mutations are found. This loop is critical for the function of the Na,K-ATPase pump since it harbours the phosphorylation and ATP-binding domains and undergoes major conformational changes during the enzymatic cycle.[9]

Interestingly, both mutations show reduced penetrance in family members of the proband. The proband's father (II-2), who has the T415M mutation (associated with complete loss of cellular rescue), only had non-migrainous headaches. His grandson (IV-2) who also carries the T415M mutation suffers from migraine with aura. However, he is still young (8 years old) and can still develop hemiplegic migraine. Although aunt II-1 who carries the I286T mutation (associated with partial cellular rescue) was diagnosed with hemiplegic migraine and migraine with aura, the proband's mother (II-3) also carrying the I286T mutation only had a few isolated visual auras without headache. Clinical variation and reduced penetrance in FHM2 families has been reported before.[10,11] In this respect, it is interesting to note that Todt *et al*[12] identified two *ATP1A2* variations that are possibly involved in the susceptibility to common forms of migraine.

Importantly, the patient described here shows that compound heterozygosity for *ATP1A2* is compatible with life. Apparently, *ATP1A2* mutations of various severities can cause FHM, but probably other mechanisms are still able to compensate partially for the loss of functional sodium–potassium pumps. In knock-out *Atp1a2* mice, however, homozygosity of two totally non-functional alleles is incompatible with life as pups die immediately after birth because they are unable to start breathing due to altered neuronal activity in respiratory neurons.[13]

The presence of two *ATP1A2* mutations in the proband causes a more severe phenotype compared to the milder FHM phenotype of her aunt, who carries only the I286T mutation. Hemiplegic migraine in the proband occurs at a lower age of onset, with a higher frequency and a longer duration of attacks that are always associated with hemiplegia. Although most likely due to the presence of two dysfunctional *ATP1A2* alleles, (part of) the difference in severity between these two individuals may reflect clinical variability that is not uncommon in FHM.[14] Similarly, other genetic modifiers and environmental factors play a role in the expression of disease.

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Chapter 4

The novel p.L1649Q mutation in the *SCN1A* epilepsy gene is associated with Familial Hemiplegic Migraine: genetic and functional studies

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MUTATION IN BRIEF

The Novel p.L1649Q Mutation in the *SCN1A* Epilepsy Gene Is Associated With Familial Hemiplegic Migraine: Genetic and Functional Studies

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Familial hemiplegic migraine (FHM) is a severe subtype of migraine with hemiparesis during attacks. We scanned 10 families with FHM without mutations in the *CACNA1A* (FHM1) and *ATP1A2* (FHM2) genes. We identified the novel p.L1649Q mutation (c.4946T>A) in $Na_v1.1$ sodium channel gene *SCN1A* (FHM3) in a North American kindred with FHM without associated ataxia or epilepsy. Functional analysis of the mutation, introduced in the highly homologous human SCN5A, revealed markedly slowed inactivation and a two-fold faster recovery from fast inactivation predicting enhanced neuronal excitation. Our findings establish the role of neuronal $Na_v1.1$ sodium channels in FHM and reinforce the involvement of ion channel dysfunction in the pathogenesis of this episodic brain disorder. © 2007 Wiley-Liss, Inc.

KEY WORDS: familial hemiplegic migraine; FHM; migraine; *SCN1A*; epilepsy

INTRODUCTION

Familial hemiplegic migraine (FHM) is a rare monogenic form of migraine with hemiparesis during aura. Mutations in three genes for FHM have been identified, in the *CACNA1A* calcium channel gene (MIM# 601011) for FHM1 (MIM# 141500) (Ophoff et al., 1996), the *ATP1A2* Na_vK-ATPase gene (MIM# 182340) for FHM2 (MIM# 602481) (De Fusco et al., 2003) and, recently, the p.Q1489K mutation (c.4465C>A; p.Gln1489Lys) in the *SCN1A* sodium channel gene (MIM# 182389) for FHM3 (MIM# 609634) (Dichgans et al., 2005). All three gene products are intimately involved in the modulation of ion fluxes across neuronal and glial cell membranes, suggesting that FHM, and possibly also common types of migraine, are cerebral ionopathies (Ferrari and Goadsby,

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2006). The p.Q1489K *SCN1A* mutation is remarkable as it represents the first among more than 150 mutations in this gene that is not associated with either severe myoclonic epilepsy of infancy (SMEI, MIM# 607208) or generalized epilepsy with febrile seizures (GEFS+, MIM# 604233) (Meisler et al., 2005; Mulley et al., 2005). The mutation spectrum in SMEI differs from that in GEFS+ as the majority of SMEI mutations occurred de novo. Approximately half of the SMEI mutations are nonsense or frameshift mutations resulting in protein truncation and consequent loss-of-function. Almost 40% of SMEI mutations are missense mutations, with functional consequences that range from complete loss-of-function, gain-of-function to minimal functional effects (Rhodes et al., 2004). The milder GEFS+ phenotype is associated with missense mutations only, showing either loss- or gain-of-function effects (George, 2005; Barela et al. 2006). Functional studies of the p.Q1498K mutation expressed and analyzed in the highly homologous human SCN5A revealed a more rapid recovery from fast inactivation (Dichgans et al., 2005). A limitation in that study was that the mutation was found in three families of common ancestry leaving the possibility of an isolated finding rather than a prominent FHM gene. In order to firmly establish the *SCN1A* gene as a gene for FHM3, independent confirmation in other families is necessary.

Here we performed mutation scanning in the *SCN1A* gene in 10 FHM families that were negative for mutations in the *CACNA1A* and *ATP1A2* genes. We identified the novel *SCN1A* p.L1649Q mutation (c.4946T>A) in a large kindred with pure FHM without epilepsy and show that this mutation severely interferes with voltage-gated sodium channel functioning.

SUBJECTS AND METHODS

Patients

We investigated 10 families with pure FHM (without associated epilepsy or ataxia) and without mutations in the *CACNA1A* (FHM1) and *ATP1A2* (FHM2) genes. Two to seven affected members were available per family. Diagnoses were made according to the IHS criteria (Headache classification subcommittee of the international headache society, 2004). All subjects gave written informed consent. Detailed information on the clinical characteristics of the *SCN1A* mutation carriers is shown in Table 1. Clinical diagnosis was made blinded for the genetic data.

Genetic analysis

Genomic DNA was isolated from peripheral blood using a standard salting out extraction method (Miller et al., 1988). All 26 exons of *SCN1A* were amplified by polymerase chain reaction (PCR), and primer details are available from the authors upon request. For several exons, primers were improved compared to our original paper (Dichgans et al., 2005), for instance the alternatively spliced exon 5N, reported by Tate et al. (2005) is now included in the scan. All PCR products were analyzed for mutations by direct sequencing. DNA numbering for *SCN1A* is based on cDNA reference sequence AB093548.1. Nucleotide numbering uses the A of the ATG translation initiation codon as nucleotide +1. Mutation nomenclature follows guidelines of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/).

Mutagenesis, Cell Culture, and Electrophysiology

As in the first study, we used the closely related *SCN5A* cDNA because of known difficulties in stability of recombinant bacteria with *SCN1A* cDNA (Dichgans et al., 2005). p.L1636Q, which corresponds to p.L1649Q in *SCN1A* was introduced by site-directed mutagenesis into full-length human *SCN5A* cDNA subcloned in pCDNA3.1 (QuikChange XL Kit, Stratagene, La Jolla, CA, USA). *SCN5A-L1636Q* and *SCN5A-WT* cDNA constructs were transfected into human tsA201 cells using the calcium phosphate method and were each co-expressed with accessory human sodium channel subunit β1 (ratio of cDNA 2:1) and CD8 cDNA. Before recording, DMEM medium was exchanged with bath solution and anti-CD8 coated microbeads (Dynabeads M-450 CD8, Oxoid, Basingstoke, UK) were added to the cell suspension. The bath solution contained (in mM): 110 Na-glutamate, 35 NaCl, 4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 mM HEPES (pH 7.3). Macroscopic sodium currents were recorded using the whole-cell configuration of the patch clamp technique, filtered at 10 kHz by a low-pass Bessel filter, and acquired by a patch clamp L/M-EPC7 amplifier (List Medical Electronics, Darmstadt, Germany) interfaced with a National Instruments interface (PCI-6052E) and a custom acquisition program (GePulse). Temperature was controlled (20 ± 0.5 °C) with a Peltier device. Patch pipettes were pulled from aluminium silicate glass (Hilgenberg Gmbh, Malsfed, Germany) and fire polished with a microforge. Electrode resistance was 1.5-2.0 MΩ with a pipette solution containing (in mM): 110 CsGlu, 30 NaCl, 2 $MgCl_2$, 5 Cs-EGTA, 10 mM HEPES (pH 7.3). Access resistance was between 2 and 5 MΩ, and the cell capacitance was between 5 and 15 pF, as measured

by the compensating circuit of the amplifier. Data from cells with a current amplitude of 0.5-1.5 nA were used for the analysis of the voltage-dependent parameters. Thus, the series resistance error was less than 4 mV. The holding-potential was –120 mV and steady state activation, steady-state inactivation, time constants of inactivation (e.g. time constants τ_{fast} and τ_{slow}), and recovery from inactivation were measured using protocols, as described before (Dichgans et al., 2005). Data analysis was performed using the program Ana (available at http://www.ge.cnr.it/ICB/conti_moran_pusch/programs-pusch/software-mik.htm), and Sigma Plot (SPSS Inc., Chicago, IL, USA).

RESULTS

CLINICAL GENETIC ANALYSIS OF FHM FAMILIES

Mutation scanning of the *SCN1A* gene in the probands of the 10 families revealed one mutation in a North American family of Caucasian descent (Fig. 1A). Clinical details of mutation carriers from this family are shown in Table 1. The proband (III-4), aged 51 years, has hemiplegic migraine attacks since the age of 10 with a frequency that varies from twice a month to once a year. The attacks always start with blurred vision with dark spots, followed within minutes by spreading hemiparasthesia and hemiparesis with dysarthria and dysphasia. After 20-30 minutes, this is followed by a hemicranial throbbing headache, which is always located on the side opposite to the hemiparesis and is accompanied by nausea, vomiting, photo- and phonopobia. Six additional family members suffer from typicial hemiplegic migraine attacks as well, with an age of onset varying from 11 to 24 years. We classified individual IV-3 also as affected, despite the fact that he only has had one attack of FHM so far. We feel that because of his young age (22) subsequent attacks are still likely to occur. Besides hemiplegic attacks, individual III-2 suffers from migraine with and without aura and individual IV-3 suffers from migraine without aura. No cerebellar signs or epilepsy symptoms were reported in this family. Mutation analysis in the proband revealed a heterozygous point mutation in exon 26 (c.4946T>A; p.L1649Q), resulting in an amino acid substitution of glutamine for leucine. The mutation co-segregated completely with the hemiplegic migraine phenotype in this family and was not found in a panel of 400 control chromosomes. Sequence alignments indicated high conservation of Leucine^{1649} among several vertebrate sodium channel α1 subunits (Fig. 1B). Mutation p.L1649Q is located in the S4/D4 domain that is implicated in voltage sensing of fast inactivation (Fig. 1C-D).

Table 1. Clinical Characteristics of *SCN1A* Mutation Carriers

ID	Age at onset (yrs)	Aura symptoms during hemiplegic attacks				Hemiplegic attacks		Headache characteristics during hemiplegic attacks			
		H	S	V	A	Duration Hemiplegia	Frequency	Duration	Side	Character	Nausea/vomiting/ photophobia/ phonophobia
II-1	12	+	+	+	+	1-6 h	2/week	24-48 h	Both sides	Throbbing	+/+/+/+
II-2	11	+	+	+	+	10-60 min	4-5/year	4-72 h	Unilateral	Nagging	+/+/+/-
III-2*	21	+	+	+	+	1-14 h	3-4/year	2 days	Both sides	Pulsating	+/+/+/+
IV-2	15	+	+	+	+	10-60 min	2/year	4-72 h	Both sides	Throbbing	+/+/+/+
III-4	10	+	+	+	+	>60 min	2/month-1/year	24 h	Unilateral	Throbbing	+/+/+/+
III-5	24	+	+	+	+	15 min	5/life	3 h	Both sides	Pounding	-/-/-/-
IV-3**	19	+	+	+	+	45 min	1 till present	1.5 h	Unilateral	Throbbing	+/+/+/+

H: hemiparesis or hemiplegia; S: sensory disturbance; V: visual disturbance; A: aphasia; +: symptom consistently present in all or most attacks; -: symptom never present; *Patient III-2 also suffers from migraine with and without aura attacks. **Patient IV-3 also suffers from migraine without aura attacks.

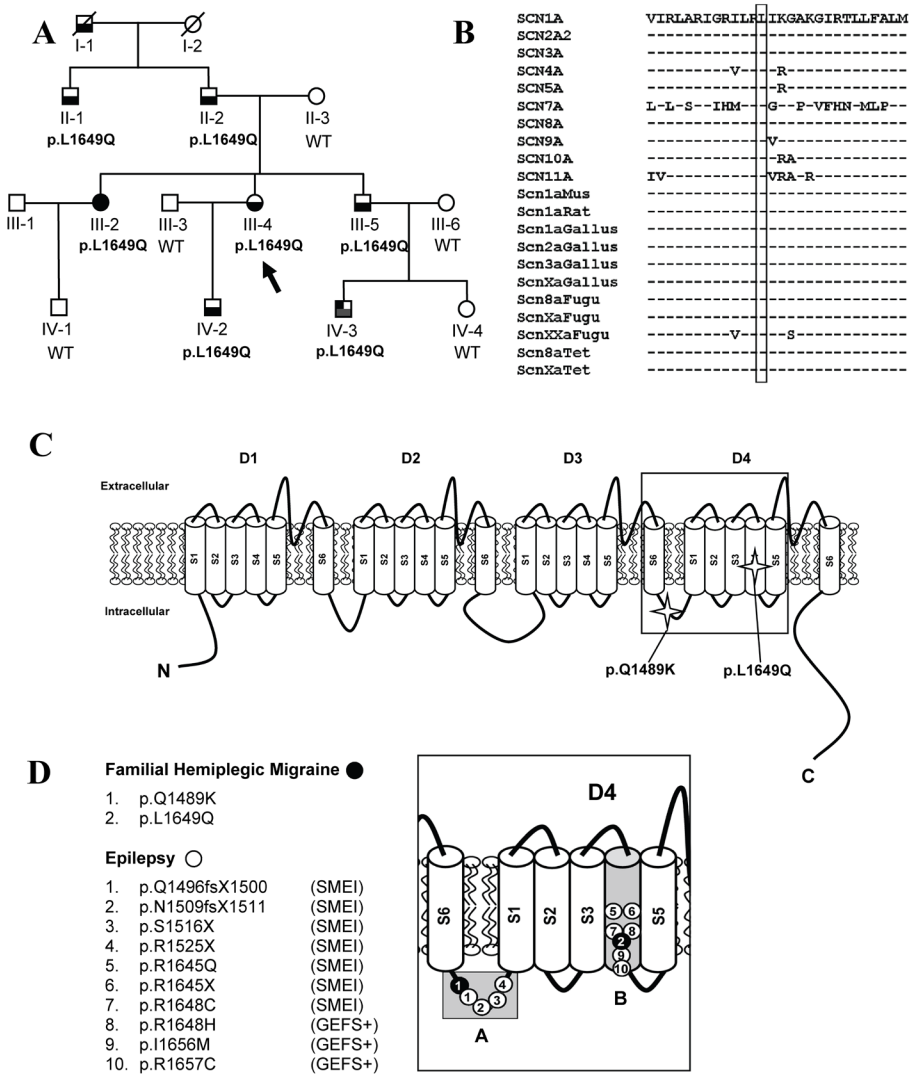

Figure 1. A: Pedigree of the FHM3 p.L1649Q family. The arrow indicates the proband. Symbols present: FHM: black lower half; MA (migraine with aura): right upper square; MO (migraine without aura): left upper square; WT: wild-type; p.L1649Q heterozygous carrier of the pathogenic *SCN1A* mutation. Patient IV-3 (aged 22, gray lower half) had one typical (fulfilling the IHS criteria) hemiplegic migraine attack until present. **B:** Alignment of the amino acid sequence from several vertebrate sodium channel α subunits, showing complete conservation of the mutated amino acid Leucine[1649]. **C:** Topology of SCN1A, encoding the α1-subunit of a neuronal $Na_v1.1$ sodium channel. The subunit consists of 4 repeat domains (D1-D4), which contain 6 transmembrane domains (S1-S6). The location of the novel mutation p.L1649Q in the voltage sensor domain S4/D4 is depicted, as well as the previously identified p.Q1489K FHM3 mutation. **D:** Over 150 mutations have been identified for severe myoclonic epilepsy in infancy (SMEI) or generalized epilepsy with febrile seizures plus (GEFS+). For clarity, only SMEI and GEFS+ mutations are shown that are located in the (A) inactivation gate and (B) S4/D4 voltage sensor domain where FHM3 mutations were identified (all mutations are based on *SCN1A* cDNA reference sequence: AB093548.1). For review papers with all *SCN1A* epilepsy mutations see Meisler et al. (2005) and Mulley et al. (2005).

Functional Consequences of the FHM3 Mutation

TsA201cells expressing construct *SCN5A-L1636Q*, which is equivalent to mutation p.L1649Q in SCN1A showed typical voltage-dependent sodium inward currents, similar to cells transfected with construct *SCN5A-WT* (Fig. 2A), and with similar current density (Table 2). Mutant channels activated with the same voltage dependence as WT channels (Table 2). However, the time course of inactivation was slower for the mutant (Fig. 2A). Quantitative analysis of the inactivation time-course revealed that, at all tested voltages (-50 mV to 30 mV), both time constants of the double exponential fits (τ_{fast} and τ_{slow}) were two- to four-fold larger for the mutant compared to wild-type (Fig. 2B-C). The contribution of the fast component relative to the slow component (also expressed as the ratio C_{fast}/C_{slow}) of inactivation was reduced (Fig. 2D). Both effects lead to an overall slower fast inactivation of mutant channels (Fig. 2A). On the other hand, slow inactivation was unaffected (Table 2). The voltage dependence of steady state fast inactivation was shifted by ~ 10 mV towards more positive voltages and recovery from fast inactivation, measured after a 500 ms conditioning pulse to –10 mV, was 150% faster in the mutant than in the wild-type channels. Altogether, these functional analyses show that this mutation severely interferes with the fast inactivation process.

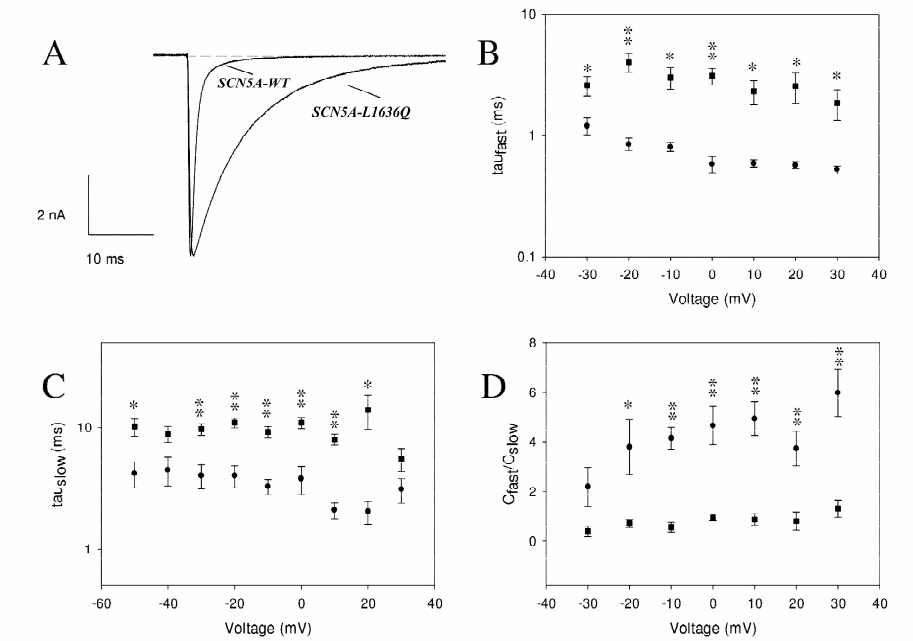

Figure 2. Electrophysiological properties of wild-type and mutant sodium channels. **A:** Macroscopic sodium currents were recorded using the whole-cell configuration of the patch clamp technique. Depicted are current traces recorded at -10 mV showing slowed inactivation of mutant channels. **B-D:** Time constants of inactivation (tau_{fast} and tau_{slow}) and their respective contributions (C_{fast} and C_{slow}) were obtained from a bi-exponential fit to the decaying current phase as described earlier (Dichgans et al., 2005). B: fast time constant of inactivation (tau_{fast}). C: slow time constant of inactivation (tau_{slow}). D: ratio of the contribution of the fast and the slow component of inactivation (C_{fast}/C_{slow}). At -50 mV and -40 mV the fast component was negligible. Mean values ± SEM are given for *SCN5A-WT* (filled circles; n=6) and mutant *SCN5A-L1636Q* (filled squares; n=6). Values marked with asterisks are significantly different between wild-type and mutant (* $p<0.05$; ** $p<0.01$) with Student's unpaired *t*-test.

Table 2. Activation and inactivation parameters for wild-type and mutant sodium channels

PARAMETERS	*SCN5A-WT*	*SCN5A-L1636Q*
Current density (nA/pF)	0.24 ± 0.04 (30)	0.21 ± 0.03 (28)
Steady-state activation		
Voltage of half maximal activation (V*a*, mV)	-44.2 ± 5.1 (7)	-41.4 ± 1.5 (6)
Slope factor (k*a*)	6.1 ± 1.8	6.0 ± 1.5
Reversal potential (V*rev,* Mv)	45.6 ± 5.2	41.7 ± 7.0
Steady-state fast inactivation		
Voltage of half maximal inactivation (V*fi,* mV)	-77.4 ± 3.1 (8)	-67.8 ± 2.7 (5) **
Slope factor (k*fi*)	7.2 ± 1.4	7.5 ± 1.1
Steady-state slow inactivation		
Voltage of half maximal slow inactivation (V*si,* mV)	-99.1 ± 10.2 (3)	-92.8 ± 0.7 (3)
Slope factor (k*si*)	12.6 ± 3.3	13.0 ± 2.1
Recovery from inactivation (-120 mV)		
Fast time constant (τ_{fast}) (ms)	7.1 ± 1.6 (4)	4.6 ± 1.0 (5) *
Slow time constant (τ_{slow}) (ms)	322 ± 200	108 ± 48
Onset of slow inactivation		
Fast time constant (τ_{fast}) (s)	3.8 ± 1.4 (9)	5.1 ± 0.9 (4)
Slow time constant (τ_{slow}) (s)	302 ± 105	217 ± 98

Data are mean ± SD. Numbers in brackets indicate number of experiments. Values marked with asterisks are significantly different between wild-type and mutant (* $p<0.05$; ** $p<0.01$) with Student's unpaired *t*-test.

DISCUSSION

We here firmly establish that certain mutations in the *SCN1A* epilepsy gene may cause FHM. First, the p.L1649Q SCN1A mutation completely co-segregated with a pure FHM phenotype (without epilepsy) in our family (Fig. 1A) and was not found in the control panel. Second, Leucine^{1649} is highly conserved among several vertebrate sodium channel α1 subunits (Fig. 1B). Third, the mutation is located in the S4 segment of domain 4 that acts as a voltage sensor and is known to play an important role in channel gating (Fig. 1C-D) (Kuhn and Greeff, 1999; Ulbricht, 2005). Finally, our functional studies of the mutation introduced in the highly homologous human SCN5A revealed clear functional effects: i) an overall slower inactivation of sodium channels; ii) a depolarizing shift by ~ 10 mV in the voltage dependence of the steady state inactivation; and iii) an accelerated recovery from fast inactivation (Table 2). Most likely, the p.L1636Q SCN5A mutation directly interferes with the inactivation process, as do other mutations in the S4/D4 domain (Ulbricht, 2005), even though we cannot fully exclude a contribution of an altered interaction with the β1 subunit (Ko et al., 2005). Although this study convincingly showed causality for p.L1649Q in FHM3, in future studies, these findings should be confirmed in SCN1A, or even better in a knockin mouse model.

The previously identified FHM3 mutation p.Q1489K (Dichgans et al. 2005) was also introduced in the highly homologous *SCN5A* cDNA, which allows comparison of the functional consequences of both FHM3 mutations.

This mutation is located in the cytoplasmic linker between domains III and IV (Fig. 1C-D) and revealed a two-fold to four-fold accelerated recovery from fast inactivation (Dichgans et al., 2005). Thus, both the p.Q1489K and the p.L1649Q mutation lead to impaired fast inactivation and predict enhanced neuronal excitation. This fits very well with our current understanding of the pathogenesis of FHM (Moskowitz et al., 2004; Ferrari and Goadsby, 2006). The *CACNA1A* gene encodes neuronal $Ca_v2.1$ calcium channels that modulate the release of neurotransmitters. FHM1 *CACNA1A* mutations were shown to cause gain-of-function effects in cellular models (Plomp et al., 2001; Pietrobon et al., 2005) and in a knockin mouse model (van den Maagdenberg et al., 2004). In the transgenic model, FHM1 mutations increase the release of glutamate and other neurotransmitters (A. Tottene, A. van den Maagdenberg, and D. Pietrobon, unpublished observations) and reduce the threshold for cortical spreading depression (CSD) (van den Maagdenberg et al., 2004). CSD has been convincingly shown to be the underlying mechanism for the migraine aura (Lauritzen, 1994) and, based on animal experiments, may also be responsible for triggering the headache phase of migraine attacks by activating the trigeminovascular system (Bolay et al., 2002). The *ATP1A2* gene encodes a Na,K-ATPase in glial cells. FHM2 *ATP1A2* mutations were shown to have altered kinetics or loss-of-function effects in cellular studies, predicting reduced re-uptake of both K^+ and glutamate from the synaptic cleft into glial cells (De Fusco et al., 2003; Segall et al., 2005). Voltage-gated sodium channels are involved in the generation and propagation of action potentials in excitable tissues. FHM3 *SCN1A* mutations changed $Na_v1.1$ channel inactivation kinetics, predicting enhanced neuronal excitation leading to increased release of neurotransmitters, including glutamate. The common overall effect of FHM mutations in all three FHM genes seems to be an increase of the concentration of K^+ and glutamate in the synaptic cleft. This should translate into an enhanced propensity for CSD and may thus be responsible for triggering FHM, and possibly "normal" migraine attacks.

Migraine and epilepsy are comorbid disorders and seem to have some overlapping mechanisms related to dysfunction of ion transportation (Haut et al., 2006). It is remarkable that the vast majority of *SCN1A* mutations are associated with severe forms of epilepsy, whilst the p.Q1489K and p.L1649Q mutations cause pure FHM. The p.L1649Q FHM3 mutation is even adjacent to two "epilepsy" mutations that affect Arginine^{1648}, the p.R1648C mutation causing SMEI (Ohmori et al., 2002) and p.R1648H causing GEFS+ (Escayg et al., 2000). Both mutations have been examined in several expression systems with different outcomes all affecting channel inactivation (Meisler and Kearney, 2005). From these and our studies it is evident that the voltage sensor in domain 4 (S4/D4) is pivotal to the fast inactivation of sodium channels but that there is no simple correlation between clinical phenotype and biophysical changes induced by *SCN1A* mutations. Dedicated functional studies comparing epilepsy and migraine mutations in the same gene may further the insight into both episodic brain disorders.

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Chapter 5

Systematic Analysis of the Familial Hemiplegic Migraine Genes *CACNA1A, ATP1A2* and *SCN1A* in 39 Sporadic Patients with Hemiplegic Migraine

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Abstract

Background Familial (FHM) and Sporadic (SHM) Hemiplegic Migraine are severe subtypes of migraine associated with transient hemiparesis. For FHM, three genes have been identified: the calcium channel subunit gene *CACNA1A*, the sodium-potassium ATPase subunit gene *ATP1A2,* and the sodium channel subunit gene *SCN1A*. Their role in SHM is unknown. Establishing a genetic basis for SHM may further the understanding of its pathophysiology and its relationship with common types of migraine. It will also facilitate the often difficult differential diagnosis from other causes of transient hemiparesis.

Methods We systematically scanned 39 well-characterized patients with SHM without associated neurologic features for mutations in the three FHM genes. Functional assays were performed for all new sequence variants.

Results Sequence variants were identified in seven SHM patients: one *CACNA1A* mutation, five *ATP1A2* mutations and one *SCN1A* polymorphism. All six mutations caused functional changes in cellular assays. One SHM patient later changed to FHM because another family member developed FHM attacks.

Conclusion We show that FHM genes are involved in at least a proportion of SHM patients without associated neurologic symptoms. Screening of *ATP1A2* offers the highest likelihood of success. Because FHM gene mutations were also found in family members with “nonhemiplegic” typical migraine with and without aura, our findings reinforce the hypothesis that FHM, SHM and “normal” migraine are part of a disease spectrum with shared pathogenetic mechanisms.

Introduction

Hemiplegic migraine is a rare, often severe sub-type of migraine with aura in which attacks are associated with hemiparesis.[1] Otherwise, the aura and headache symptoms are identical to those of common types of migraine.[2] Hemiplegic migraine may run in families (familial hemiplegic migraine; FHM) or may be sporadic (SHM).[1] Clinically, FHM and SHM attacks are indistinguishable, and the vast majority of patients also have common attacks of migraine with or without aura, not associated with hemiparesis.[3]

Thus far three genes for FHM have been identified. The *CACNA1A* gene (FHM1)[4] encoding the pore-forming subunit $Ca_v2.1$ of neuronal P/Q-type calcium channels, the *ATP1A2* gene (FHM2)[5] encoding the $\alpha 2$ subunit of sodium-potassium pumps and the SCN1A gene (FHM3)[6] encoding the $\alpha 1$ subunit of neuronal sodium channels.

Although clinically indistinguishable,[3] it is unknown whether and to what extent SHM and FHM are pathophysiologically related and whether and to what extent FHM genes are also involved in SHM. Previous studies identified mutations in the *CACNA1A* gene in SHM patients.[7-11] Most of these patients showed cerebellar signs, suggesting an involvement of the *CACNA1A* gene in SHM with associated cerebellar and other neurological signs or symptoms, such as cerebral edema and coma after minor head trauma. In contrast, the role of the FHM genes in "pure" SHM without associated neurological symptoms is less clear. One *CACNA1A* mutation (R583Q)[8] and one *ATP1A2* (R383H)[12] mutation were reported in such patients.

Investigating the involvement of FHM genes in sporadic patients with hemiplegic migraine is important as it may further the insight into the pathophysiology of SHM and the relationship with other types of migraine. Moreover, understanding and establishing the genetic basis of SHM may help clinicians in diagnostic and therapeutic decision-making. Many patients are initially misdiagnosed and mistreated. We therefore set out to systematically search for mutations in the known FHM genes in a large set of 39 clinically well-characterized patients with "pure" SHM, who had no interictal neurological symptoms.

Methods

Patients

Sporadic hemiplegic migraine was diagnosed according to the criteria of the International Headache Society.[1] Patients with interictal neurological symptoms, in particular ataxia, were excluded as these patients have a high a priori probability of carrying a *CACNA1A* mutation.[7-11] All available family members were directly interviewed and their headache was diagnosed according to the IHS criteria. In addition to newly recruited SHM patients we included 25 of the 27 patients from our previous study in which only the FHM1 *CACNA1A* gene was investigated.[8] Two patients from that study were excluded because of associated symptoms; one had ataxia and carried the T666M mutation, the other patient had childhood epilepsy and did not carry a *CACNA1A* mutation. Approval was obtained by local ethical committees in accordance to national legislation; all patients gave informed consent.

Mutation scanning

Genomic DNA was isolated from peripheral leukocytes using a standard salting out extraction method. The *CACNA1A, ATP1A2* and *SCN1A* gene, were screened for mutations by sequencing.[6,8,13] In brief, all exons and flanking intronic regions were amplified by PCR,

using genomic DNA as a template. Direct sequencing was done by Cycle Sequencing (Prism Big Dye Terminators Cycle Sequencing kit, Applied Biosystems, Foster City, CA, USA) using the dideoxy termination method and an ABI3700 automated sequencer (Applied Biosystems, Foster City, CA, USA). For each exonic variant identified, 150 healthy controls were screened, by restriction enzyme analysis or direct sequencing. Detailed information is available from the authors upon request.

Functional analysis

Functional analysis of mutations in the $Ca_v2.1$-α1 calcium channel subunit was not performed, as the single *CACNA1A* mutation found in this study was thoroughly investigated before.[14] Functional analysis of *ATP1A2* variants was performed by survival assays. Human Na,K-ATPase α2-subunit cDNA was subcloned into a modified pCDNA3.1 vector.[15] To distinguish endogenous Na,K-ATPase activity from that of transfected Na,K-ATPase, we used a cDNA encoding ouabain-resistant wild-type (*ATP1A2*-WT).[15,16] Mutations E120A, E492K, P786L, R834X and R908Q were introduced in the ouabain-resistant wild-type α2-subunit construct by site-directed mutagenesis (Quikchange; Stratagene, La Jolla, CA, USA). HeLa cells (5 x 105) were transfected with plasmid DNA of either *ATP1A2-WT* or *ATP1A2-mutant* (*ATP1A2-E120A, ATP1A2-E492K, ATP1A2-P789L, ATP1A2-R834X, ATP1A2-R908Q*) using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). Two days after transfection, two third of the cells was harvested for immunoblotting and the α2-subunit protein was detected using specific polyclonal antibody HERED.[15,16] The remaining one third of the cells was seeded on 10 cm petri dishes and subsequently 1 μM ouabain was added to the culture medium. After 5 days of ouabain challenge, colonies were stained with 1% methylene blue in 70% methanol, scanned, and analyzed with Image Pro Plus (MediaCybernetics, Silver Spring, MD, USA). Each transfection was performed 7 - 15 times. In case of partial survival, statistical significance was tested using Student's t-test ($p < 0.05$).

For functional analysis of the *SCN1A* variant, we used the closely related SCN5A cDNA, because of the known stability problems of recombinant bacteria with SCN1A cDNA.[6,17] R1914G, which corresponds to *SCN1A* R1928G, was introduced by site-directed mutagenesis into full-length human SCN5A cDNA subcloned in pCDNA3.1 (QuikChange XL Kit, Stratagene, La Jolla, CA, USA). *SCN5A-R1914G* and *SCN5A-WT* cDNA's were transfected into human tsA201 cells and were each co-expressed with accessory human sodium channel subunit β1.[6] Macroscopic sodium currents were recorded using the whole-cell configuration of the patch clamp technique.[6] Steady state activation, steady-state inactivation, time constants of inactivation (e.g. time constants τ_{fh} and τ_{sh}), as well as recovery from inactivation (e.g. τ_{fast} and τ_{slow} time constants) were measured using protocols, as described before.[6]

Results

Patients:
Thirty-nine patients with "pure" SHM were included, 37 originated from Western Europe (mostly The Netherlands or Germany) and two came from the United States (table 1). As expected [3,18] some of the patients exhibited basilar-type migraine symptoms during the attacks, but they were all free of interictal signs or symptoms. Age at onset of hemiplegic attacks ranged from 4 to 42 years. The number of attacks varied from 2 per lifetime to more than 200 per year. Likewise, duration of hemiparesis was very variable, from several minutes to 1 week. Four patients reported loss of consciousness during attacks; two patients reported triggering of attacks by minor head trauma. In one patient, the initial diagnosis was later changed to FHM, when a family-member developed hemiplegic migraine attacks. Notwithstanding, this patient was kept in the SHM group as he fulfilled the inclusion criteria of SHM at the time of clinical presentation. In ~70% of the families of our 39 SHM patients, attacks of common non-hemiplegic migraine with aura (one third), without aura (one third), or both (one third) were present in one or more first degree relatives.

Table 1 *Clinical and genetic characteristics of SHM patients*

Patient	Mutation in HM gene	Age of onset (year)	Freq of attacks (per year)	Total duration of attack	Duration of paresis	BAM symptoms	Unconsciousness during attacks	Attacks triggered by minor head trauma	MO or MA in first degree family members
1	No	27	3	1-2 days	min	no	no	no	MO
2	No	20	4	4 hrs-2 days	0.5-2 hrs	no	no	no	MA
3	No	12	?	days	hrs	no	no	no	MA
4	No	18	24	15-60 min	15 min	no	no	no	MO
5	No	20	0-3	8 hrs	1 hr	no	no	no	no
6	*ATP1A2* - R834X	10	1	4 days	2 days	no	no	yes	MO
7	No	22	12	12 hrs	0.5-2 hrs	no	no	no	MO and MA
8	No	9	12-30	1-3 days	1 hr-1 day	no	no	no	MA
9	No	25	24	Hrs-3 days	0.5-1 hr	yes	no	no	MO
10	No	12	4-36	1-2 days	5-10 min	no	yes	no	no
11	No	19	2 in lifetime	20 min-1 hr	20 min	no	no	no	MA
12	*ATP1A2 - E120A*	13	12-250	1 day	1 hr	no	no	no	no
13	No	25	48	10-24 hrs	?	?	no	no	MO and MA
14	No	13	2-6	Few hrs	0.5 hr	no	no	no	MO and MA
15	No	29	2-100	Min-hrs	Min-hrs	yes	yes	yes	no
16	No	4	24	1 day	Few hrs	yes	no	no	MA
17	No	10	Once	1 day	1 hr	no	no	no	MO and MA
18	No	20	0-2	1 wk-1 mnth	1 wk-1 mnth	?	no	no	MO
19	*ATP1A2 - P786L*	5	0.5	3 wks	6 days	no	no	no	no
20	No	16	12	1 day	1 hr	no	no	no	MO and MA
21	No	13	Once	Few days	few days	no	no	no	MO and MA
22	No	13	2-52	1 day	0.5-1 hr	no	no	no	no
23	*ATP1A2 - E492K*	19	2	?	?	no	no	no	MA
24	No	32	2-12	days	hrs-days	no	no	no	no
25	*CACNA1A – R583Q*	13	2	1 day	0.5	no	no	no	MA
26	No	34	8	0.5?	hrs?	no	no	no	no
27	No	37	36-48	3-48hrs?	Hrs-48hrs	no	no	no	MO or MA?
28	*SCN1A - R1928G*	38	2 in lifetime	Few hrs	1.5 hrs	no	yes	no	MO and MA
29	No	42	2 in lifetime	3-4 hrs	0.5hrs	no	no	no	migraine unspecified
30	No	11	30 in lifetime	"hrs"	1hr	no	no	no	MA and migraine unspecified
31	No	17	24	up to 4hrs	3-4hrs	no	no	no	MA in grandmother
32	No	22	up to 50	hrs	"hrs"	no	no	no	no
33	No	15	4 to 10	1 hr	15-30min	no	no	no	migraine unspecified
34	*ATP1A2 - R908Q*	5	2 to 5	2hrs	30-90min	yes	no	no	no
35	No	27	5 in lifetime	up to 7hrs	up to 7 hrs	no	no	no	MO and MA
36	No	16	4	1.5 - 3hrs	1-3hrs	no	no	no	no
37	No	4	4	"hrs"	24-72hrs	no	no	no	no
38	No	8	2-12	"hrs"	15-60min	no	no	no	no
39	No	35	5	Few hrs	15min-3h	no	yes	no	MA

MO = migraine without aura; MA = migraine with aura

Genetic and functional findings

Sequencing of all exons and flanking intronic sequences in the 39 index cases revealed seven sequence variants which were present in seven probands (table 2 and figure 1): one in *CACNA1A*, five in *ATP1A2*, and one missense variant in the *SCN1A* gene. None of the sequence variants was present in 150 healthy controls (data not shown).

Table 2 *DNA variants identified in pure SHM patients*

Gene	Amino acid change	Nucleotide change	Abnormality in functional test
CACNA1A	R583Q	nt 2021G>A	Electrophysiological consequence
ATP1A2	E120A	nt 463A>C	Partial cell survival
ATP1A2	E492K	nt 1578G>A	Partial cell survival
ATP1A2	P786L	nt 2604C>T	No cell survival
ATP1A2	R834X	nt 2461C>T	No cell survival
ATP1A2	R908Q	nt 2827G>A	No cell survival
SCN1A	R1928G	nt 5782C>G	No electrophysiological consequence

Used reference sequences: X99897 (*CACNA1A*), NM_000702 (*ATP1A2*), AB093548 (*SCN1A*).

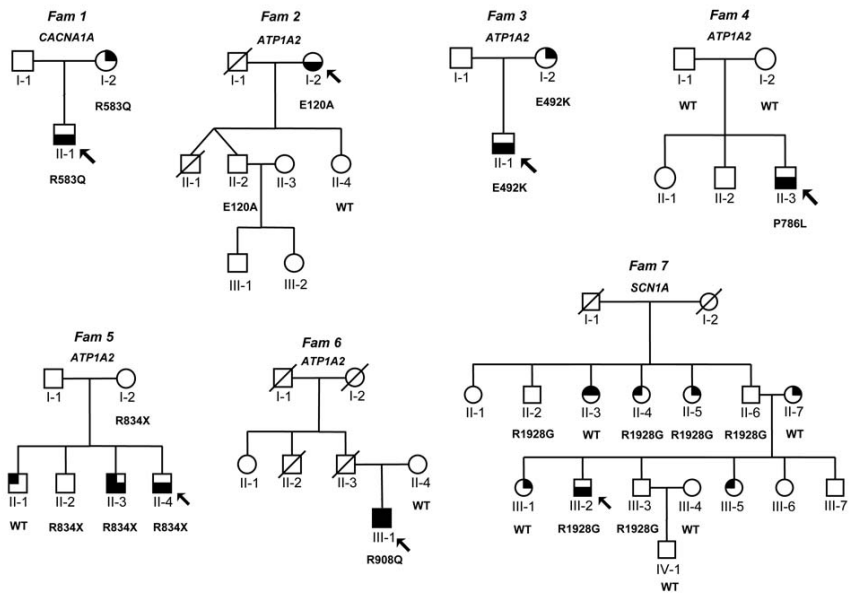

Figure 1. Pedigrees of SHM cases with a mutation in one of the FHM genes (*CACNA1A, ATP1A2* and *SCN1A*). The following symbols are used to indicate the diagnosis: FHM: filled lower half; MA: right upper quadrant; MO: left upper quadrant. Circle: female; square: male. Arrows indicate probands. Individuals homozygous for the wild-type allele are indicated by WT; individuals heterozygous for a DNA variant are indicated by the respective variant. Fam = family.

CACNA1A

The single variant in the *CACNA1A* gene (R583Q) has been reported as part of our earlier study. [8] R583Q was present in the index case and his unaffected mother, who had migraine with aura but no hemiplegic attacks indicating incomplete penetrance. R583Q has previously been identified in families with FHM and shown to affect $Ca_v2.1$ Ca^{2+} channel gating in functional studies. [14] Thus, R583Q can be considered causative in our case.

ATP1A2

The five DNA variants in *ATP1A2* included four missense variants (E120A, E492K, P786L, R908Q) and one nonsense mutation (R834X). P786, R908 and R834 are completely conserved across multiple homologs and orthologs whereas E120 and E492 are less well conserved (figure 2). The P786L mutation was not present in the proband's parents. False paternity was excluded in this case. Thus P786L represents a *de novo* mutation. E120A, E492K, and R834X were all present in one or more relatives who had no hemiplegic attacks thus suggesting incomplete penetrance. R908Q was not present in the proband's mother but DNA from additional family members was not available (figure 1).

Functional consequences of all five *ATP1A2* variants were investigated using survival assays in HeLa cells as previously reported for FHM2 mutations.[5,16] The survival assays test for the ability of mutant alleles to compensate for the loss of endogenous Na,K pump function (figure 3C and D). Loss of endogenous Na,K-ATPase activity is achieved by ouabain treatment. Because of an altered ouabain binding site the transfected wild-type (*ATP1A2-WT*) and mutant (*ATP1A2-E120A*, *ATP1A2-E492K*, *ATP1A2-P786L*, *ATP1A2-R834X*, *ATP1A2-R908Q*) Na,K-ATPase α2 subunits are ouabain-insensitive. Western blot analysis showed that the constructs were expressed at comparably levels (figure 3A and B). In the survival assay, cells expressing the wild-type construct survived ouabain treatment. In contrast, *ATP1A2* mutants gave no (*ATP1A2-P786L*, *ATP1A2-R834X*, *ATP1A2-R908Q*) or partial (*ATP1A2-E120A* ($p = 0.03$), *ATP1A2-E492K* ($p = 0.002$)) cell survival, indicating a clear functional consequence for all mutants (figure 3C and D).

SCN1A

DNA variant R1928G in the *SCN1A* gene was present in the index and five additional family members two of whom had non-hemiplegic migraine. R1914G (which is equivalent to *SCN1A* R1928G) was introduced to highly homologous human *SCN5A* (*SCN5A-R1914G*) and functionally tested for its biophysical properties by patch-clamp experiments in transiently transfected human tsA201 cells. [6] Cells expressing the *SCN5A-R1914G* showed no significant difference in current density, steady state activation, steady state inactivation and recovery from inactivation, when compared to wild-type (table 3). These results indicate that the variant may be a DNA variant without a biologically significant effect on $Na_v1.1$ channel functioning.

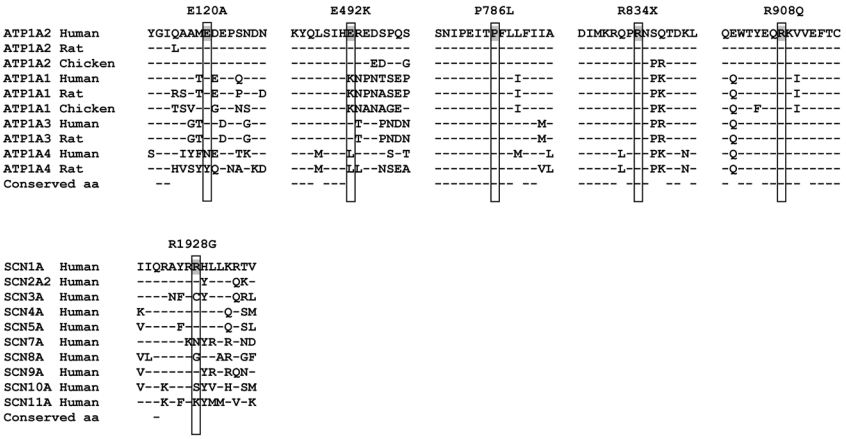

Figure 2. Alignments of novel *ATP1A2* and *SCN1A* DNA variants identified in SHM patients
Conservation of mutated amino acids (highlighted in gray) is depicted in boxes. Dashed lines indicate conserved amino acids. Proteins were obtained from GenBank. Human: P50993 (ATP1A2), P05023 (ATP1A1), P13637 (ATP1A3), Q13733 (ATP1A4); Rat: P06686 (ATP1A2), P06685 (ATP1A1), P06687 (ATP1A3), Q64541 (ATP1A4); Chicken: P24797 (ATP1A2), P09572 (ATP1A1). Human: P35498 (SCN1A), Q99250 (SCN2A2), Q9NY46 (SCN3A): P35499 (SCN4A), Q14524 (SCN5A), Q01118 (SCN7A), Q9UQD0 (SCN8A), Q15858 (SCN9A),Q9Y5Y9 (SCN10A), Q9UI33 (SCN11A).

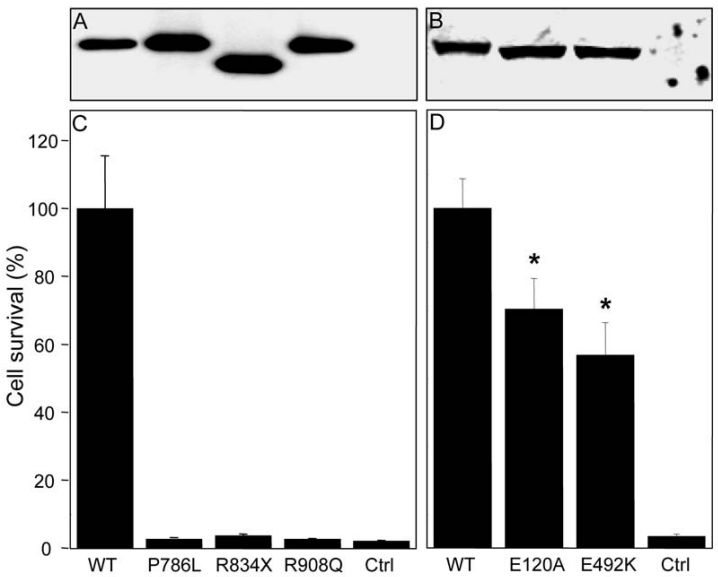

Figure 3. Ouabain survival assay of novel *ATP1A2* DNA variants identified in SHM patients.
A and B: Western blot analysis of HeLa cells transfected with WT or mutant ATP1A2 cDNA. C and D: Ouabain sensitivity of cells transfected with either wild-type or mutant ATP1A2 cDNA. Bars represent cell survival after 5 days of ouabain treatment (error bars: SEM). *Partial survival is significantly lower then for WT ($p<0•05$). Mutants P786L, R834X and R908Q gave no survival Ctrl = control..

Table 3 Activation and inactivation parameters for wild-type and mutant sodium channel subunit

	SCN5A/WT	*SCN5A*/R1914G
Currrent density (nA/pF)	0.24 ± 0.04 (30)	0.14 ± 0.09 (15)
Steady state activation		
Voltage of half maximal activation (Va, mV)	-44.2 ± 5.1 (7)	-40.4 ± 3.7 (5)
Slope factor (k*a*)	6.1 ± 1.8	6.2 ± 0.78
Reversal potential (V*rev,* mV)	45.6 ± 5.2	35.4 ± 3.2
Steady-state inactivation		
Voltage of half maximal fast inactivation (V*fi,* mV)	-77.4 ± 3.1 (8)	-76.5 ± 5.9 (5)
Slope factor (k*fi*)	7.2 ± 1.4	8.1 ± 1.5
Recovery from inactivation (-120 mV)		
Fast time constant (τ_{rf}) (ms)	7.1 ± 1.7 (4)	.6 ± 2.1 (4)
Slow time constant (τ_{rs}) (ms)	323 ± 200	310 ± 144

Electrophysiology was performed for wild-type SCN5A-WT and mutant SCN5A-R1914G, equivalent to SCN1A R1928G, in transiently transfected tsA201 cells. Values are mean +/- SD. In brackets the number of recorded cells is given.

Discussion

We screened 39 patients with "pure" SHM without ataxia or other additional neurological features for mutations in the three known FHM genes. In seven patients we found a sequence variant (table 2). None was found in 300 control chromosomes. Six of these showed obvious functional changes and can be considered causal mutations. These results indicate that genes for FHM are involved in at least a proportion of patients with "pure" SHM. Our findings have important pathogenetic, clinical, and diagnostic implications.

With our findings, a sensible approach to genetic testing in SHM has become available to confirm the often difficult and clinically important diagnosis of SHM.[19] As SHM patients with *de novo* mutations may represent the founder of a new family with highly disabling FHM, genetic confirmation of the diagnosis may have consequences for genetic counseling. When genetic testing is considered in a patient with "pure" SHM, the *ATP1A2* gene should be screened first. We found an *ATP1A2* sequence variant in 5 of the 7 SHM cases with a confirmed sequence variant corresponding to 13% of the overall SHM sample. This is a strikingly higher prevalence compared to a previous study of the *ATP1A2* gene that included patients with SHM, but provided no specific clinical details.[12]

Our findings are in line with earlier smaller studies showing that the yield of *CACNA1A* mutations in SHM patients is low in the absence of ataxia.[20,21] In contrast, *CACNA1A* mutations were found in 50% of SHM patients with associated cerebellar signs.[7,9] The present study is the first to evaluate the role of the recently identified FHM3 gene[6] in SHM. The likelihood of finding *SCN1A* mutations in "pure" SHM seems however very low.

Most *ATP1A2* mutations in this study were also found in asymptomatic relatives and in relatives with non-hemiplegic migraine. They thus showed reduced penetrance as has also been noticed for *ATP1A2* mutations associated with FHM2.[22-24] This might explain why mutations in the *ATP1A2* gene are relatively common among sporadic patients. In contrast, all *SCN1A* mutations previously identified in FHM families showed complete penetrance.[6,17] This might relate to the low yield of mutations in this gene in our sample of sporadic cases.

Although we found FHM gene mutations in 18% of our patients with pure SHM, we failed to find mutations in the majority of our patients. It is quite likely that when additional

genes for FHM will be discovered, greater proportions of patients with SHM will prove to have mutations in FHM genes. Until then, a diagnosis of SHM remains based on the exclusion of other causes of recurrent hemiparesis, careful physical examination, detailed personal- and family-history, and a regular follow-up. In one patient (with *ATP1A2* mutation R834X; family 5: figure 1) we had to change the initial diagnosis of SHM to FHM when an additional family member developed hemiplegic migraine attacks several years after our initial investigation. A diagnosis of pure SHM is very likely when transient hemiparesis occurs in the course of a typical attack of migraine with aura, when there are no interictal abnormalities, and when "normal" attacks of migraine with or without aura are present in first-degree relatives.[25] Approximately ~70% of pure SHM cases and 60% of the mutation carriers had first-degree relatives with common types of migraine.

Our findings provide genetic evidence that FHM genes are also involved in SHM and thus extend and reinforce the growing clinical, epidemiological, genetic, and pathophysiological evidence that FHM and SHM share neurobiological mechanisms.[26] Moreover, as the vast majority of hemiplegic migraine patients also have "normal" attacks of migraine without hemiparesis, both diseases can be considered extremes of the pathogenetic migraine spectrum with shared common pathways with "normal" migraine with and without aura.[26-29]

We identified five sequence variants in *ATP1A2* (figure 1; families 2-6). All conferred reduced survival in cellular assays (figure 3) and therefore are likely to be causative mutations. P786L occurred *de novo*, and could thus be the founder of a new FHM family. R908Q was found in a patient whose mother did not carry the mutation. As DNA from the father was not available it could not be established whether the mutation had occurred *de novo*. E120A and E492K showed partially reduced survival (figure 3 D). Both mutations were identified in other family members who were unaffected or had non-hemiplegic migraine with aura. The single *CACNA1A* mutation (R583Q) we found, was previously shown to affect $Ca_v2.1$ Ca^{2+} currents in cellular models by changing channel gating.[14] The mutation was inherited from the mother, who has attacks of non-hemiplegic migraine with aura. The R1928G DNA variant that was identified in the *SCN1A* gene did not reveal significant effects on channel properties as investigated by electrophysiological recordings. Also, this variant poorly segregates with the migraine phenotype. It was present in five non-hemiplegic family members; only one of them has migraine with aura and another migraine without aura. R1928G may therefore be a rare sequence variant without functional consequences.

Screening of FHM genes in sporadic patients with hemiplegic migraine may help to establish the diagnosis, enable counseling and prevent unnecessary diagnostic and therapeutic trail with potentially harmful drugs. Scanning of the FHM2 *ATP1A2* gene seems to offer the highest likelihood of success. As FHM mutations were also found in SHM and common types of migraine with or without aura, our findings reinforce the growing evidence that FHM, SHM, basilar-type migraine, and "normal" migraine are part of a disease spectrum with at least some shared pathogenetic pathways. Unraveling these pathways may help to identify novel migraine prophylactic drugs.

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Chapter 6

Practical lessons from genetic family studies in migraine: should we still collect large migraine families or not?

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Abstract

Migraine is a complex disease, influenced by genetic and environmental factors. Over the last decade, many chromosomal loci have been reported for common forms of migraine, using classical linkage approaches. Despite great research efforts no gene variants have been identified. Here we report on two of our own studies to map migraine genes using two different approaches; the first is an outbred linkage approach with MO families, the second is a family-based association approach with severe MA patients from a genetic isolate. Our results support that, most likely, the intrinsic genetic heterogeneity in migraine families seriously hampers the identification of migraine loci and ultimately migraine genes. In fact, they suggest that future genetic studies should also use alternative strategies. Recent successes in genetic studies using genome-wide association involving hundreds to thousands of patients and controls have been reported for several complex diseases. Such well-powered association studies should be considered for common migraine too. For this, patient recruitment should focus primarily on very large numbers of well-characterized migraine patients instead of large migraine families.

Introduction

Migraine is a paroxysmal neurovascular disorder affecting up to 6% of males and 18% of females in the general population.[1, 2] Clinical diagnosis is made on the basis of patient history and evaluation of attack types according to standardized diagnostic criteria as defined by the International Headache Society (IHS).[3, 4] Attacks of migraine without aura (MO) are characterized by severe, often unilateral, throbbing headache that is aggravated by physical activity and is accompanied by other disabling neurological symptoms like vomiting, nausea, photophobia and/or phonophobia. In one-third of the migraine patients the headache phase is preceded or accompanied by, primarily visual, aura-symptoms; migraine with aura (MA).[3, 4]

Family and twin studies have clearly indicated that migraine is a complex disorder with involvement of both genetic and environmental factors.[5-9] The identification of migraine genes, until now, is only successful for a rare, autosomal dominant, subtype of migraine with aura; familial hemiplegic migraine (FHM). In FHM, the aura is accompanied by transient hemiparesis. For FHM three genes have been identified: the *CACNA1A* gene (FHM1) encoding the α1 subunit of neuronal $Ca_V2.1$ (P/Q-type) calcium channels, the *ATP1A2* gene (FHM2) encoding the α2 subunit of sodium-potassium pumps and the *SCN1A* gene (FHM3) encoding the α1 subunit of neuronal voltage-gated $Na_V1.1$ sodium channels.[10-12] All three genes are involved in a unifying pathway of ion translocation. Notably, FHM is a genetically heterogeneous disease and initial locus identification was successful only in single, very large, families.

Assuming that common migraine is even more genetically heterogeneous than FHM, it is perhaps not surprising that gene identification for the complex migraine types has not been successful. Part of the problem may come from the approach that is used; genome-wide scans in single or multiple migraine families and standard linkage analysis using clinical end diagnosis (i.e. migraine without or with aura) were performed. Over the last decade, loci were identified in single migraine families to chromosomes 6p12-p21 and 14q21-q22 in a Swedish MO/MA family and an Italian MO family, respectively.[13, 14] In other studies loci were identified by analyzing large groups of migraine families together. A locus on chromosome 11q24 was identified in Canadian MA families.[15] One finding that seemed more promising was a locus on chromosome 4q21-24 - in fact the only replicated locus thus far - that was identified using families from the genetic isolates of Finland and Iceland.[16, 17] Surprisingly, replication in the Icelandic study was only significant for women with a relaxed definition of MO, whereas initial linkage was obtained for MA. A recent more promising approach seems mapping of disease loci making use of endophenotypes or trait components instead of migraine end diagnoses. Using latent class analysis, a promising novel locus was identified on chromosome 5q21 in Australian twins.[18] By trait component analysis, a novel locus was identified on chromosome 17p13 in Finnish MA families.[19]

These linkage studies showed that chromosomal loci are difficult to replicate and that for none of the loci the causal gene variant has been identified. Here we report on two of our own studies to map migraine genes using two different approaches, the first with an outbred linkage approach with MO families and the second with a family-based association approach with severe MA patients from a genetic isolate. We would argue that it is time to consider other gene identification strategies. We will also discuss the impact this will have on the collection of clinical material for gene identification studies.

Material and methods

Dutch migraine without aura families

From our set of 55 well-defined Dutch families with common forms of migraine, seven families were selected for a classical genome-wide scan. Ten additional migraine families were selected for subsequent fine-mapping efforts. Only families were selected in which nearly all patients had only attacks of MO. The selected families are all two-, three- or four-generation pedigrees and comprised of one very large family (1) and sixteen medium-sized families (2-17) (Figure 1). In these families MO appeared to be inherited as an autosomal dominant trait. To avoid additional genetic heterogeneity, all branches of the families in which a spouse was diagnosed with migraine were excluded from the linkage analysis. After this exclusion, the seven families that were used for the genome scan comprised a total of 204 individuals. The ten additional families (8-17) comprised of 128 individuals. All individuals were carefully examined personally by experienced neurologists from the out-patient Headache Clinic of the Leiden University Medical Centre using a semi-structured validated migraine questionnaire according to the IHS criteria. [3,4] The project was approved by the Committee of Medical Ethics of the Leiden University Medical Centre.

Migraine with aura patients from the Central Valley of Costa Rica

In Costa Rica, patients with migraine with aura (MA) were ascertained from the Central Valley through three different sources: (1) referrals by neurologists, (2) outpatient clinics of Tibas, Santa Ana and Pavas and (3) news/media/adds/flyers. All patients and family members were personally interviewed using a semi-structured questionnaire by a Spanish-speaking research nurse (KC), trained by a Spanish-speaking migraine specialist (EEK) of the Leiden University Medical Centre. Patient's response was documented in Spanish to give the patient the best opportunity to accurately describe his or her attacks. Patients were asked to make drawings of their visual aura's. The final diagnosis was made in Leiden after converting the Spanish questionnaires into an electronic version. Migraine diagnosis was made according to criteria of the International Headache Society [3, 4] The following clinical criteria were used for inclusion of patients with severe, early-onset, migraine with aura (MA) from the CVCR: (1) a diagnosis of MA according to IHS criteria, (2) onset of the disease before the age of 25, (3) a frequency of at least 1 attack per month. For a haplotype sharing strategy it is very important that patients belong to the genetic isolate. Therefore, only patients were included who had at least six out of eight great-grandparents that where born in the Central Valley. Finally we chose to include only those patients with both parents (or at least one parent and a sibling) willing to provide blood samples. For this study, 170 trios were selected of which 124 consisted of a patient and both parents, whereas 46 trio's had one missing parent, but an additional sibling. The study was approved by the Committee of Medical Ethics of the Leiden University Medical Centre and by the Costa Rican government.

DNA Analysis

Genomic DNA was isolated from lymphocytes using standard methods. [20] For both studies, a genome-wide scan was performed by the NIH-funded Marshfield Medical Research Foundation (http://research.marshfieldclinic.org/genetics). For the *Dutch migraine family study*, 149 samples were tested for in total 392 highly polymorphic repeat markers with an average spacing of ~ 9 cM (Kosambi). For the *Costa Rican migraine study*, 170 trios were tested for in total 732 autosomal highly polymorphic STR markers with an average spacing

of ~5 cM (Kosambi).

For subsequent fine-mapping of interesting regions on chromosome 4q21 and 6q23.3, DNA samples, including two CEPH samples (1331-1, 1331-2) for standardization of allele labeling, were genotyped for several polymorphic repeat markers. Markers were retrieved either from NCBI (http://ncbi.nlm.nih.gov) or newly developed by downloading genomic sequences from NCBI and identifying larger di- or tetranucleotide repeats and flanking unique sequences. For all markers, standard PCRs were performed using a PTC200 thermal cycler (Bio-Rad Laboratories, Foster City, CA, USA). PCR products were analysed on an ABI3700 sequencer (Applied Biosystems, Foster City, CA, USA) and genotypes were assigned using GENESCAN and GENOTYPER software (Applied Biosystems, Foster City, CA, USA). Two researchers scored genotypes independently.

Statistical Analysis

Prior to the linkage analysis of the *Dutch migraine family study*, pedigree genotype data were checked for possible sample switches using the Graphical Relationship Representation (GRR) program. [21] The loop of family 2 was broken between the married-in brother and sister in all analyses. Errors of Mendelian inheritance were tested with the UNKNOWN program of the LINKAGE 5.1 software package. [22] Given the apparent dominant inheritance pattern of MO in our families, the data was analyzed using model-based linkage analysis. For the linkage model, used in all analyses, the disease frequency was set at 0.05 based on current data of MO prevalence. [2] Furthermore, the penetrances were set at 0.10 for non-carriers - and 0.70 for carriers of one or two copies of the disease allele. Linkage analysis was performed with the MLINK program of the LINKAGE 5.1 software package using an affected-only approach. [22,23] Two-point LOD scores, single marker vs. disease, were calculated for every marker for the individual families, and for the data of all families combined. Furthermore, HLOD scores were calculated for every marker with the combined data of all families under the assumption of two-locus heterogeneity using the program HOMOG. [23,24]

For the *Costa Rican migraine study*, genotype quality checks were performed using PEDCHECK. [25] In addition, each marker was tested for deviation of Hardy-Weinberg equilibrium (HWE) with the HWE program. [26] Family-based association analysis was performed by a likelihood ratio based transmission disequilibrium test (TDT), [27] included in the AUTOSCAN package. [28] In TDT, parental alleles that are not transmitted to affected offspring served as (pseudo)controls, thereby circumventing the potential problem of population stratification.

An important part of each genetic linkage or association study is to estimate power to successfully detect an effect of a particular size. Power calculations are generally rough estimations because various assumptions have to be made concerning, marker heterozygosity, disease allele frequency, penetrance and effect size. For the Dutch migraine family study power was estimated with simulation studies using SLINK and MSIM programs. [29-30] For every MO family 1000 replicates were generated and analyzed using the linkage model shown above with an 'average' genetic marker having five alleles, linked at a recombination rate of 5% and unlinked. The maximum LOD scores and LOD score distributions obtained from the simulations were evaluated. The maximum expected LOD scores for two-point analysis with FHM families 1 and 2 were 4.51 and 3.49, respectively, showing genome-wide significance. The remaining five MO families were too small to produce significant linkage in a genome wide screen, but are expected to be informative in combination with the other MO families.

For the Costa Rican migraine study power was estimated from linkage disequilibrium (LD) mapping simulation studies in founder populations done by Service et al. [31] Two methods, ancestral haplotype reconstruction (AHR) and multipoint LD LR test (MLD) were tested in 100 replicate populations of respectively 200 or 400 patient- and control chromosomes. Results showed that power for detection of a specific risk locus was adequate (80%) in a sample of 100 trios and excellent (close to 100%) in a sample of 200 trio's, in a scenario in with 20% of chromosomes in the patient sample represent true disease chromosomes descended from a common ancestor, applying a marker screen with density between 4-6 cM and using a threshold of p=0.01. From these simulation studies, that used a similar study design and population, but a different analysis method, we concluded that our sample had sufficient power.

Results

Recruitment of patients in an outbred population and a genetic isolate

For the *Dutch migraine family study*, initially seven and, in the fine-mapping phase, another 10 multigenerational families were selected, in which MO was the predominant migraine subtype (Figure 1). In these families migraine seemed transmitted in an autosomal dominant fashion and therefore were ideal for a standard, family-based, linkage approach. Of all 332 family members that were interviewed for this study, 178 suffered from migraine. By far the majority exclusively suffered from MO attacks, 12 patients also suffered from MA attacks. Only in 6 patients migraine attacks were diagnosed exclusively as attacks of MA. DNA samples of 149 family members (of families 1 to 7) were used for a 9 cM genome-wide scan with 392 polymorphic genetic markers to identify migraine loci.

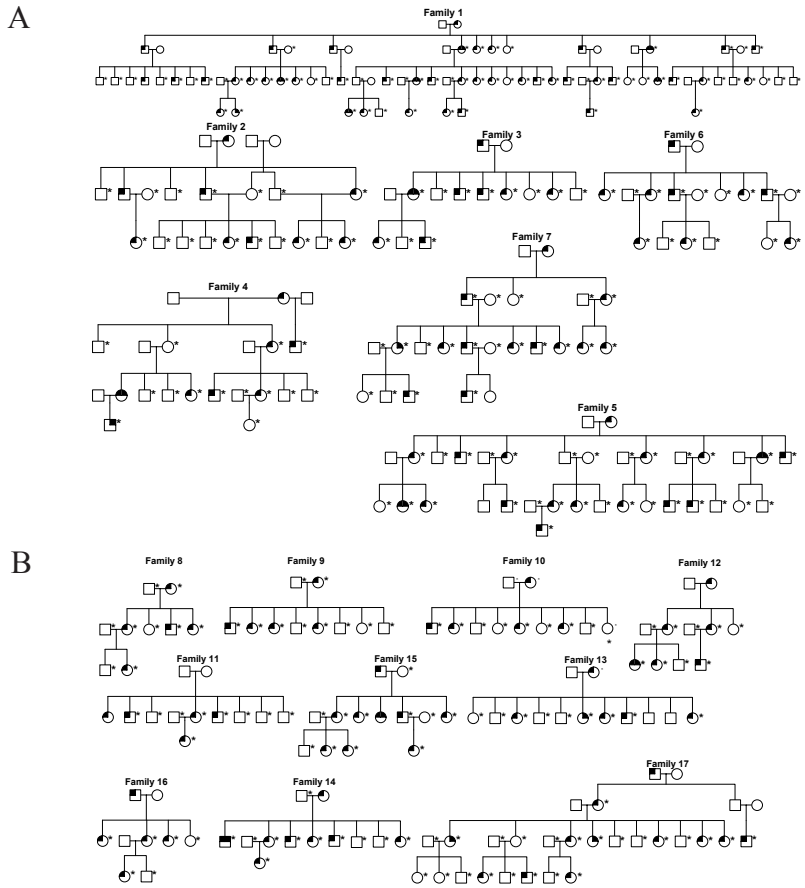

Figure 1: Pedigrees of Dutch MO families that were used for linkage analysis.

(A): seven Dutch migraine families used for the genome wide screen (1-7); (B): ten additional migraine families (8-17) used for fine-mapping of the chromosome 4q21 locus. Symbols represent the following: left upper quadrant: migraine without aura; right upper quadrant: migraine with aura; square: male; circle: female. Genotyped persons are indicated by an asterix.

For the second study, the *Costa Rican migraine study*, we aimed to minimize even further clinical heterogeneity by using a well-validated, semi-structured questionnaire in the Spanish language. Special care was taken to apply stringent criteria to select clinically homogenous patients with early-onset, severe, migraine with aura that has a higher genetic load than migraine without aura of the first study. In this study we also minimized genetic heterogeneity by selecting migraine patients from the Central Valley of Costa Rica, a well-known genetic isolate. Only those patients were recruited that had at least six of their eight great-grandparents born in the Central Valley. Notably, detailed genealogical analysis for the first 50 MA patients revealed that the information provided by the patient on their great-grandparents was reliable and that family relationships between patients within the first four generations did not occur. For this study we included in total 170 trio's, of which 124 consisted of a patient and both parents, whereas 46 trio's consist of a patient, a sib and one parent. The majority of patients was female (83,5%). The mean age at onset was 13 years, the mean attack frequency was 4 attacks per month and the mean percentage of attacks with aura was 89%. We aimed at including patients with an early age of onset; only 10 patients were included with an age of onset older than 25.

Genome-wide scan analysis

Two-point linkage analyses of all markers for the *Dutch migraine family study* did not yield any chromosomal location with significant, or even suggestive, evidence for linkage when the families were analyzed separately (data not shown). Next, two-point linkage analyses were performed in all 7 families combined. Heterogeneity analysis, taking into account that not all families need to be linked to a single locus, was performed as well. None of the markers showed significant or even suggestive evidence for linkage. The highest LOD score of 1.64 was found for marker D4S2361 at 93 cM on chromosome 4q21, which was previously implicated in migraine. [16, 17] Two additional markers, D4S2367 (LOD = 1.13) at 78 cM and D4S1647 (LOD = 0.88) at 105 cM, in the area 4q13-28 on chromosome 4 also showed positive LOD score values. The positive results in chromosomal region 4q21 prompted us to perform fine-mapping of this region in a larger set of MO families. A set of 10 additional medium-sized MO families were selected and together with the seven initial families genotyped for eleven additional polymorphic repeat markers. Two-point parametric linkage analyses were performed for all families combined both under locus homogeneity and locus heterogeneity, as described above. Table 1 shows the two-point linkage analysis results for these chromosome 4 markers. The highest LOD score of 0.97 was again found for marker D4S2361 at 93 cM on chromosome 4q21. Three flanking markers centromeric to D4S2361 showed also positive LOD scores from 0.30 to 0.74.

Table 1: Two-point linkage results for chromosome 4q21 fine-mapping markers for all 17 families combined.

Chromosome	Marker	Position in cM[a]	Position in Mb	LOD score	θ max[b]	HLOD score	α[c]
4	D4S3042	83	77	0.30	0.20	0.30	1.00
4	D4S3243	88	81	0.56	0.14	0.63	0.53
4	D4S2932	90	83	0.74	0.10	0.77	0.58
4	D4S2361*	93	85	0.97	0.00	0.98	0.89
4	D4S2409	96	87	0.01	0.18	0.14	0.40
4	D4S2929	96	89	0.01	0.40	0.03	0.12
4	D4S3373	unknown	95	0.07	0.40	0.07	1.00
4	D4S1647*	104	97	0.18	0.36	0.18	1.00
4	D4S411	109	105	0.32	0.30	0.32	1.00
4	D4S3240	114	110	0.25	0.34	0.25	1.00
4	D4S2297	117	115	0.20	0.30	0.45	0.29

[a] Sex-averaged map position in cM (Kosambi) based on the Marshfield '98 marker map.

[b] The optimal recombination proportion θ for which the maximum LOD score was found under homogeneity.

[c] Estimated proportion of families adding to the HLOD score.

[*] Marker from initial genome-wide scan.

Because of recent successes in migraine using trait components (i.e. individual clinical symptoms of migraine) instead of end diagnosis as affection status, we reanalyzed our genome-wide scan of seven MO families. Based on the underlying hypothesis is that these traits are closer to the molecular background of the disorder than the clinical classification, which represents a consensus among clinicians, we hoped to find better evidence for linkage. Altogether nine individual traits were analysed independently as phenotypes in the linkage analysis: attack length, pulsation, unilaterality, aggravation by physical exercise, intensity of pain, photophobia, phonophobia, nausea and/or vomiting. Two-point linkage analysis for each trait for all families combined was performed both under locus homogeneity and locus heterogeneity. Table 2 shows that slightly higher LOD scores were obtained: the highest observed LOD score of 1.96 was found for the unilaterality trait on chromosome 6q23.3 (D6S1009, 26.7 cM, θ=0.08, HLOD=1.96, α=1.0).

Table 2: Highest LOD score for the various trait components in the combined dataset of all families analyzed under homogeneity and heterogeneity.

Trait	Chromosome	Marker	Position in cM[a]	LOD score	θ max[b]	HLOD score	α[c]
Attack length (4-72h)	13	D13S1807	47	1.56	0.16	1.62	0.69
Age of onset (<20 years)	1	D1S1679	171	1.42	0.18	1.42	1.00
Unilateral headache	6	D6S1009	138	1.96	0.08	1.96	1.00
Pulsating headache	13	D13S1807	47	1.39	0.20	1.39	1.00
Intensity moderate/severe	13	D13S1807	47	1.34	0.20	1.34	1.00
Aggravated by physical activity	1	D1S1728	109	1.22	0.22	1.22	1.00
Nausea and/or vomiting	2	D2S1780	10	1.33	0.18	1.33	1.00
Photophobia	12	D12S2070	125	1.36	0.18	1.36	1.00
Phonophobia	4	D4S2417	182	1.36	0.18	1.36	0.99

[a] Sex-averaged map position in cM (Kosambi) based on the Marshfield '98 marker map.

[b] The optimal recombination proportion θ for which the maximum LOD score was found under homogeneity.

[c] Estimated proportion of families adding to the HLOD score.

Despite our efforts to homogenize clinical heterogeneity in our Dutch migraine families, genetic heterogeneity most likely is extensive as the Netherlands are considered an outbred population.

In an attempt to control also genetic heterogeneity in linkage studies in migraine, we performed a gene identification study in the genetic isolate of Cost Rica. For the *Costa Rican migraine study*, a 5 cM genome-wide scan for 170 MA trios was performed. Family-based association analysis was performed for each marker with a likelihood ratio-based transmission disequilibrium test (TDT). Figure 2 shows a graphical representation of the TDT results for all markers per chromosome. Again, the linkage results were disappointing. None of the reported migraine loci was replicated. In total 29 markers showed individual significance levels of $p<0.05$, of which seven markers showed p-values <0.01, on chromosomes 1, 6, 9, 15 and 22. A more promising, marker D6S1009, located on chromosome 6q23.3, showed the lowest p-value of 0.0012. Interestingly, this marker also showed the highest LOD score in the trait analysis of the *Dutch migraine family study*. Fine-mapping of the chromosome 6q23.3 locus was performed by testing eight additional markers in the 10 Mb flanking region around marker D6S1009 in all 170 trios (data not shown). Although our initial finding was quite promising, none of the additional fine mapping markers yielded significant association. Therefore most likely, the significant p-value of D6S1009 in the initial analysis represents a false positive result.

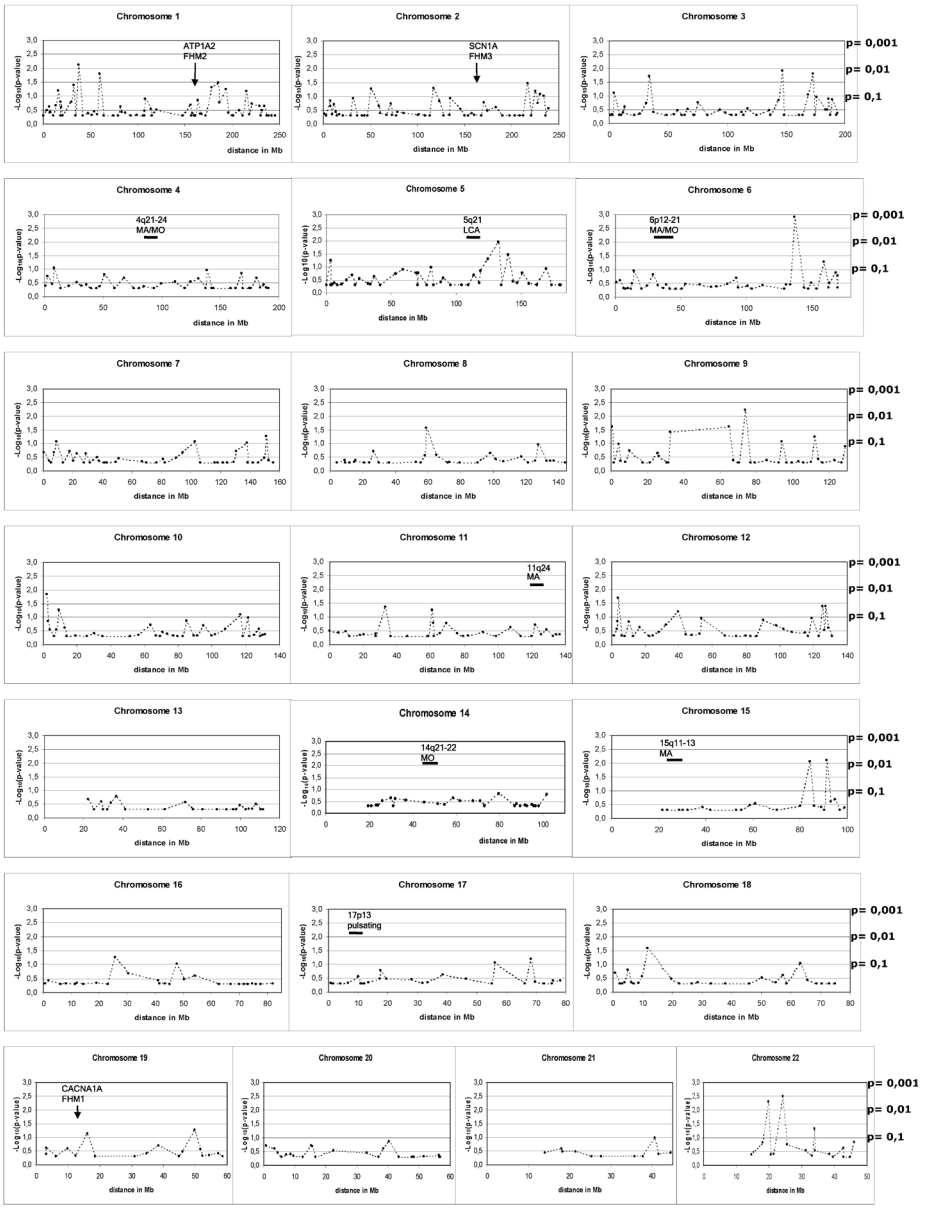

Figure 2: Graphical representation of the TDT results for all markers of the genome-wide scan per chromosome.

The –log10 (*p*-value) results on the y–axis are plotted against the physical distance of the markers (0= start of chromosome) on the x-axis. Arrows and bars indicate the position of reported migraine loci.

Discussion

Here, we report the results of two genome-wide scans to map susceptibility loci for common migraine, the first with an outbred linkage approach with MO families and the second with a family-based association approach with severe MA patients from a genetic isolate. The *Dutch migraine family study* did not yield any significant loci. Using the alternative approach of trait component analysis did not improve our results. The highest evidence for linkage was found on chromosome 4q21 that overlaps with the Finnish MA and Icelandic MO locus.16,17 In our study, marker D4S2361 (93 cM) showed the highest peak at 4q21 with a LOD score of 1.64 in the seven original families combined. Subsequent testing of additional markers and expanding the number of MO families to 17 only reduced possible evidence for linkage (0.98 for marker D4S2361). Although our results consistently hint towards the involvement of the known chromosome 4q21 locus in our MO families, there is not enough evidence to statistically prove this. Our inability to obtain better support for the chromosome 4 locus clearly demonstrates the intrinsic problem that classical linkage studies face in mapping disease loci and gene variants for complex diseases: because of genetic and clinical heterogeneity adding of additional families is not helpful, especially in outbred populations such as the Netherlands.

The *Costa Rican migraine study* aimed to reduce genetic heterogeneity and this strategy should make locus identification easier. In contrast to outbred populations, genetically isolated populations have a reduced genetic variability, due to a limited number of founders, genetic drift and the absence of migration. [32, 33] Also LD in young genetically isolated populations extends over much larger regions than in outbred populations. [34] Another advantage is that isolates generally have a more uniform environment and culture and often extensive genealogical records. Genetic isolates have been extremely helpful in the identification of monogenic disorders, especially of rare recessive diseases. Many genes for monogenic diseases have been identified in Finland. [35-38] Also the Central Valley of Costa has been successful for identification of disease loci/genes. [39-41] However, the high expectations that genetically isolated populations would also allow gene variant identification for complex diseases still have to be proven true. [32, 33] Also, in the present study in migraine in the Central Valley of Costa Rica no chromosomal regions were identified with significant, or even suggestive, evidence of association to migraine. Strongest evidence for possible association was found for a region on chromosome 6q23.3, but fine-mapping did not provide additional support for our initial association findings. Also for this study the initial linkage peaks most likely were false positive results.

What conclusions can be drawn from these attempts to identify migraine genes? Clearly both studies did not yield the positive results we anticipated from the estimated sufficient power of these studies. One possible contributing factor causing failure to identify major migraine loci could be the large gaps in the Marshfield STR marker set, especially at the telomeric regions. Therefore currently available high-density SNP arrays might be a better choice for genotyping and provide new prospects for LD-based and haplotype association studies. Another factor could be diagnostic problems and clinical heterogeneity. There are no biological or radiological markers to confirm a migraine diagnosis and misdiagnosed patients can have severe effects especially on linkage analysis studies. The largest causal factor for failing most likely is insufficient power due to an underestimated genetic heterogeneity of the disease. If anything our studies suggests that migraine is genetically a very heterogeneous disorder and that we should reconsider whether classical linkage studies are still worthwhile.

Performing linkage analysis in large multigenerational migraine families with a seemingly clear autosomal dominant inheritance pattern, even with 44 affected patients like were studied here in one family, is no guarantee for success. Even within a single family migraine is apparently very complex with multiple genetic factors involved and undesired phenocopies that hamper linkage analysis. If no such complexity would exist, our simulation studies indicated that we would have identified a gene locus in of our large migraine families. Why then were other studies successful and able to identify novel loci in single migraine families? Perhaps the researchers were fortunate and investigated rare examples of migraine families with a very clear diagnosis and lack of phenocopies. However, the consequence would be that high-penetrant gene mutations cause the disease in these peculiar families. In that respect, it is surprising that for none of these families the gene mutation has been identified. Migraine loci that were identified in studies analyzing multiple migraine families pose other problems. Certainly, genetic heterogeneity was a factor in these studies, which is also indicated by the fact that only modestly significant LOD scores were reported. The issue that most loci have not been replicated could be explained in part by the fact that different populations were used, with different genetic backgrounds. However, it could also be an indication that not all findings are true linkage signals.

The search for migraine susceptibility genes will remain challenging due to the genetic, as well as clinical complexity of the disease. Studies should therefore also aim to reduce clinical heterogeneity, by optimizing migraine diagnosis and its sub-classifications and develop new phenotyping strategies to stratify study samples into less heterogeneous groups. In line with this two new approaches to phenotyping for genetic purposes have recently been applied for migraine studies: latent class analysis (LCA) [18] and trait analysis [19]. How can we further learn from previous experience and improve gene identification strategies in migraine? Especially, recent breakthroughs in powerful and affordable high-throughput SNP genotyping technologies seem very promising for the identification of gene variants of complex diseases. Genome-wide association (GWA) studies in large case-controls cohorts yield gene variants for an ever increasing number of several complex disorders, including Crohn's disease, obesity, type 1 and type 2 diabetes, psoriasis, coronary heart disease, amyotrophic lateral sclerosis (ALS), restless legs syndrome (RLS) and prostate and breast cancer. [42-50] These studies yielded genes or regions previously unrecognized and in many cases genes with unknown function in the particular disease. Interestingly, a substantial number of significant associations was identified in regions without annotated genes, indicating that investigation of noncoding DNA with possible regulatory functions will be critical to unravel the role of these regions. Importantly, these studies showed that most individual SNPs that were identified had rather modest effects in the population, with odds ratios below 1.5. It is evident from these odds ratios that for complex disorders in order to be successful, in GWA studies, large numbers (>1000) of well-phenotyped patients and ethnically-matched controls are needed. It seems only logical that similar approaches should be applied to migraine. With respect to collection of patient material, this would require that efforts of clinical migraine researchers should be directed towards collecting multiple, very large groups of well-diagnosed patients and controls that are carefully selected to ensure ethnic homogeneity. Future advances in development of rapid and affordable SNP technologies, biostatistics, phenotyping strategies and the recognition of the importance of large sample sizes holds renewed promises for the identification of susceptibility genes for migraine. Identification of susceptibility genes and their pathways will contribute to a better understanding of the migraine pathophysiology and can ultimately lead to novel drug targets

and improved prophylactic treatment.

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Chapter 7

General Discussion

Migraine is a common episodic neurovascular headache disorder characterised by attacks of severe headache and autonomic and neurological symptoms. The etiology of migraine remains largely unknown. Identification and characterisation of susceptibility genes for migraine is an important step in understanding the pathophysiological mechanisms underlying this disease and might give novel leads for drug development. This thesis focuses on genetic studies ranging from a rare Mendelian form of migraine – Familial Hemiplegic Migraine (FHM) – to the complex forms of migraine – migraine with or without aura (MA and MO). FHM is considered a good model to study the genetics of migraine mainly because of its clinical similarities with the common forms of migraine. Until now three FHM genes have been identified, *CACNA1A*, *ATP1A2* and *SCN1A*; the latter two genes were identified in the course of the research for this thesis. Several chromosomal loci have been mapped by investigating patients with common forms of migraine, but no genes have been identified yet. Migraine susceptibility genes may also come from investigating migraine in patients with comorbid Mitochondrial Encephalopathy, Lactic Acidosis and Stroke-like episodes MELAS, Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leucoencephalopathy (CADASIL) and Retinal Vasculopathy with Cerebral Leukodystrophy (RVCL) and will be discussed below.

7.1 FHM mutations: genetic and clinical spectrum and their functional consequences

7.1.1 CACNA1A

Since the discovery in 1996 of the *CACNA1A* FHM1 gene, encoding the pore-forming α_{1A} subunit of P/Q-type voltage-gated neuronal calcium channels, 18 different FHM1 mutations have been reported. Figure 1 shows a schematic overview of all *CACNA1A* mutations associated with FHM. The FHM1 mutations are exclusively missense mutations and are predominantly aggregated in the transmembrane domains (S4-S6) and the S5-S6 linkers. The S4 domains act as voltage sensors, while the S5, S5-S6 linker and S6 segments are involved in ion conductance and selectivity. The most recurrent *CACNA1A* mutations are T666M and R583Q, both associated with hemiplegic migraine with cerebellar signs. Mutations S218L, R1347Q and I1710T have been found in a few families, whereas all other FHM1 mutations have only been identified in only one family.

Clinical heterogeneity

Particularly interesting is the clinical heterogeneity that is observed for *CACNA1A* mutations, even within a given family. [1, 2] In addition to hemiplegic migraine, mutation carriers can have cerebellar ataxia, epilepsy and coma. One example is the mutation I1710T, described in chapter 2, which causes FHM, cerebellar ataxia and childhood epilepsy. [3] In this family, epileptic seizures occurred independently from the FHM attacks, which had not been described before in FHM families. A *de novo* I1710T mutation was also identified in a 14-year-old girl with a severe phenotype of attacks of coma, hemiplegia and status epilepticus, again supporting that this mutation is associated with an epilepsy phenotype. [4]

Two other mutations have been associated with particularly severe phenotypes. Mutation Y1384C was identified *de novo* in a patient with prolonged attacks of migraine with hemiplegia, coma, seizures, mental retardation, permanent cerebellar ataxia with cerebellar atrophy, and right-sided brain atrophy. [5] Diffusion MRI showed reversible hemispheric oedema (swelling caused by an abnormal accumulation of fluid) and decrease of cerebral water mobility during a long-lasting attack of hemiplegic migraine associated with coma.

Similar hemispheric swelling was reported as part of the clinical features associated with the S218L mutation, which was identified by our group in two FHM families with delayed severe cerebral oedema and coma after a minor head trauma.[6] In one patient the attack after a minor head trauma caused uncontrollable cerebral oedema and was even fatal. Neuropathological examination of the patient's brain showed Purkinje cell loss in the cerebellar cortex. There was marked swelling and deformity of the dendrites of many of the remaining Purkinje cells. Thus, FHM1 mutations can lead to very severe phenotypes.

More than half of the FHM1 mutations are associated with ataxia, a motor-coordination disorder. So far, it is unknown why certain mutations are associated with ataxia and others are not. There is no clear correlation with location or amino acid alterations. Therefore the pathogenic mechanism must be much more complex and, perhaps even, specific for each mutation. The most frequent FHM1 mutation associated with ataxia, T666M, was reported in 22 families. Kors and colleagues compared the detailed clinical features of five T666M families.[2] There was a remarkable heterogeneity between T666M families. In one family, some affected members with the mutation had attacks with confusion but without hemiparesis. In another family, patients had also progressive cognitive dysfunction. Interestingly, although all other reported T666M families had chronic cerebellar ataxia, one of these families displayed pure FHM without ataxia. It adds to the consensus that a straightforward genotype-phenotype relation for *CACNA1A* mutations does not exist.

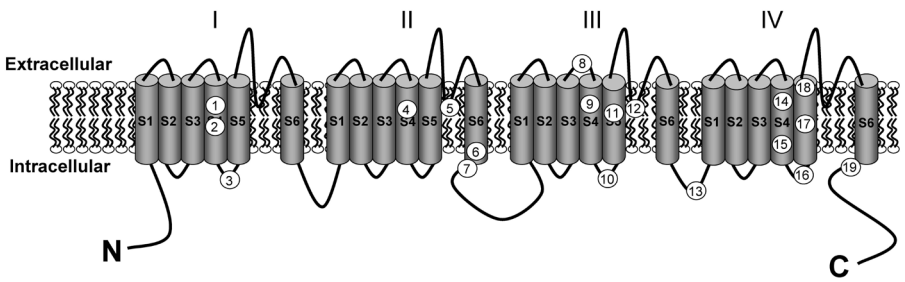

Figure 1: Schematic representation of the α1A subunit of the P/Q-type Ca^{2+} channel, encoded by the *CACNA1A* gene.

Each of four domains (I-IV) has six transmembrane segments (S1-S6). The numbered circles indicate FHM1 mutations, shown in Table 1.

Functional consequences

The functional consequences of 11 FHM1 mutations have been studied in heterologous expression systems (*Xenopus* oocytes, mammalian cell lines, or cultured neurons) by transiently expressing recombinant $Ca_v2.1$ channels (Table 1).[7-12]

$Ca_v2.1$ channels are opened by voltage changes at the plasma membrane. Using electrophysiological methods, the range of parameters of channel opening, closing and reopening can be studied of wildtype and mutant $Ca_v2.1$ channels that are endogenous to the cell or that are expressed after transfection. Studies in heterologous expression systems have shown that the FHM1 mutations alter biophysical properties of human $Ca_v2.1$ channels in a complex way (Table 1). Effects were different depending on the mutation, but also on the expression system used and whether single-channel or whole-cell measurements were performed. An important consistent gain-of-function effect for all FHM1 mutations was an enhanced single-channel Ca^{2+} influx over a broad voltage range, reflecting an increased

channel open probability, mainly due to a shift to lower voltages of channel activation. However in transfected cells, there were conflicting results in whole-cell current density because the FHM1 mutation produced apparent alterations in the density of functional channels in the membranes. For instance, the density of functional R192Q mutant $Ca_v2.1$ channels in transfected neurons is decreased, but increased in transfected HEK293 cells. [8, 10] A much more reliable manner to study the exact functional consequences of gene mutations are knock-in mouse models carrying human pathogenic mutations. Our group generated knock-in (KI) mouse models, carrying either the human FHM1 R192Q mutation [13] or the S218L mutation (van den Maagdenberg, van de Ven, personal communication). Unlike the natural *Cacna1a* mutant mouse models, that show severe phenotypes of ataxia and/or epilepsy, transgenic KI R192Q mice exhibit no evident clinical phenotype or overt structural abnormalities. This seems more in line with R192Q patients that, except for attacks of hemiplegia, otherwise appear without easily noticeable clinical symptoms. In patients the R192Q mutation causes FHM attacks and attacks without hemiparesis, but no epilepsy or ataxia. Interestingly, extensive functional analysis revealed multiple gain-of-function effects as a consequence to a lower threshold of channel activation and increased channel open probability, resulting in a lower threshold for CSD induction and an increased velocity of propagation in the R192Q KI mice. [13] These findings support the concept that CSD plays an important role in the pathogenesis of FHM and probably common forms of migraine, especially MA. *Cacna1a* knockout mice that do not express $Ca_v2.1$ calcium channels have been generated and show a severe phenotype of epilepsy and ataxia that is lethal. [14-16] Therefore, knockout mice seem less suitable to investigate migraine-related mechanisms as the knock-in mice. [17]

Other diseases associated with CACNA1A

CACNA1A mutations also cause episodic ataxia type 2 (EA-2) and spinocerebellar ataxia type 6 (SCA-6). EA-2 is characterized by recurrent episodes of severe truncal and gait ataxia (unsteadiness and limb incoordination) as well as dysarthria lasting typically from 15 minutes to a few hours. [33] Attacks are often associated with vertigo, nausea, and migraine headaches. Interictal findings include mainly gaze-evoked nystagmus and mild permanent cerebellar ataxia although in some patients ataxia can also be slowly progressive. [33] Over 50% of reported EA-2 mutations are nonsense or splice-site *CACNA1A* mutations that predict truncated proteins; the remaining proportion is missense mutations. [33]

In contrast to FHM1 mutations, electrophysiological studies have shown that most EA-2 mutations cause a complete or marked loss-of-function of the $Ca_v2.1$ channel. [34-39] Both missense and truncating EA-2 mutations cause loss-of-function due to either decreased single channel conductance or to reduced current density or both, depending on the mutation. Interestingly, EA-2 mutations have been reported to cause abnormal channel trafficking in addition to altered channel kinetics, [39] but also act as dominant-negative mutations. [40, 41] It is still not clear whether *in vivo* (i.e. in neurons) haploinsufficiency or dominant-negative effects cause EA-2 symptoms.

SCA-6, resulting in late-onset cerebellar ataxia, is characterized by atrophy of cerebellar Purkinje cells. SCA-6 is a polyglutamine disorder caused by small expansions of the CAG repeat located in the 3'-end of the *CACNA1A* gene. [42, 43] An interesting, but unresolved, issue is whether SCA-6 is caused by dysfunction of $Ca_V2.1$ Ca^{2+} channels or by toxic gain-of-function effects of the extended glutamine expansions, as is suggested for other glutamine-

repeat disorders. Electrophysiological evidence supported former hypothesis by showing that the polyglutamine expansion interferes with the Ca^{2+} channel and reduces Ca^{2+} influx. [44, 45]

Table 1: *CACNA1A* FHM1 mutations and their respective position and reported functional consequences.

Mutation	Phenotype (reference)	Position	Functional consequences (reference)
1. R192Q	FHM (18)	I S4	a) No detectable functional changes (7) b) Increased current density and open probability (8); increased open probability (10) c) Decreased maximal Cav2.1 current density (10) d) Increased current density in cerebellar neurons; enhanced neurotransmission at neuromuscular junction, reduced threshold and increased velocity of CSD (13)
2. R195K	FHM (1)	I S4	
3. S218L	FHM + delayed (fatal) cerebral edema and coma (3, 19)	I S4-S5	b) Increased open probability ; increased whole cell current density (12) c) Increased open probability ; increased Cav2.1 current density (12)
4. R583Q	FHM + ataxia (1, 20, 21, 22, 23)	II S4	a) Slowed recovery from inactivation; shifted voltage range of activation to more negative voltages (9)
5. T666M	FHM + ataxia (1, 2, 18, 21, 23, 24-29)	II S5-S6	a) Slowed recovery from channel inactivation (7) b) Decreased density of functional channels and their unitary conductance (8); increased single-channel Ca2+ influx (10) c) Increased single-channel Ca2+ influx and decreased maximal current density (10)
6. V714A	FHM (18)	II S6	a) Accelerated recovery from channel inactivation (7) b) Increased open probability and rate of recovery from inactivation and decreased density of functional channels (8); increased single-channel Ca2+ influx (10) c) Increased single-channel Ca2+ influx and decreased maximal current density (10)
7. D715E	FHM + ataxia (24)	II-III loop	a) Shifted voltage range of activation to more negative voltages; faster current inactivation (9)
8. K1335E	FHM (1)	III S3-S4	a) Shifted voltage range of activation to more negative voltages; shifted voltage range of inactivation to more negative voltages, expressed with β3; accelerated inactivation; delayed recovery from inactivation (11)
9. R1347Q	FHM + ataxia (29, 30)	III S4	
10. C1369Y	FHM (23)	III S4-S5	
11. Y1384C	HM + coma/cerebellar atrophy (5)	III S5	
12. V1457L	FHM (31)	III S5-S6	a) Shifted voltage range of activation to more negative voltages (9) b) Increased channel open probability and deceased conductance and density of functional channels (10) c) Increased single-channel Ca2+ influx and decreased maximal current density (10)
13. C1534S	FHM + ataxia (29)	III-IV loop	
14. R1667W	FHM + ataxia (1)	IV S4	
15. L1682P	FHM + ataxia (32)	IV S4	
16. W1683R	FHM + ataxia (1)	IV S4-S5	a) Shifted voltage range of activation to more negative voltages; shifted voltage range of inactivation to more negative voltages; delayed recovery from inactivation (11)
17. V1695I	FHM (1)	IV S5	a) Slowed inactivation, expressed with β1 ; delayed recovery from inactivation (11)
18. I1710T	FHM + epilepsie/ataxia (3, 4)	IV S5	
19. I1811L	FHM + ataxia (18)	C-terminus	a) Accelerated recovery from channel inactivation (7) b) Increased open probability and rate of recovery from inactivation and decreased density of functional channels (8); increased single-channel Ca2+ influx (10) c) Increased single-channel Ca2+ influx and decreased maximal current density in neurons (10)

Functional consequences investigated using: a) *Xenopus laevis* oocytes, b) HEK cells, c) Granule cell neurons isolated from $Ca_v2.1$ knockout mice, d) R192Q KI mice

7.1.2 ATP1A2

In 2001, the second FHM gene was identified, *ATP1A2*, which encodes the α_2 subunit of sodium-potassium ATPases. Figure 2 and table 1 show the 39 FHM2 mutations, their respective position and functional consequences. Almost all FHM2 mutations are missense mutations and the majority is located in the large intracellular loop, which harbours important regulatory domains for ion transport, e.g. ATP-binding and phosphorylation sites. Three truncating mutations and one mutation that elongate the wildtype protein by 27 amino acids (X1021R) have been reported. Most *ATP1A2* mutations have been identified in single families; only three recurrent mutations (T376M, A606T and R763H) have been found occurring twice or three times in different families.

Clinical heterogeneity

In clear contrast to *CACNA1A* were ataxia is a common feature, only the G301R *ATP1A2* mutation has been associated with cerebellar signs. Interestingly, this mutation is one of the few located in a transmembrane domain. Future genotype-phenotype studies will have to show whether or not ataxia is observed only for transmembrane domain mutations. Like with *CACNA1A*, a large proportion of *ATP1A2* mutations is associated with additional clinical features such as epilepsy, prolonged hemiplegia, mental retardation and coma (Table 2). In chapter 3.1, we describe an *ATP1A2* mutation that was found in a Dutch-Canadian family in which FHM and benign familial infantile convulsions (BFIC) segregate. In this family all available affected family members with FHM, BFIC or both, carry the R689Q *ATP1A2* mutation. Interestingly, mouse studies have shown that *Atp1a2* is differentially expressed during life: in neurons during late gestation and the early neo-natal period and primarily in astrocytes in the adult stage. This may provide an attractive explanation for the different clinical effects in the FHM/BFIC family. Subsequently the role of *ATP1A2* in pure BFIC was investigated in two large sets of BFIC families, but no mutations were found. [46, 47] Therefore it seems that *ATP1A2* is not a major gene for pure BFIC without associated features like hemiplegic migraine. In knockin mice, one would be able to assess whether the R689Q mutation is specifically associated with early childhood epilepsy. Nevertheless, our results show that other genes in the same pathway as *ATP1A2* would be good candidates for BFIC. Until now, no other genes for BFIC have been identified. As mutations were identified in in the potassium channel genes *KCNQ2* and *KCNQ3* in the related disorder benign familial neonatal convulsions (BFNC), [48, 49] ion-dysfunction seems a likely mechanism for BFIC.

Inspection of the clinical penetrance in FHM families with *ATP1A2* and *CACNA1A* mutations suggests that the penetrance is lower for *ATP1A2* mutations. [23, 50] Our findings show that *ATP1A2* mutations are also more prevalent in sporadic hemiplegic migraine patients (SHM) (chapter 5). An interesting hypothesis is that there is more functional redundancy for α-subunits of sodium-potassium pumps than for α1 subunits of $Ca_v2.1$ calcium channels. This hypothesis seems supported by our results described in chapter 3.4 that show that having functional defects in both *ATP1A2* alleles is not a lethal condition and can apparently be compensated by back-up mechanisms.

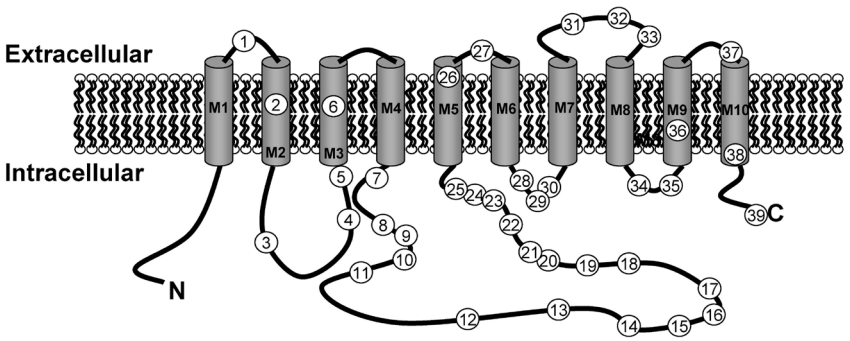

Figure 2: Schematic representation of the α_2 subunit of the Na, K-ATPase encoded by the *ATP1A2* gene. The sodium-potassium pump has ten transmembrane domains (M1-M10). The numbered circles indicate FHM2 mutations, also shown in table 2.

Functional studies of FHM2 ATP1A2 mutations

For several *ATP1A2* mutations either cell survival assays or more detailed studies into mutant Na,K-ATPase kinetics have been performed (Table 2). Na,K-ATPase activity is necessary for cell survival. In cell survival studies, the endogenous Na,K-ATPase activity is inactivated by application of ouabain to the culture medium of mammalian cells that express either wildtype or mutant *ATP1A2* cDNAs that were made insensitive to ouabain by mutagenesis of specific amino acids. Wildtype ouabain-insensitive *ATP1A2* gene product can rescue cell survival, but when mutant ATP1A2 protein lacks that ability, this is prove of diminished Na,K-ATPase function. Cell survival assays are a fast and useful tool to prove altered Na,K-ATPase functioning, and thus causality of the mutation in disease, but detailed kinetic studies that investigate Na,K-ATPase activity, catalytic turnover and ion-affinities, are required to investigate the exact consequences of *ATP1A2* mutations. Therefore, if no effect of the mutant on cell survival is obtained, this does not exclude that the mutant protein has other defects in pump functioning. For instance, the Finnish T345A mutation did not show any effect on cell survival, but a detailed kinetic study showed a substantial decrease in affinity for K^+. [67] Also, mutations R689Q and M731T showed almost no effect in survival assays, but showed a reduced catalytic turnover and increased apparent affinity for extracellular K^+. [68] For other *ATP1A2* mutations the survival assay better revealed the functional consequences, as kinetic studies for the L746P and W887R mutations exhibited total inhibition of pump activity. [67, 70]

There are no *Atp1a2* knockin models available yet, but α2-subunit deficient (*Atp1a2*-null) mice have been generated.[71-74] These mice die immediately after birth because of severe motor deficits and absent respiration. *Atp1a2*-null fetuses of 18.5 days revealed selective neuronal apoptosis in the amygdala and piriform cortex in response to neural hyperactivity.[72] *Atp1a2*-null mice on 129sv genetic background displayed frequent generalized seizures and died within 24 hrs after birth.[74] Epilepsy is also a feature of the clinical phenotype in humans with *ATP1A2* mutations. *Atp1a2* knockin models will certainly be usefull to further study the functional consequences of FHM2 mutations and provide more insight into the FHM pathogenesis. The most informative mutations for generating this *Atp1a2* knockin model will be mutations T345A, R689Q or M731T for which other effects than loss-of-function effects have been shown.

Table 2: *ATP1A2* FHM2 mutations and their respective position and reported functional consequences.

Mutation	Phenotype (reference)	Position	Functional consequences (reference)
1. E120A	SHM (51)	M1-M2	a) Partial effect on cell survival (51)
2. V138A	FHM (23)	M2	
3. R202Q	FHM (23)	M2-M3	
4. T263M	FHM (52)	M2-M3	
5. I286T	FHM (53)	M2-M3	a) Partial effect on cell survival (53)
6. G301R	FHM+ seizures/prolonged coma/cerebellar signs (54)	M4-M5	
7. T345A	FHM (55)	M4-M5	a) Normal cell survival; decreased K^+ affinity in kinetic studies (67)
8. T376M	FHM (52)	M4-M5	
9. T378N	FHM/AHC (alternating hemiplegia of childhood) (56, 57)	M4-M5	a) No cell survival (57)
10. R383H	SHM (58)	M4-M5	
11. T415M	FHM (53)	M4-M5	a) No cell survival (53)
12. E492K	SHM (51)	M4-M5	a) Partial effect on cell survival (51)
13. R548H	BM (basilar migraine) (59)	M4-M5	
14. R593W	FHM (60)	M4-M5	a) Partial effect on cell survival (60)
15. A606T	FHM; HM + prolonged hemiplegia episodes (52, 61)	M4-M5	
16. G615R	FHM + mental retardation (62)	M4-M5	a) No cell survival (62)
17. V628M	FHM (60)	M4-M5	a) Partial effect on cell survival (60)
18. R689Q	FHM + BIFC (benign familial infantile convulsions) (63)	M4-M5	a) Partial effect on cell survival (67); reduced catalytic turnover and increased apparent affinity for extracellular K^+ (68) b) Strong decrease of functional expression of Na, K pumps (69)
19. E700K	FHM (64)	M4-M5	
20. N717K	HM + prolonged hemiplegia episodes (61)	M4-M5	
21. D718N	FHM + seizures/mental retardation (58)	M4-M5	
22. M731T	FHM (63)	M4-M5	a) Normal cell survival (67); reduced catalytic turnover and increased apparent affinity for ATP and extracellular K^+ (68) b) Strong decrease of functional expression of Na, K pumps (69)
23. R763H	FHM (58, 65)	M4-M5	
24. R763C	FHM (23)	M4-M5	
25. L764P	FHM (66)	M4-M5	a) No cell survival (66, 67) b) Strong decrease of functional expression of Na, K pumps (69); complete loss of catalytic function (70)
26. P786L	SHM (51)	M5	a) No cell survival (51)
27. P796R	FHM (58)	M5-M6	
28. M829R	FHM (52)	M6-M7	
29. R834X	FHM (51)	M6-M7	a) No cell survival (51)
30. R834Q	FHM (52)	M6-M7	
31. W887R	FHM (66)	M7-M8	a) No cell survival (66, 67) b) Strong decrease of functional expression of Na, K pumps (69); complete loss of catalytic function (70)
32. E902K	FHM + coma (58)	M7-M8	
33. R908Q	SHM (51)	M7-M8	a) No cell survival (51)
34. Del 935K-940S, ins I	FHM (52)	M8-M9	
35. R937P	FHM (52)	M8-M9	
36. Del nt2897-2898 >FS	FHM (52)	M9	
37. P979L	FHM + coma/seizures/mental retardation (58)	M9-M10	
38. R1002Q	HM + prolonged hemiplegia episodes (61)	M10	
39. X1021R	FHM (58)	C-terminus	

Functional consequences investigated using: a) HeLa cells b) *Xenopus laevis* oocytes

7.1.3 SCN1A

Recently, missense mutation Q1489K was identified in the *SCN1A* in three German FHM families of common ancestry, designating this gene as FHM3. [75] In chapter 4 we describe the first confirmation that *SCN1A* indeed is a FHM gene. Figure 3 shows the location of both FHM3 mutations. Mutation L1649Q was found in a family with pure FHM. [76] Three patients in the German FHM3 families, in addition to hemiplegic migraine also had epileptic seizures during infancy. Both FHM3 *SCN1A* mutations are highly penetrant. Electrophysiological studies were performed with these mutations that were introduced in highly homologous human SCN5A. They revealed a depolarizing shift in the voltage dependence of steady state inactivation, an overall slower inactivation and an accelerated recovery from fast inactivation for the L1649Q mutation. The Q1489K mutation only exhibited accelerated recovery from fast inactivation. Both effects severely interfere with the fast inactivation process and are predicted to enhance neuronal excitation and release of neurotransmitters.

Intriguing is the fact that over 200 mutations in *SCN1A* had been associated with either severe myoclonic epilepsy of infancy (SMEI) or generalized epilepsy with febrile seizures (GEFS+). [77, 78] The mutation spectrum in SMEI differs from that in GEFS+ as the majority of SMEI mutations occurred *de novo*. Approximately half of the SMEI mutations are nonsense or frameshift mutations resulting in protein truncation and consequent loss-of-function. The remaining SMEI mutations are missense mutations, with functional consequences that range from complete loss-of-function, gain-of-function to minimal functional effects. [79] The milder GEFS+ phenotype is associated with missense mutations only, showing either loss- or gain-of-function effects. [80, 81]

It is at present unclear why certain *SCN1A* mutations cause FHM and no epilepsy. Both FHM3 mutations are located in the vicinity of epilepsy mutations. Interestingly, functional consequences of two epilepsy mutations affecting amino acid 1648, which is adjacent to L1649Q have also been studied in different expression systems and revealed that they too affect channel inactivation. [82-85] Clearly, no simple correlation between the clinical phenotype and the biophysical consequences of *SCN1A* mutations exist. Direct, detailed comparison of the various mutations in functional studies might shed light to this important question. In addition, comparison of knockin mice with either epilepsy or FHM *SCN1A* mutations is needed. Recently, a knock-out (*Scn1a*-null) and a knock-in (introducing SMEI loss-of-function nonsense mutation R1407X into the *Scn1a* gene) *SCN1A* mouse model were generated. [86, 87] Homozygous *Scn1a*-null mice developed ataxia and die on postnatal day (P)15. Heterozygous knock-out mice have spontaneous seizures and sporadic deaths beginning after P21, with a notable dependence on genetic background. It was concluded that a dramatic loss of sodium current in hippocampal GABAergic inhibitory interneurons in these mice most likely explained the epileptic phenotype. Both homozygous and heterozygous knock-in mice developed epileptic seizures within the first postnatal month. Apparently, $Na_v1.1$ channels play critical roles in the spike output from parvalbumin-positive (PV) interneurons and, furthermore. It was suggested that the specifically altered function of the inhibitory circuits may contribute to epileptic seizures in the mice. Both transgenic models may be very helpful to address the question how haploinsufficiency of $Na_v1.1$ sodium channels causes epilepsy.

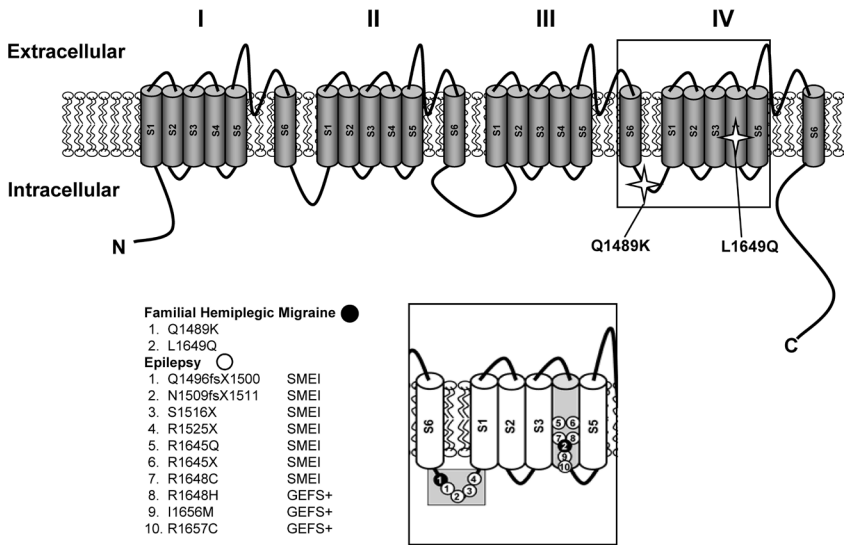

Figure 3: Schematic representation of the α1 subunit of voltage-gated $Na_V1.1$ sodium channels encoded by the *SCN1A* gene.

Each of four domains (I-IV) has six transmembrane segments (S1-S6). The stars indicate both FHM3 mutations. The SMEI and GEFS+ mutations located in the regions where the FHM3 mutations were identified (in grey) are depicted in the lower box.

7.2 Is there a common pathway for all three FHM genes?

Functional consequences of mutations in all three FHM genes fit well in a common pathway of a hyperexitable neuronal system with a lower treshold for cortical spreading depression (CSD). [88] CSD is a slowly propagating (3-5 mm/min) wave of sustained strong neuronal depolarization that spreads across the cortex and is followed by prolonged nerve cell depression.[89] Figure 4 shows the functional roles of the proteins coded by the FHM genes within a glutamatergic synapse. Mutations in the FHM1 calcium channel gene cause increased neuronal release of neurotransmitters, and in cortical neurons, the neuroexcitatory amino acid glutamate (Pietrobon D, et al. *unpublished data*) that can induce, maintain, and propagate CSD. Mutations in the FHM2 sodium potassium pump gene predict a reduced re-uptake of K^+ and, as a consequence, glutamate from the synaptic cleft into the glia cell. Mutations in the FHM3 sodium channel gene may also result in hyperexcitability and most likely increased release of neurotransmitters at the synaptic cleft. The overall predicted result for all three mutated FHM genes is increased levels of glutamate and K^+ in the synaptic cleft resulting in an increased propensity for CSD. This would easily explain the increased susceptibility of FHM (and likely MA) patients to get aura's. More controversial, however, is whether the enhanced tendency for CSD might also be responsible for triggering the headache phase, e.g. by activation of the trigeminovascular system as was suggested in animal studies. [90]

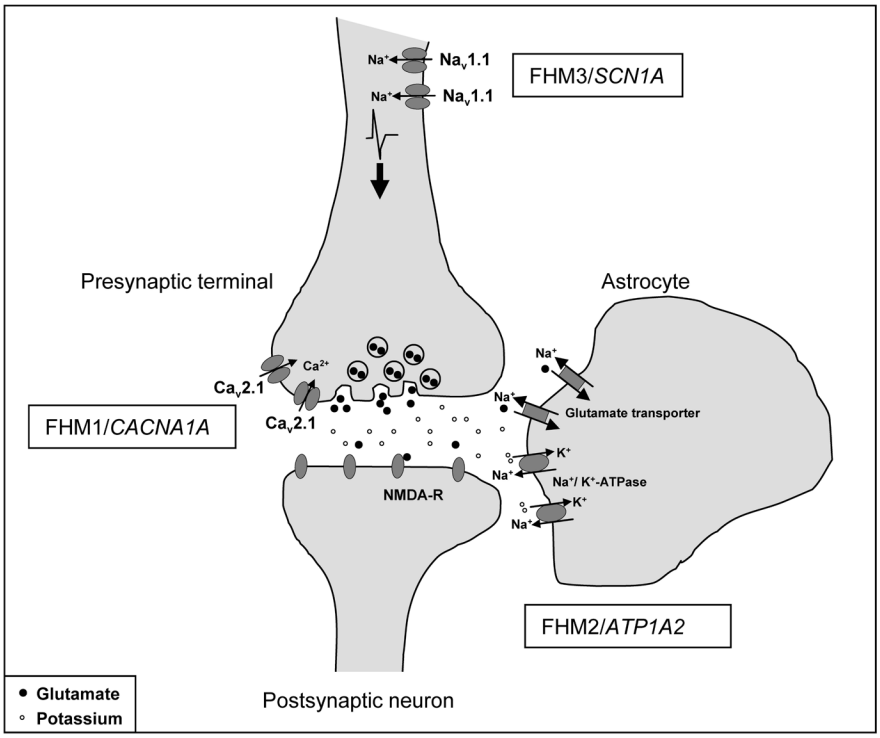

Figure 4: Functional roles of the proteins encoded by the three FHM genes within a glutamatergic synapse.

$Na_v1.1$ channels (FHM3/*SCN1A*) are essential for the generation and propagation of action potentials. After depolarization of the plasma membrane at the nerve terminal, calcium enters the neuron through $Ca_v2.1$ channels gating calcium ions (FHM1/*CACNA1A*) resulting in subsequent release of glutamate into the synaptic cleft. Glutamate can bind and activate postsynaptic NMDA receptors. Synaptic activity is terminated, in part, by astrocytic uptake of glutamate via glutamate transporters driven by sodium gradients, which are maintained by activity of Na,K-ATPase (FHM2/*ATP1A2*).

7.3 What is the role of FHM genes and pathways in SHM and common migraine?

An interesting question remains whether the FHM genes also play a role in sporadic and common forms of migraine. In chapter 5 we investigated the role of all three FHM genes in the sporadic hemiplegic migraine and concluded that mutations in FHM genes are involved in a small, but relevant, proportion (~15%) of SHM patients. Mutations in *ATP1A2* (E120A, E492K, P786L, R834X, R908Q) were more prevalent than in *CACNA1A* (R583Q). No mutations were identified in the *SCN1A* gene. These results clearly indicate that other genetic, and non-genetic factors must be involved in SHM. Part of the SHM patients may even have a unique aggregation of specific genetic factors, each with a relatively small susceptibility to migraine. The observation that SHM patients often have many relatives with the common forms of migraine seems in agreement with this concept. This strengthens the idea that hemiplegic migraine and common forms of migraine share similar pathophysiological

mechanisms. Many studies tried to asses the role of FHM1 and FHM2 genes and loci in the common forms of migraine (Table 3).[91-108] Studies using linkage or association analyses assessed the involvement of the FHM1 and FHM2 loci in migraine, while several mutation analysis studies investigated whether FHM1 or FHM2 mutations can be identified in patients with common migraine. Linkage and association studies gave contradictory results, especially for *CACNA1A*, and only one mutation screening identified two possible *ATP1A2* mutations in a large set of patients. Unfortunately, many of these studies were underpowered and were therefore less meaningful. Clearly, larger well-designed studies are needed to accurately investigate the role of these genes in common migraine. Such studies may reveal that the FHM1 and FHM2 genes are not major susceptibility genes for common migraine. Detailed studies investigating the role of the FHM3 *SCN1A* gene in migraine still have to be performed. Notably, our genome-wide scans of migraine families and trios, described in chapter 6 did not yield any support for the involvement of any of the known FHM loci in migraine with or without aura. Although the FHM genes may after all not play a major role in common migraine, their identification suggested that the pathway of ion dysfunction is important in common migraine. This is for instance illustrated by the fact that drugs that counteract disturbed ion and neurotransmitter levels in migraine patients (and similarly in patients with epilepsy) are efficacious. Research on mutant FHM genes pointed not only towards an increased susceptibilty for CSD in MA, but may also help answering the question whether migraine with and without aura share underlying pathophysiological mechanisms. Recent animal studies have shown that drugs belonging to a wide range of pharmacological classes (e.g. anti-epiletics and blockers of calcium channels and serotinergic, beta-adrenergic, and histaminergic receptors) with migraine prophylactic activity in both MA and MO patients, all share anti-CSD activity.[109] CSD inhibition may thus be a promising model system for the development of preventive medicines.

Table 3: Involvement of FHM loci/genes in various migraine types

FHM locus	Phenotype	Study design	Results	No. of families or cases	Reference
19p13	MA+MO	Linkage analysis with micro-satellite markers	Negative	4 families	91
			LOD: 1.38	28 families	92
			LOD=1.41; λ_{ab}=1.56 (MA); LOD=1.22; λ_{ab}=1.22 (MA+MO)	36 families (189 affected sibs)	93
			LOD=1.92	4 families	94
		Linkage analysis + case-control association study with micro-satellite markers	Negative; no association	82 families; 177 cases and 182 controls	95
		Case-control association study with intragenic SNPs in *INSR* and *CACNA1A* genes	INSR: p=0.002-0.008; CACNA1A: p=0.03	827 cases and 765 controls	96
	MA	Linkage analysis with micro-satellite markers	LOD=4.97 (D19S592 10cM telomeric to *CACNA1A)*	16 families	97
		Linkage analysis + TDT with micro-satellite markers	Negative	64 families	98
		Linkage analysis with micro-satellite markers	LOD=0.16	72 families	99
CACNA1A	MA+MO	Mutation screen all exons	No mutations	1 (identical to reference 94)	95
		Mutation screen exon 4, 13, 16, 17, 27, 36	No mutations	143 cases	100
	MA+MO with vertigo	Mutation screen all exons	No mutations	9 cases	101
	MA	Mutation screen exon 4, 17, 36	No mutations	15 cases	102
		Mutation screen 22 exons	No mutations	12 cases	103
CACNA1A/ATP1A2	MA+MO+BM	Mutation screen all exons	No mutations	18 MA, 25 MO, 7 BM cases	104
19p13/1q23/ *CACNA1A/ATP1A2*	MA	Linkage analysis with micro-satellite markers + mutation screen all exons	No linkage; no mutations	34 families	105
1q23/*ATP1A2*	MA	Linkage analysis with micro-satellite markers + mutation screen all exons	Linkage; no mutations	21 families	106
ATP1A2	MA	Mutation screen all exons	C515Y and E174K mutations	45 probands	107
		Mutation screen all exons + case-control single marker and haplotype-based association study with *ATP1A2* SNPs	No mutations; no association	45 probands; 284 cases and 241 controls	108
	MA+BM	Mutation screen all exons	R548H mutation	1 family	59

MA: migraine with aura, MO: migraine without aura, BM: basilar-type migraine.

7.4 Genetic heterogeneity of migraine and strategies to facilitate gene mapping

The identification of genes underlying common migraine proved particularly challenging and still is in its early stages. Already in FHM, there is extensive genetic heterogeneity with so far three genes identified and many, often smaller, families without the causal gene identified. It is tempting to speculate that, at least in part, FHM in seemingly smaller families might be caused by hemiplegic migraine genes with a lower disease penetrance, thereby reducing the number of HM patients in a family and hampering genome-wide linkage mapping.

Logically, common migraine is even more heterogeneous than FHM, which makes gene identification in the common forms a difficult task. This thesis describes two genome-wide scans that were performed to map migraine genes, but did not yield novel migraine loci or genes (chapter 6). Eight published genome-wide scans yielded several loci with significant evidence for linkage to migraine with or without aura. [110-117] Only the locus on chromosome 4q21-24 has been replicated. Interestingly, replication was performed in Icelandic MO patients, whereas initial linkage was obtained in a large set of Finnish MA samples.

In addition to these linkage studies, many case-control association studies using a classic candidate gene approach have been reported. [118] Migraine has been mostly associated with DNA variants in genes involved in neurotransmitter function, vascular function, or hormone function. Most of the association studies are single findings awaiting confirmatory replication. Only for the *MTHFR* gene, encoding the 5, 10-methylenetetrahydrofolate reductase, associations were replicated in several populations, including in a Dutch population-based study, [119] although it has to be mentioned that a recent, well-powered, Finnish study could not replicate this finding. [120] Still, a meta-analysis of all published studies investigating the association between polymorphisms in the *MTHFR* gene and migraine showed that for migraine with aura there was a significant association. [121] Replication is vital to show that associations are not mere false-positive results caused by for instance biases in study design, subpopulation structure, multiple testing issues, and/or lack of power.

Lack of consistent findings in identifying migraine susceptibility loci is probably an indication of high clinical and genetic heterogeneity in migraine; a common feature of complex genetic disorders. One approach to decrease genetic complexity is the use of heritable endophenotypes or quantitative traits, like plasma lipid concentrations, glucose levels, blood pressure or cognitive traits. [122] The underlying hypothesis is that these endophenotypes are closer to the molecular background of the disorder than the clinical classification, which represents a consensus among clinicians. Although there is some debate about its usefulness, this approach led to the mapping of loci for several complex diseases, like asthma, hypertension, schizophrenia and autism. [123-126] Using individual lipid traits, familial combined hyperlipidaemia (FCHL) was linked to chromosome 1q21 and subsequently the upstream transcription factor 1 (*USF1*) gene was successfully identified as the underlying gene for FCHL. [127]

Most migraine studies have used the diagnostic criteria of the International Headache Society (IHS), which might be an oversimplified way of diagnosis and might lead to too heterogeneous sets of patients for genetic analysis. Two endophenotyping strategies have recently been applied for migraine to reduce heterogeneity and improve power to detect true linkage signals. These new phenotyping approaches of migraine cohorts should be considered as research tools for geneticists, rather than novel diagnostic approaches. *Latent*

class analysis (LCA) is an empirical approach that produces classes of patients on the basis of their patterns of symptoms formed according to heritability estimates. This approach was used in two Australian studies of twins or families, and provided new loci on chromosome 5q21 and 18p11 by using a subtype of patients with a severe migraine phenotype named 'LCA-severe'. [115, 116] The second strategy is *trait component analysis*, which uses individual traits instead of the traditional end diagnosis. Trait component analysis was performed in a study with Finnish MA families and was also used in chapter 6 of this thesis. The individual traits of the IHS criteria, like age at onset, attack frequency, photophobia and others, were used as subphenotypes in linkage analysis. The Finnish study found linkage with several traits; the most significant one for pulsation on chromosome 17p13. [117] Therefore endophenotyping strategies can be useful for stratifying a heterogeneous set of patients and subsequently detecting novel migraine loci.

Another strategy to reduce heterogeneity is the use of genetically isolated populations. These populations are shown to have a reduced genetic heterogeneity for rarer variants. [128, 129] But perhaps more important for complex disease studies, patients from these populations share a more uniform genetic and environmental background. In addition, isolated populations exhibit extended linkage disequilibrium (LD), which facilitates gene mapping. For studies of complex traits, preferably younger population isolates (i.e. 10-20 generations old) are investigated that originated from a relatively small number of founders and that underwent rapid population expansion. Good examples of such isolates are populations in eastern Finland, Costa Rica, Quebec, Newfoundland and some areas in the Netherlands. The most extensive example of the systematic use of population history, genealogical records and nationwide healthcare registries in genetic research is the DeCode project in Iceland. Although perhaps not representing an ideal population isolate, [130] the excellent genealogical records and the possibility to create extensive pedigrees with shared ancestors have led to the identification of multiple potential disease loci and genes (www.decode.com). We applied the genetic isolate approach in chapter 6, which describes a genome-wide family-based association study for migraine with aura in the Central Valley of Costa Rican (CVCR) population. The 2.6 million residents of the CVCR descend mainly from a small group of Spanish and Amerindian founders who lived in the 16th and 17th centuries; by the beginning of the 18th century, the CVCR had a single population that then grew rapidly, without subsequent immigration, for almost 200 years. The CVCR population has the typical characteristics of a young genetic isolate; it displays a concentration of rare autosomal recessive diseases [131] and haplotype sharing in CVCR was shown to extend for distances up to several centimorgans (cM). [132] The *Costa Rican migraine study* (chapter 6) did not yield any significant migraine loci, probably due to a higher genetic heterogeneity than estimated that led to insufficient power of our sample size. Nevertheless, genetic isolates do have advantages to map genes compared to outbred populations, even for complex diseases.

Recently our group has started to collaborate with the Erasmus Medical Centre to map migraine genes in a Dutch genetically isolated population, the GRIP population. This population was founded in the middle of the 18th century by ~150 individuals and remained isolated until the last few decades. An extensive genealogic database including more than 63.000 individuals is available. GRIP has been proven successful for mapping genes, for example for Parkinson and type two diabetes. [133, 134] The Erasmus Rucphen Family (ERF) Study is part of the GRIP program and extensive genealogy and availability of phenotyping and genotyping data for more than 2500 ERF persons makes it a very promising study to identify genes for many different diseases. We have identified over 350 migraine cases

in the ERF cohort and genome-wide linkage and association studies will be performed in order to map migraine genes. Because of the availability of extensive phenotype data of all persons in the ERF study, we will also be able to study the clinical and genetic correlation of migraine with other co-morbid disorders, like depression.

7.5 Migraine and other genetic diseases

The relation between migraine and other genetic disorders might provide valuable insights into other possibly involved pathophysiological pathways in migraine. Migraine is part of the clinical spectrum of several disorders. Most apparent is the occurrence of migraine in several vascular disorders. For instance, about 30% of patients with Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leucoencephalopathy (CADASIL) have migraine, mainly migraine with aura, as presenting clinical feature. CADASIL is caused by mutations in the *NOTCH3* gene, encoding the Notch3 receptor. Notch3 receptors play a key role in the development, survival and function of vascular smooth muscle cells and are thought to be essential for the maintenance of healthy muscle cells in the small arteries and arterioles in the brain. Although a few genetic studies were performed to investigate the role of the *NOTCH3* gene in migraine, they either focused only on CADASIL mutation hotspots and/or functional polymorphism T6746C in an association study with too low power. [135, 136]

Another vascular disorder that is associated with migraine is autosomal dominant Retinal Vasculopathy with Cerebral Leukodystrophy (RVCL). Previously, our group reported a large Dutch family with autosomal dominant vascular retinopathy, migraine and Raynaud's phenomenon (HVR). [137, 139] Of the 20 originally described patients in this family, approximately 70% suffer also from migraine. This family, together with two additional families with overlapping clinical features was linked to chromosome 3p21. [139] Recently, our consortium identified the causal gene, *TREX1*, encoding a 3'-5' repair exonuclease 1. [140] *TREX1* plays a role in apoptotic single-stranded DNA damage induced by for instance the killer lymphocyte protease granzyme A. *TREX1* mutations were also shown to cause Aicardi-Goutieres syndrome and familial chilblain lupus. [141-143] Recently, TREX1 mutations were reported in a small proportion of patients with Systemic Lupus Erythematosus (SLE) and Sjögren's syndrome.[144] Future studies are needed to elucidate possible role of *TREX1* in migraine and other neurovascular diseases, like CADASIL, Raynaud's phenomenon, vascular dementia, ischemic stroke and autoimmune diseases, like Rheumatoid arthritis (RA) and Scleroderma. Interestingly, migraine was also reported as an associated feature in a large family with a mutation in the transforming growth factor beta receptor 2 gene *TGFBR2*. In this family, 10 of the 14 mutation carriers not only suffered from familial aortic dissection and several other blood vessel abnormalities, but also had migraine. [145]

A completely different pathway that might be involved in migraine pathophysiology is impairment of mitochondrial oxidative metabolism. Migraine has been associated with Mitochondrial Encephalopathy, Lactic Acidosis and Stroke-like episodes (MELAS), caused by mutations in several mtDNA genes. [146] Interestingly, migraine was also reported as one of the additional phenotypes associated with *POLG* (encoding mitochondrial γ polymerase) mutation carriers. [147-149] Further studies are still needed to clarify if migraine is associated with mutations in mtDNA or in nuclear genes that code mitochondrial proteins.

Finally, a pathway that might also be involved in migraine is circadian rhythm. Migraine is associated with a family that has a mutation in the human *CSNK1D* gene, encoding casein kinase I delta, a serine/threonine-specific protein kinase. [150] This family suffers from

familial advanced sleep phase syndrome (FASPS), which is a human behavioural phenotype characterized by early sleep times and early-morning awakenings. Transgenic mice carrying the same mutation have a shorter circadian period, but even more interesting with respect to migraine pathophysiology also a reduced threshold for cortical spreading depression. [151]

7.6. Future Perspectives

This thesis focused on elucidating the role of genetic factors in migraine. Compared with many other complex diseases, like diabetes and psychiatric disorders, molecular genetics of migraine has been studied for a relatively short time. So far, three FHM genes have been identified and the genetic spectrum of mutations causing FHM as well as the clinical spectrum associated with such mutations was extended in this thesis. Screening of known FHM genes by sequencing should not only be considered as a valid diagnostic tool, but is also crucial to increase the knowledge about genotype-phenotype correlations and to provide novel scientific leads for further functional characterization studies. Although three FHM genes that function in a common pathway have been identified, it is still interesting to map additional FHM genes in future studies, as they may unravel novel pathways involved in migraine pathophysiology. Already highlighted possible pathways are vascular function, mitochondrial metabolism, circadian rhythm, hormonal and neurotransmitter function. For genome-wide linkage studies large FHM families are required. Therefore, substantial efforts should be made to search for new extended FHM families with reliable diagnoses, a crucial feature for success. Recently, the power of these linkage studies has certainly improved with the advanced technology of dense SNP arrays designed for linkage mapping. Compared to the classic panels of microsatellite markers, these SNP panels have a more uniform distribution over the genome, high call rates, information content, and genotyping accuracy. In addition to the identification of new FHM genes and their pathways, further research is required that investigates the role of genes and pathways involved in other genetic disorders that are associated with migraine, like CADASIL, since every innovative pathway might provide new leads to potential drug targets.

After the initial high hopes and disappointing results of many genetic studies for complex diseases, future research looks promising again. Rapid advances in genotyping technology [152], drastic reductions in genotyping costs, the sequencing of the human genome [153] and the recent completion of the HapMap project (showing the genetic variation found among four different human populations) [154] have now made whole genome association analysis feasible. Until now, all the genetic association studies for common migraine have been performed using a candidate-gene approach and many were severely underpowered. In stead of a few hundred patients, preferably at least 1000 or more should be used in these studies to detect low-risk variants and to prevent spurious associations. Recently the largest genome-wide association study (GWAS) thus far (2,000 cases of seven common diseases and 3,000 shared controls) has been reported by the Wellcome Trust Case Control Consortium. [155] In addition several other GWAS reports recently have provided compelling genome-wide significant evidence for associations with a variety of diseases, including Crohn's disease, obesity, type 1 and type 2 diabetes, psoriasis, coronary heart disease, amyotrophic lateral sclerosis (ALS), restless legs syndrome (RLS) and prostate and breast cancer. [156-163] These studies yielded gene variants or regions previously unrecognized and in many cases with unknown function. Also, a substantial proportion of associations was identified in regions without annotated genes, showing that investigation of non-coding associations with possible

regulatory functions will be critical to unravel the role of these regions. Importantly, these studies showed that individual SNPs that were identified had very modest effects in the population, with odds ratios below 1.5, with a few exceptions for instance in age-macular degeneration.

For migraine, advances in clinical, neurophysiological, radiological, and/or biochemical biomarkers will facilitate unequivocal diagnoses and subsequent genetic analysis. Recently our group has started an innovative project comparing metabolites, peptides and proteins in cerebrospinal fluid (CSF) of migraine patients and controls. Looking even further into the future, the most comprehensive approach towards understanding the genetic background of complex diseases would be complete genome resequencing in a large population of cases and controls. This would cover the complete spectrum of coding and non-coding variation and in contrast to the current SNP assays, would make it possible to test both common and rare variants for their role in disease. Already advances in rapid and affordable complete genome sequencing technologies are being made; therefore likely this approach will become feasible soon. Still, a challenging task will be to translate genetic insights into knowledge about disease pathophysiology and new therapeutic avenues. Therefore, advanced tools will be required to investigate functional consequences of coding and non-coding DNA changes and copy number variations.

Concluding, over the last decade, we have acquired considerable knowledge on how migraine attacks begin. Identification of additional migraine genes and their pathways will certainly contribute to better diagnoses, understanding of the disease, and identification of drug targets that can lead to improved prophylactic treatment.

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Summary

This thesis is focused on elucidating the role of genetic factors in migraine. To this end genetic studies were performed in rare monogenic familial hemiplegic migraine (FHM), sporadic hemiplegic migraine (SHM), as well as in families with multifactorial common migraine: migraine without (MO) or with aura (MA). For FHM, three genes have been identified and mutations in these genes are presented in this thesis. The *CACNA1A* gene (FHM1) encodes the α1 subunit of neuronal P/Q-type calcium channels, the *ATP1A2* gene (FHM2) encodes the α2 subunit of sodium-potassium pumps and the *SCN1A* gene (FHM3) encodes the α1 subunit of the neuronal voltage-gated sodium channels. All three genes are involved in a unifying pathway of ion translocation. Only chromosomal loci, but no genes, have been identified for common migraine. Genetic studies in an outbred clinic-based population and an isolated population were also subject of investigation in this thesis.

Chapter 2 further expands on the clinical spectrum associated with mutations in the FHM1 *CACNA1A* gene. Mutation I1710T was identified in a family with childhood epilepsy, familial hemiplegic migraine and cerebellar ataxia. Epilepsy in the I1710T mutation carriers occurred independently from the FHM attacks. This is the first example of an FHM1 mutation associated with hemiplegic migraine and childhood epilepsy. These results strengthen the clinical and genetic relation between epilepsy and migraine and hint to shared pathophysiological mechanisms.

A major part of this thesis involves the identification and functional analysis of FHM2 *ATP1A2* mutations. In **chapter 3** seven novel *ATP1A2* mutations are described and for five mutations functional consequences using cellular survival assays are demonstrated. Section 3.1 provides the first confirmation of FHM2 as an important FHM gene. One of the two novel *ATP1A2* mutations that were presented, R689Q, was associated with FHM and benign familial infantile convulsions (BFIC). In section 3.2, an *ATP1A2* mutation is described causing severe episodic neurological deficits and permanent mental retardation in a young girl, emphasizing the severity of clinical phenotypes associated with FHM mutations. Section 3.3, describes the identification of the first *de novo ATP1A2* mutations whereas section 3.4 provides the first and until now only case of compound heterozygosity for FHM mutations. The proband of this FHM family, who has two allelic *ATP1A2* mutations that both cause dysfunctional Na,K-pumps, demonstrates that having two affected *ATP1A2* alleles is compatible with life. In this chapter, considerable clinical variation was revealed associated with *ATP1A2* mutations and interesting mutational mechanisms were highlighted.

Recently, the FHM3 *SCN1A* gene was identified and in **chapter 4** mutation scanning was performed in 10 FHM families that were negative for mutations in *CACNA1A* and *ATP1A2*. In one large FHM family, without associated epilepsy or ataxia, a novel mutation, L1649Q, was identified. Electrophysiological investigations showed that this mutation severely affects voltage-gated $Na_v1.1$ channel functioning. Our results firmly established that the *SCN1A* "epilepsy" gene is indeed also implicated in FHM.

In **chapter 5,** the role of all three known FHM genes was investigated in sporadic hemiplegic migraine (SHM). Thirty-nine well-characterized SHM patients were systematically scanned

for mutations in these FHM genes. For all novel sequence variants that were identified functional assays were performed. Thus, it was clearly demonstrated that FHM genes are involved in a considerable sub-population of SHM patients, but that other genetic and non-genetic factors must be involved as well. In clinical practice, mutation scanning of the *ATP1A2* gene offers the highest likelihood of success in cases of "pure" SHM not associated with other neurological symptoms. As FHM gene mutations were also found in family members with "non-hemiplegic" typical migraine with and without aura, our findings reinforce the hypothesis that FHM, SHM and "normal" migraine are part of a disease spectrum with shared pathogenetic mechanisms.

In **chapter 6** the genetics of common migraine was investigated with two genome-wide scan studies. The first study was performed in seven large, well-characterized, Dutch families with apparent autosomal dominantly inherited migraine without aura (MO). No significant evidence for involvement of novel or known loci was found. Notably, suggestive linkage to the previously reported locus on chromosome 4q21 was reduced, not increased, by testing additional families; probably because more genetic heterogeneity was introduced into the analysis. In an attempt to reduce the genetic heterogeneity, for the second study we selected severe early onset migraine with aura patients that have a higher genetic load, from the genetically isolated population from the Central Valley of Costa Rica, which is expected to have reduced genetic heterogeneity. Even this strategy did not yield any positive results. Therefore, despite earlier published positive linkage results in migraine, both studies reveal intrinsic problems of migraine with respect to clinical and genetic heterogeneity. These results prompted us to reconsider existing gene identification strategies in common migraine and suggested that patient recruitment should also focus on very large numbers of well-characterized migraine patients.

Taken together, this thesis contributes to a better understanding of the role of genetic factors involved in various types of migraine. The genetic spectrum of mutations causing FHM was extended in these studies as well as the associated clinical spectrum. However, from this thesis it is also clear that gene mapping in common migraine is challenging. Different study designs were used and two genome-wide scans were performed, but did not yield novel migraine genes. It has to be emphasized that, compared with many other complex diseases, molecular genetics of migraine has been studied for a relative short time. Specific features of migraine (i.e. high prevalence, absence of biomarkers, sometimes high age of onset), and the probable contributions of many susceptibility genes with small effects, will continue to make the search for migraine susceptibility genes challenging. The continuing development of optimal study designs, like endophenotyping, and recent breakthroughs in powerful high-throughput genotyping technologies, accessibility of public Web-based resources, like HapMap, will certainly improve the search for genetic factors in migraine.

Nederlandse samenvatting

Dit proefschrift is gericht op de opheldering van de rol van genetische factoren in migraine. Hiervoor werden genetische studies uitgevoerd in families met zeldzame familiaire hemiplegische migraine (FHM), sporadische hemiplegische migraine (SHM), maar ook in families met de frequente vormen van migraine: migraine zonder aura (MO) en migraine met aura (MA). Voor FHM zijn drie genen geïdentificeerd en mutaties in deze genen zijn beschreven in dit proefschrift. Het *CACNA1A* gen (FHM1) codeert voor de α1 subeenheid van neuronale P/Q-type calcium kanalen, het *ATP1A2* gen (FHM2) codeert voor de α2 subeenheid van natrium-kalium pompen en het *SCN1A* (FHM3) gen codeert voor de α1 subeenheid van neuronale voltage-gereguleerde natrium kanalen. Deze FHM genen zijn alle drie betrokken in een overeenkomstige pathway van ionentransport. Voor de frequente vormen van migraine zijn slechts chromosomale gebieden, maar nog geen genen gevonden. Genetische studies in een kliniek-gebaseerde populatie en in een genetisch geïsoleerde populatie worden ook beschreven in dit proefschrift.

Hoofdstuk 2 breidt het klinische spectrum geassocieerd met mutaties in het FHM1 *CACNA1A* gen verder uit. Mutatie I1710T werd geïdentificeerd in een familie met een vorm van epilepsie op jonge leeftijd, familiaire hemiplegische migraine en cerebellaire ataxie. Epilepsie in de I1710T mutatie dragers trad onafhankelijk van de FHM aanvallen op. Dit was het eerste voorbeeld van een FHM1 mutatie die geassocieerd was met hemiplegische migraine en een vorm van epilepsie bij kinderen. Deze resultaten versterken de klinische en genetische relatie tussen epilepsie en migraine en wijzen op overlappende pathofysiologische mechanismen.

Een groot gedeelte van dit proefschrift beschrijft de identificatie en functionele analyse van FHM2 *ATP1A2* mutaties. In **hoofdstuk 3** worden zeven nieuwe *ATP1A2* mutaties beschreven en voor vijf mutaties worden functionele consequenties met cellulaire survival-assays aangetoond. Sectie 3.1 geeft de eerste bevestiging dat het *ATP1A2* gen een belangrijke rol speelt in FHM. Eèn van de twee nieuwe mutaties die beschreven werden, R689Q, was geassocieerd met FHM en benigne familiale infantiele convulsies (BFIC), een vorm van epilepsie bij jonge kinderen. In sectie 3.2 wordt een *ATP1A2* mutatie beschreven die zeer ernstige episodische neurologische afwijkingen en permanente mentale retardatie in een jong meisje veroorzaken. Dit benadrukt de ernst van bepaalde klinische fenotypes die geassocieerd kunnen zijn met FHM mutaties. Sectie 3.3 beschrijft de identificatie van de eerste *de novo ATP1A2* mutaties, en sectie 3.4 laat het eerste en tot nu toe enige geval van 'compound heterozygosity' voor FHM mutaties zien. De proband van deze FHM familie, die twee allelische *ATP1A2* mutaties heeft die beiden disfunctionele natrium/kalium-pompen veroorzaken, laat zien dat het hebben van twee aangedane *ATP1A2* allelen toch verenigbaar is met leven. Dit hoofdstuk laat een substantiële klinische variatie zien die geassocieerd is met *ATP1A2* mutaties en demonstreert interessante mutatie mechanismen.

Recentelijk werd het FHM3 *SCN1A* gen geïdentificeerd en in **hoofdstuk 4** wordt een mutatie scanning beschreven die uitgevoerd is in 10 FHM families die negatief waren voor mutaties in de *CACNA1A* en *ATP1A2* genen. In een grote FHM familie, zonder geassocieerde epilepsie of ataxie, werd een nieuwe mutatie, L1649Q, gevonden. Elektrofysiologische studies toonden aan dat deze mutatie het functioneren van de voltage-gereguleerde $Na_v1.1$

kanalen ernstig beïnvloedde. Deze resultaten bekrachtigen dat het *SCN1A* 'epilepsie' gen inderdaad ook een rol speelt bij FHM

In **hoofdstuk 5** werd de rol van de drie bekende FHM genen bestudeerd in 39 goed-gekarakteriseerde patiënten met sporadische hemiplegische migraine (SHM). Voor alle nieuwe sequentie variaties die werden gevonden, werden functionele studies uitgevoerd. De resultaten lieten duidelijk zien dat de FHM genen in een aanzienlijk deel van de SHM patiënten een rol spelen, maar ook dat andere genetische en niet-genetische factoren bij SHM betrokken moeten zijn. In de klinische praktijk biedt mutatie scanning van *ATP1A2* de beste kans op succes voor 'pure' SHM patiënten die geen andere neurologische symptomen hebben. Omdat FHM mutaties ook werden gevonden in familieleden met 'niet-hemiplegische' typische migraine met of zonder aura, versterken onze resultaten de hypothese dat FHM, SHM en 'normale' migraine onderdeel zijn van een ziektespectrum met gedeelde pathogenetische mechanismen.

In **hoofdstuk 6** werd de genetica van de frequente vormen van migraine onderzocht met behulp van twee genome-wide scans. De eerste studie werd uitgevoerd in zeven families met een duidelijke autosomale dominante overerving van migraine zonder aura (MO). Er werd geen significant bewijs gevonden voor koppeling aan een nieuw of een al eerder gerapporteerd chromosomaal gebied. Opmerkelijk was dat suggestief bewijs voor koppeling aan een eerder gerapporteerd gebied op chromosoom 4q21-24 niet verbeterde, maar juist verslechterde, door het toevoegen van families, waarschijnlijk doordat hierdoor meer genetische heterogeniteit geïntroduceerd werd in de analyse. In een poging deze genetische heterogeniteit te verminderen werden voor de tweede studie patiënten geselecteerd met een hogere genetische belasting, namelijk met ernstige migraine met aura uit de genetisch geïsoleerde populatie van de Central Valley of Costa Rica. Zelfs deze strategie leverde geen positieve resultaten op. Ondanks eerder gerapporteerde positieve koppelingsresultaten, laten deze studies de intrinsieke problemen van migraine zien met respect tot klinische en genetische heterogeniteit. Deze resultaten sporen aan om de huidige genidentificatie-strategieën voor de frequente vormen van migraine opnieuw onder de loep te nemen en geven aan dat het verzamelen van patiënten zich ook zou moeten richten op grote aantallen van individuele goedgekarakteriseerde migraine patiënten.

Concluderend, dit proefschrift draagt bij tot een beter inzicht in de rol van genetische factoren die betrokken zijn bij verschillende vormen van migraine. Zowel het genetische als het klinische spectrum van mutaties die FHM veroorzaken werd in deze studies uitgebreid. Het is echter ook duidelijk dat het vinden van genen voor de frequente vormen van migraine erg moeilijk is. Verschillende studiebenaderingen werden gebruikt, maar deze leverden geen nieuwe migraine-genen op. Er moet benadrukt worden dat in tegenstelling tot andere complexe genetische ziekten, de moleculaire genetica van migraine nog maar een relatief korte tijd bestudeerd wordt. Specifieke kenmerken van migraine (bv. hoge prevalentie, gebrek aan biomarkers, soms een hoge beginleeftijd, etc) en de waarschijnlijke bijdrage van vele genetische factoren met kleine effecten, zullen de zoektocht naar migraine-genen uitdagend houden. De voortdurende ontwikkeling van nieuwe studiebenaderingen en de recente doorbraken in high-throughput genotypering technologieën, zoals Illumina, en de toegankelijkheid tot publieke Web-gebaseerde databanken, zoals HapMap, zullen de zoektocht naar genetische factoren voor migraine zeker verbeteren.

List of abbreviations

AHC	alternating hemiplegia of childhood
ATP	adenosine tri-phosphate
BFIC	benign familial infantile convulsions
CD-CV	common disease-common variant
CD-RV	common disease-rare variant
cDNA	copy deoxyribonucleic acid
CGRP	calcitonin gene-related peptide
cM	centiMorgan
CSD	cortical spreading depression
CVCR	Central Valley of Costa Rica
DNA	deoxyribonucleic acid
DZ	dizygotic
EA-2	episodic ataxia type 2
EEG	electroencephalogram
FHM	familial hemiplegic migraine
GEFS+	generalised epilepsy with febrile seizures
HGP	human genome project
HWE	Hardy-Weinberg equilibrium
IHS	International Headache Society
LD	linkage disequilibrium
LOD	logarithm of odds
MA	migraine with aura
MO	migraine without aura
MRI	magnetic resonance imaging
MZ	monozygotic
PCR	polymerase chain reaction
SCA6	spinocerebellar ataxia type 6
SHM	sporadic hemiplegic migraine
SMEI	severe myoclonic epilspsy of infancy
SNP	single nucleotide polymorphism
TDT	transmission disequilibrium test
TGVS	trigeminal vascular system
TNC	trigeminal nucleus caudalis
WT	wild-type

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Curriculum Vitae

Kaate Raymond Josepha Vanmolkot werd op 4 april 1978 geboren in Heerlen. In 1996 behaalde zij haar VWO diploma aan de RK Scholengemeenschap Serviam te Sittard. In datzelfde jaar begon zij met de studie Biologie aan de Katholieke Universiteit Nijmegen. In het jaar 1997 werd het propedeutische examen behaald. Tijdens de doctoraal fase liep zij twee onderzoeksstages, waarvan de eerste bij de afdeling Moleculaire Dierfysiologie aan de Katholieke Universiteit Nijmegen onder begeleiding van Dr. V. Schoonderwoert en Prof. Dr. G. Martens. De tweede stage werd verricht bij de afdeling Antropogenetica aan de Katholieke Universiteit Nijmegen onder begeleiding van Dr. H. van Bokhoven en Prof. dr. H. Brunner. Van april 2001 tot mei 2006 was zij werkzaam als assistent in opleiding bij de afdeling Humane Genetica van het Leids Universitair Medisch Centrum onder leiding van Prof. dr. M.D. Ferrari, Prof. dr. R.R.Frants en Dr. A.M.J.M. van den Maagdenberg. De resultaten van het onderzoek staan beschreven in dit proefschrift. Vanaf mei 2006 is zij werkzaam op hetzelfde onderzoek als postdoctoraal onderzoeker bij de afdeling Humane Genetica van het Leids Universitair Medisch Centrum.