Department of Clinical Chemistry,
Research Program in Molecular Medicine, Biomedicum Helsinki, and
Department of Biological and Environmental Sciences, Division of Genetics
University of Helsinki
Finland

ASSIGNMENT OF GENETIC LOCI AND VARIANTS PREDISPOSING TO MIGRAINE WITH AURA AND EPISODIC ATAXIA TYPE 2

Mari Kaunisto

ACADEMIC DISSERTATION

To be publicly discussed, with the permission of the Faculty of Biosciences,
University of Helsinki, in Lecture Hall 2 of Biomedicum Helsinki, Haartmaninkatu 8,
on November 4th, 2005, at 12 noon.

Helsinki 2005

Supervisors

Docent Maija Wessman, PhD
Academy Research Fellow
Department of Clinical Chemistry and
Folkhälsan Research Center
University of Helsinki,
Helsinki, Finland

Professor Aarno Palotie, MD, PhD
Department of Clinical Chemistry and
The Finnish Genome Center
University of Helsinki,
Helsinki, Finland

Reviewers

Docent Tiina Paunio, MD, PhD
Department of Molecular Medicine
National Public Health Institute and
Department of Psychiatry
Helsinki University Central Hospital,
Helsinki, Finland

Associate Professor Joanna Jen, MD, PhD
David Geffen School of Medicine
University of California, Los Angeles,
California, USA

Opponent

Docent Pentti Tienari, MD, PhD
Department of Neurology
Helsinki University Central Hospital and
Neuroscience Programme, Biomedicum Helsinki,
University of Helsinki,
Helsinki, Finland

ISBN 952-91-9269-X (paperback)
ISBN 952-10-2709-6 (PDF)

Yliopistopaino, Helsinki 2005

“When you reach for the stars, you may not quite get one, but you won't come up with a handful of mud either.”

-Leo Burnett

"Simple vs. Complex Traits: the real definition:
Simple: things we have deluded ourselves into thinking we understand.
Complex: things we're pretty sure we don't understand."

-Eleanor Feingold

To my dear family

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data are presented.

I Wessman M, Kallela M, Kaunisto MA, Marttila P, Sobel E, Hartiala J, Oswell G, Leal SM, Papp JC, Hämäläinen E, Broas P, Joslyn G, Hovatta I, Hiekkalinna T, Kaprio J, Ott J, Cantor RM, Zwart JA, Ilmavirta M, Havanka H, Färkkilä M, Peltonen L, Palotie A (2002). A susceptibility locus for migraine with aura on chromosome 4q24. *American Journal of Human Genetics* 70:652-662.

II Kaunisto MA*, Tikka PJ*, Kallela M, Leal SM, Papp J, Korhonen A, Hämäläinen E, Harno H, Havanka H, Nissilä M, Säkö, E, Ilmavirta M, Kaprio J, Färkkilä M, Ophoff R, Palotie A, Wessman, M (2005). Chromosome 19p13 loci in Finnish migraine with aura families. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics* 132:85-89.

III Kaunisto MA, Kallela M, Hämäläinen E, Kilpikari R, Havanka H, Harno H, Nissilä M, Säkö E, Ilmavirta M, Liukkonen J, Teirmaa H, Törnwall O, Jussila M, Terwilliger J, Färkkilä M, Kaprio J, Palotie A, Wessman M. Testing of variants in the *MTHFR* and *ESR1* genes in 1798 individuals fails to confirm the association to migraine with aura. *Manuscript*.

IV Kaunisto MA, Harno H, Vanmolkot KRJ, Gargus JJ, Sun G, Liukkonen E, Kallela M, van den Maagdenberg AMJM, Frants RR, Färkkilä M, Palotie A, Wessman M (2004). A novel missense *ATP1A2* mutation in a Finnish family with familial hemiplegic migraine type 2. *Neurogenetics* 5:141-146.

V Kaunisto MA, Harno H, Kallela M, Somer H, Sallinen R, Hämäläinen E, Miettinen PJ, Vesa J, Orpana A, Palotie A, Färkkilä M, Wessman M (2004). Novel splice site *CACNA1A* mutation causing episodic ataxia type 2. *Neurogenetics* 5:69-73.

* These authors contributed equally to the respective work.

Study V also appears in the thesis of Hanna Harno (2005).

The original publications have been produced with the permission of the copyright holders.

ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
AHC	alternating hemiplegia of childhood
ASP	affected sib-pair
ATP	adenosine triphosphate
BFIC	benign familial infantile convulsions
bp	base pair
CD-CV	common disease – common variant hypothesis
cDNA	complementary DNA
CEPH	Centre d'Etudes du Polymorphisme Humain
cM	centiMorgan
CSD	cortical spreading depression
cSNP	coding SNP
DNA	deoxyribonucleic acid
DZ	dizygotic
EA(-2)	episodic ataxia (type 2)
EMLOD	expected maximum LOD score
FHM	familial hemiplegic migraine
GABA	gamma-amino butyric acid
h^2	heritability
HLOD	LOD score under heterogeneity
HRR	haplotype relative risk
htSNP	haplotype tagging SNP
HWE	Hardy-Weinberg equilibrium
ICHD	International Classification of Headache Disorders
IHS	International Headache Society
kb	kilobase
LCA	latent class analysis
LD	linkage disequilibrium
LOD	logarithm of odds
MA	migraine with aura
Mb	megabase
MO	migraine without aura
MODY	maturity onset diabetes of youth
MRI	magnetic resonance imaging
MZ	monozygotic
NPL	nonparametric linkage
OMIM	Online Mendelian Inheritance in Man
OR	odds ratio
PCR	polymerase chain reaction
rCBF	regional cerebral blood flow
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase PCR
SCA(-6)	spinocerebellar ataxia (type 6)
SNP	single nucleotide polymorphism
TDT	transmission/disequilibrium test
VNTR	variable number of tandem repeat
WHO	World Health Organization

ABSTRACT

Migraine with aura (MA) is a common headache disorder with a population prevalence of approximately 5%. This subtype of migraine is characterized by neurological, typically visual, symptoms that precede the headache. Twin and family studies have provided strong evidence of genetic factors in MA susceptibility and have shown that the mode of inheritance is most likely multifactorial. Furthermore, mutations in the *CACNA1A* and *ATP1A2* genes have been shown to cause a rare monogenic subtype of MA, familial hemiplegic migraine (FHM). The FHM1 gene, *CACNA1A*, is particularly interesting since mutations in it can also cause another related neurological phenotype, episodic ataxia type 2 (EA-2), also under investigation in this thesis.

This study aimed at identifying genetic loci and variants that are involved in the etiology of MA. We have collected almost 700 Finnish families segregating this disorder and performed a genome-wide scan in 50 of these families. Strong evidence of linkage to chromosomal area 4q21-24 was obtained. Encouragingly, this finding has been subsequently replicated in the Icelandic population. The actual causative variant has, however, not yet been identified despite substantial efforts.

In an alternative study approach, the role of certain candidate loci and genes previously associated with migraine has been investigated in our large family and patient samples. A susceptibility locus on chromosome 19p13 contains two migraine-associated genes, *CACNA1A* (the FHM1 gene) and *INSR* encoding an insulin receptor. In the present study, this susceptibility locus was studied in 72 Finnish MA families but no evidence of linkage was seen. Furthermore, the association of genetic variants in candidate genes *MTHFR* (coding for a methylenetetrahydrofolate reductase) and *ESR1* (coding for an estrogen receptor) with migraine was studied in 898 unrelated MA patients and 900 healthy controls. The previously migraine-associated polymorphisms showed no association with MA while some nominal evidence of association between MA and five other *ESR1* variants was seen.

In addition, the first Finnish families with FHM and EA-2 phenotypes were identified and studied in this thesis. The FHM family was shown to have a novel missense mutation in the *ATP1A2* gene whereas the EA-2 phenotype was caused by a splice site mutation in the *CACNA1A* gene.

INTRODUCTION

The human brain performs all the necessary functions that make us who we are, ranging from sensing, locomotion, and emotion to learning and memory. The cellular and molecular networks needed for these functions are extremely complex and delicately controlled. Because of this complexity, the nervous system seems to be especially vulnerable to mutations and the number of neurological diseases caused by genetic defects is high. Migraine, a severe disabilitating headache disorder affecting approximately 15% of population, is one the most common neurological disorders (Rasmussen *et al.* 1991). Migraine with aura (MA), with a population prevalence of 5%, is characterized by neurological (typically visual) symptoms that precede the headache attacks (Russell *et al.* 1995a). Familial hemiplegic migraine (FHM), on the other hand, is a rare dominantly inherited subtype of MA distinguished by the presence of unilateral motor aura symptoms (Headache Classification Committee of the International Headache Society 2004).

Recent methodological advances and determination of the human genome sequence have facilitated the identification of mutations that cause diseases with Mendelian inheritance. However, the progress in understanding diseases with complex inheritance, where multiple genes and environmental factors contribute to the liability, has been relatively slow. Although twin and family studies have clearly shown that genetic factors have a major role in migraine susceptibility (Russell and Olesen 1995; Mulder *et al.* 2003), the underlying gene variants have not yet been identified. As a result, most that is known of the genetics of migraine comes from studies of the monogenic FHM. During the last decade, at least this migraine subtype has been added to the growing list of channelopathies, i.e. diseases caused by mutations in ion channel genes. The two genes implicated in this disease, *CACNA1A* coding for a voltage-gated Ca^{2+} channel and *ATP1A2* coding for a Na^{+},K^{+}-ATPase, are both involved in ion translocation (Ophoff *et al.* 1996a; De Fusco *et al.* 2003). In addition to FHM, *CACNA1A* mutations can also lead to another dominantly inherited neurological phenotype, episodic ataxia type 2 (EA-2) (Ophoff *et al.* 1996a). Recurrent attacks of generalized cerebellar incoordination lasting up to several hours are the main symptom of EA-2, although vertigo, nausea and migraine headaches are also often present.

In this thesis, the genetics of these three related neurological disorders, the multifactorial (MA) and monogenic (FHM) forms of migraine, and EA-2, were investigated in Finnish families.

REVIEW OF THE LITERATURE

1 CURRENT STATUS OF HUMAN GENETIC RESEARCH

During the first five years of the new millennium we human geneticists have seen a remarkable progress in our field. The completion of the human genome sequence in 2001 is naturally the biggest achievement (Lander *et al.* 2001; Venter *et al.* 2001). Gaps in the draft sequence were mostly filled by a finishing project, results of which were published last year (International Human Genome Sequencing Consortium 2004). The current sequence is estimated to cover 99% of the euchromatic genome with an overall error rate less than 1/100,000 nucleotides (International Human Genome Sequencing Consortium 2004). However, the completion of the genome sequence has raised even more questions than answers. It is now realized that the next major goal is to characterize the variance within the genome and, indeed, millions of single nucleotide polymorphisms (SNPs) have been described and deposited in public databases (Sherry *et al.* 1999; Sachidanandam *et al.* 2001). Issues related to this goal are the initiation of the International HapMap Project, the aim of which is to determine linkage disequilibrium (LD) patterns across the human genome, and the rapid progress in SNP genotyping techniques (Syvänen 2001; The International HapMap Consortium 2003).

As a result of the Human Genome Project, the predicted number of genes in the human genome has been constantly dropping and the current estimate is only around 20,000-25,000 protein-coding genes (International Human Genome Sequencing Consortium 2004). Surprisingly, this number does not differ much from the number of identified genes in, for example, the roundworm *Caenorhabditis elegans* (The *C. elegans* Sequencing Consortium 1998). Differential gene regulation and alternative splicing of gene transcripts are now widely accepted causes of the complexity needed to produce human-beings. Future research will thus likely focus more on the study of transcriptomes and proteomes instead of genomes. However, studies concerning the human genome can still surprise us. Large-scale copy number variations (insertions and deletions ranging in size from 100 kb to 2 Mb) that do not cause any apparent phenotype were shown to be much more common than previously thought (Iafrate *et al.* 2004; Sebat *et al.* 2004). Furthermore, a 900 kb inversion polymorphism in chromosome 17 with an impact on natural selection has been recently identified (Stefansson *et al.* 2005).

One of the main challenges of human genetics is to identify genetic factors underlying human diseases. Numerous genes for diseases with Mendelian inheritance have been identified using traditional genome-wide scanning and subsequent positional cloning. In August 2005, the Online Mendelian Inheritance in Man (OMIM) database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM) contained 1781 phenotypes that have a known molecular basis but the number of genes involved is somewhat lower due to allelic diseases. Identification of complex disease genes is, however, clearly more difficult than originally envisioned. It is hoped that establishing large multinational research projects in molecular epidemiology, such as the MORGAM project (Evans *et al.* 2005) and the GenomEUtwin (Peltonen 2003), will help in the understanding of complex diseases.

2 SEARCHING FOR DISEASE GENES

2.1 The gene-mapper's toolbox

2.1.1 Patients

Individuals with the phenotype of interest are crucial for identifying disease genes. A careful and thorough diagnostic procedure is an essential prerequisite for a study to succeed. In general, extended families segregating the trait, parent-affected child trios, affected sib-pairs, or case-control samples can be collected. Each of these study designs has its advantages and pitfalls and typically a combination of some of these is utilized. Extended pedigrees are superior when searching for high-penetrance variants while case-control samples may possibly be better suited for identifying low-penetrance variants (Freimer and Sabatti 2004).

2.1.2 Genetic markers

In principle, any polymorphic feature with Mendelian inheritance can be used as a marker. **Table 1** summarizes the types of genetic variation present in humans. The earliest genetic markers used were blood groups and the electrophoretic mobility variants of serum proteins (Strachan and Read 1996). Biallelic restriction fragment length polymorphisms (RFLPs) were the first deoxyribonucleic acid (DNA) markers (Botstein *et al.* 1980). Identification of the multiallelic variable number of tandem repeat (VNTR) markers that include minisatellites (moderately sized arrays of tandemly repeated DNA sequences) and microsatellites (typically di-, tri- or tetranucleotide repeats) made genetic mapping much easier (Nakamura *et al.* 1987; Weber and May 1989). Several microsatellite maps of the human genome have been published, the newest of which is the deCODE Genetics map containing precise information

on 5136 microsatellites (Weissenbach *et al.* 1992; Gyapay *et al.* 1994; Dib *et al.* 1996; Kong *et al.* 2002).

Table 1. Genetic variation in humans (excluding chromosomal rearrangements).

Type of variation	Size (repeat size)	Predicted number in the human genome	Variability	Reference
SNPs	1 bp	11 million (MAF 1%)	Diallelic	Kruglyak and Nickerson 2001
Microsatellites	<150 bp (1-13 bp)	>600,000 1 nt repeats 120,000 2-4 nt repeats 280,000	Multiallelic	Lander *et al.* 2001
Small diallelic insertions/deletions	1 bp - a few kb	>700,000 (~85% 1-4 nt)	Diallelic	Bhangale *et al.* 2005
Transposable elements	280 bp - a few kb	>3 million; ~2000 polymorphic	Diallelic	Bennett *et al.* 2004
Minisatellites	500 bp - 20 kb (10 - >100 bp)	~6000; ~4800 polymorphic	Multiallelic	Denoeud *et al.* 2003
Large-scale variation	~8 kb - 1 Mb	NA (639 identified)	Di/multiallelic	Database of Genomic Variants*

MAF = minor allele frequency; NA = information not available; * http://projects.tcag.ca/variation/

SNPs are DNA variations in which one of the four nucleotides is substituted for another. In some cases, for example in the SNP databases, single nucleotide insertions and deletions are also considered to be SNPs. Since practically all SNPs are biallelic, the heterozygosity of these markers is lower than that of microsatellites. SNPs are, however, much more abundant than microsatellites. Two haploid human genomes are estimated to differ at 1 out of every 1,331 base pairs (Sachidanandam *et al.* 2001). The total number of SNPs having minor allele frequencies of at least 1% (the traditional definition of polymorphism) has been approximated to be 11 million, which means one SNP per every 290 bp (Kruglyak and Nickerson 2001). Another advantage of SNPs is their lower mutation rate compared to microsatellites. On the other hand, a recent report on segmental genome duplications (defined as segments of >1 kb with >90% sequence similarity) comprising ~5% of the human genome has raised concern regarding the reliability of genotyping SNPs located in these duplicated areas (Fredman *et al.* 2004).

At the moment the public databases such as dbSNP (build 124; http://www.ncbi.nlm.nih.gov/projects/SNP/) and SNPper (http://snpper.chip.org/) contain slightly above 10 million SNPs, around half of which have been validated (Sherry *et al.* 1999; Riva and Kohane 2004). Progress in this field has been exceptionally rapid, since the SNP map published in 2001 contained only 1.4 million SNPs (Sachidanandam *et al.* 2001). This is mainly due to the development of high-throughput SNP genotyping techniques (Syvänen

2001), summarized in **Table 2**. Frequency information is, however, available for only 500,000 of the SNPs in the dbSNP database. A pilot study performed in 2001 using three population samples showed that of the ~1200 SNPs studied, around half were found to be common (with minor allele frequency ≥ 20%) in any given population (Marth *et al.* 2001).

Table 2. Examples of medium- to high-throughput SNP genotyping techniques (Syvänen 2001; Hirschhorn and Daly 2005; Syvänen 2005).

Method	Description	Throughput
SNaPshot (Applied Biosystems)	PCR, primer extension & gel electrophoresis	12 SNPs in 384 samples
MassEXTEND, MassArray (Sequenom)	PCR, primer extension & mass spectrometry	7-29 SNPs in 384 samples
TaqMan assay (Applied Biosystems)	ASOH, 5'-exonuclease cleavage and FRET detection during real-time PCR	1 SNP in 384 samples
Pyrosequencing (Biotage)	PCR, primer extension & luminometric detection	1 SNP* in 96 samples
Molecular Inversion Probe assay (Parallele Biosciences)	Single base extension of a circularizable probe, generic PCR & tag array	10,000 SNPs in 1 sample
Golden Gate assay (Illumina)	Allele specific extension, ligation, generic PCR & tag array on beads	1536 SNPs in 96 samples
GeneChip 10K/100K (Affymetrix)	Generic PCR, ASOH to array	10,000-100,000# SNPs in 1 sample
Perlegen "wafers"	Long-range PCR, ASOH to array	~1.5 million SNPs in 1 sample (using 49 arrays)

PCR = polymerase chain reaction; ASOH = allele specific oligonucletide hybridization; FRET = fluorescence resonance energy transfer; * several if within a ~50 bp distance; # two arrays

2.1.3 Statistical methods

2.1.3.1 Linkage analysis

The aim of linkage analysis is to determine chromosomal locations for disease susceptibility loci by genotyping genetic markers in families with several affected individuals. When a chromosomal region is transmitted with the disease phenotype within families, this region is likely to contain the gene of interest. The basis for linkage analysis is the fact that loci located close to each other on the same chromosome are not usually separated by recombination but inherited together during meiosis (Strachan and Read 1996). 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. A recombination fraction of 0.01 corresponds to a genetic distance of 1 cM which approximates 0.9 Mb (Gyapay *et al.* 1994).

For calculating linkage, a likelihood ratio test is applied. The LOD (logarithm of odds) score 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) (Morton 1955).

$$Z(\theta) = \log_{10} \frac{L(\theta)}{L(0.5)}$$

In their classic paper, Lander and Kruglyak proposed standards for the interpretation of linkage results (1995). They determined that a genome-wide significance threshold of p = 0.05 is achieved at a pointwise p-value of $p = 4.9 \times 10^{-5}$, the corresponding LOD score being 3.3. They also suggested the use of terms "suggestive linkage" ($p \leq 1.7 \times 10^{-3}$; LOD ≥ 1.86), that is statistical evidence of linkage expected to occur once at random in a genome scan, and "nominal linkage" (p ≤ 0.05; LOD ≥ 0.5875) (Lander and Kruglyak 1995; Nyholt 2000).

Both parametric, i.e. model-based, and nonparametric, i.e. model-free, linkage analysis methods exist. In parametric analysis, performed with programs such as LINKAGE (Lathrop and Lalouel 1984), the pattern of inheritance has to be determined. Nonparametric methods are based on increased sharing of chromosomal segments between affected individuals without specifying the underlying genetic model. Affected sib-pair (ASP) analysis is a classical form of nonparametric linkage analysis (Penrose 1953). Programs developed for analyzing allele sharing between more distantly related individuals such as GENEHUNTER, GENEHUNTER-PLUS, ALLEGRO, SOLAR and MERLIN are widely used, powerful tools and some of them are also suitable for identifying loci that influence quantitative traits (Kruglyak *et al.* 1996; Kong and Cox 1997; Almasy and Blangero 1998; Gudbjartsson *et al.* 2000; Abecasis *et al.* 2002).

Since nonparametric methods do not require specification of the inheritance model, these methods are often regarded as more suitable for complex diseases. On the other hand, it has been shown that in many situations parametric and nonparametric methods are actually statistically equivalent since some assumptions have to be made anyway to reduce the complexity of the data (Göring and Terwilliger 2000; Hodge 2001). A well-known example is the equivalence of the mean test of ASP analysis and parametric linkage analysis assuming a recessive mode of inheritance (Knapp *et al.* 1994). Furthermore, the power of nonparametric methods is typically relatively low. It has been shown that even when the mode of inheritance is multifactorial, parametric analysis performed under a few different genetic assumptions with reduced penetrance is often more powerful than the use of nonparametric methods (Abreu *et al.* 1999).

2.1.3.2 Association analysis

The goal of association analysis is to determine whether a trait and a particular allele of a marker co-occur nonrandomly (Ewens and Spielman 2001). If an allele increases susceptibility to a disease, it should be present at a higher frequency among affected individuals than among controls. This can be computed by comparing the observed allele or genotype frequency distributions in cases and controls using a standard χ^2-test. In association studies, two competing hypothesis, namely H_0: the allele occurs at equal frequencies in cases and controls and H_1: the allele occurs at different frequencies, are evaluated. The statistical significance of the association is measured using a p-value, which is defined as the probability of obtaining a difference as large as observed by chance although it does not actually exist in the population. In other words, it is the risk of rejecting H_0 when it is true (type I error). Typically, if a single test is performed, a p-value ≤ 0.05 is regarded as significant. Type II error (H_0 is retained although it is false) is the major concern in replication studies. When the probability of type II error is low, the power of a study (the probability of rejecting H_0 when it is false) is high. An experiment with 80% power to detect a true difference is commonly regarded as acceptable (Lalouel and Rohrwasser 2002). One of the much debated subjects is how the correction for multiple testing (many genotyped markers and subgroup analysis) should be performed. Bonferroni correction, i.e. multiplying the established p-value with the number of tests performed, is likely to be too conservative when the markers or phenotypes studied are not entirely independent (Nyholt 2001). Permutation testing can be used to empirically evaluate the probability of having observed a particular result by chance. In this method, the empirical distribution for the χ^2-statistic under the H_0 is defined by randomly re-ordering (permuting) the case-control status for the individuals in the data set many times (Hirschhorn and Daly 2005).

Unfortunately, the increased frequency of a particular allele among cases can not alone be used as proof of causality. The possibility of a spurious association due to an inadequate sample size or a poorly matched control group has to be kept in mind (Cardon and Bell 2001). Some investigators have suggested the use of so-called genomic control, i.e. evaluation of several unlinked genetic markers, to identify population stratification and also to correct for it if present (Pritchard and Rosenberg 1999). Although population admixture in Finland is thought to be minimal, some geographical factors have to be taken into account. The differences in neutral allele frequencies between western and eastern Finland can be quite dramatic and if the prevalence of the disease in question is higher in some area, a considerable

number of the cases might be collected from the more-susceptible population subgroup (Kere 2001).

Family-based controls are thought to be one answer to the population stratification problem. The transmission disequilibrium test (TDT) and haplotype relative risk (HRR) test were the first statistical methods developed based on this idea (Falk and Rubinstein 1987; Spielman *et al.* 1993). In both tests, parental alleles that are not transmitted to affected children serve as controls. Drawbacks of these methods are the need for collecting and genotyping trios and, in the case of the TDT, a loss of power due to the fact that only information from heterozygous parents can be used. Applying these methods for late onset diseases, where parental genotypes are typically not available, is also problematic, although methods using siblings as controls have been developed (Curtis 1997). Furthermore, programs for family-based tests of association (TRANSMIT, FBAT, Pseudomarker) are available and some of them can be used to test for association in the presence of linkage (Clayton 1999; Göring and Terwilliger 2000; Horvath *et al.* 2001).

To summarize, there are two main differences between a linkage study and an association study: 1) In association analysis we are interested in the particular alleles of the markers studied while in linkage analysis the markers are only tools for locating the susceptibility region and the linked alleles can be different between families. 2) In linkage analysis co-segregation of a trait and a locus is studied using families with several affected individuals but in association analysis unrelated patients can be used. However, the main principle of these methods (excluding the situation when the causal variant is directly studied) is basically the same – because of common ancestry, patients share alleles of markers located in the vicinity of the disease gene.

2.1.4 Linkage disequilibrium and haplotype blocks

Linkage disequilibrium, the co-occurrence of particular alleles at neighboring genetic markers more often than expected by chance, is an important tool in disease gene mapping. An allele showing association to a disease is not necessarily causative but can be only located close enough to be in LD with the actual susceptibility variant. Until recently, LD mapping has mainly been used for fine-mapping of previously identified susceptibility areas and thus restricting the critical chromosomal regions (Hästbacka *et al.* 1994; Horikawa *et al.* 2000; Hugot *et al.* 2001). Using genome-wide association mapping for disease gene localization has, however, raised a lot of interest (Risch and Merikangas 1996). In both approaches, the

central assumption is that although the causal variant is not necessarily directly genotyped because of practical and economic reasons, it is associated with some of the studied markers. However, this only holds if we assume that most affected individuals share the same mutant allele (Terwilliger and Weiss 1998). Using haplotypes instead of independent SNPs in association analyses may potentially lead to increased power in certain situations, such as in the presence of multiple disease-causing alleles (Morris and Kaplan 2002; Clark 2004).

The strength of LD can be described using different statistics, the most commonly used being *D'* and r^2. Both are based on a pairwise-disequilibrium coefficient, *D*, which quantifies the difference between the observed and expected frequencies of the two-locus haplotype, the expected frequency calculated as the product of the allele frequencies. *D'* is an absolute value of *D* determined by dividing *D* by the maximum value that *D* can attain, given the allele frequencies at the loci in question. The r^2measure is calculated as D^2 divided by the product of the allele frequencies.

$$D = f(A_1B_1) - f(A_1)f(B_1)$$

$$D' = \left| \frac{D}{D_{\max}} \right|$$

$$r^2 = \frac{D^2}{f(A_1)f(A_2)f(B_1)f(B_2)}$$

Both D' and r^2 equal 1 only if the two markers have not been separated by recombination. Furthermore, for r^2 to equal 1 (known as perfect LD), the allele frequencies need to be identical (Zondervan and Cardon 2004). D' has a tendency to overestimate the magnitude of LD especially in small samples and thus the r^2 measure is possibly a more relevant measure for association studies (Ardlie *et al.* 2002; Wall and Pritchard 2003). The minimum r^2 value of useful LD is thought to be $r^2 > 1/3$ but much higher D' values are needed to indicate similarly useful levels of LD (Ardlie *et al.* 2002).

Intense research efforts have recently been turned to investigating the extent of LD across the genome. It is already clear that patterns of LD vary extensively both from one genomic region to another and among populations, with European and Asian populations showing greater LD than African populations (Reich *et al.* 2001; Ardlie *et al.* 2002). The greater extent of LD in non-African populations is probably due to population bottleneck effects that have occurred during the migration of ancestral humans out of Africa (Wall and Pritchard 2003). When studying microsatellites, background LD can be detected over distances of ≥ 1 cM especially in population isolates (Laan and Pääbo 1997; Mohlke *et al.* 2001; Service *et al.* 2001; Varilo *et al.* 2003). In the case of SNPs, LD has been reported between markers separated >100 kb

(Ardlie *et al.* 2002). On the other hand, it is quite common that the expected inverse relationship between the degree of LD and physical distance does not hold; closely located markers are not necessarily in LD because of complex population demographic factors and the history of mutation events. The average extent of useful LD (defined as r^2 >1/3) in northern European populations has been estimated to be 10-30 kb (Ardlie *et al.* 2002).

Recent studies have suggested that the human genome can be partitioned into discrete blocks of limited haplotype diversity separated by possible recombination hot-spots that break the LD (Daly *et al.* 2001; Johnson *et al.* 2001; Patil *et al.* 2001; Gabriel *et al.* 2002). The size of most of the identified blocks is in the range of 5-20 kb (Wall and Pritchard 2003). However, in many regions the overall extent of LD seems to be limited, and many of the markers studied are not in the identified blocks (Gabriel *et al.* 2002). Furthermore, defining haploblocks and their boundaries is not straightforward and several methods for this have been proposed (Wall and Pritchard 2003). Three basic approaches are to: 1) make use of pairwise disequilibrium measures and define blocks as regions over which the average LD is above some predetermined threshold (Daly *et al.* 2001; Gabriel *et al.* 2002), 2) delineate blocks on the basis of limited haplotype-diversity within the blocks (Patil *et al.* 2001; Zhang *et al.* 2002) or 3) use a minimum-description-length principle based method that combines these two variables (Koivisto *et al.* 2003).

Typically only a few (2-6) common haplotypes together constituting ~90 % of all the observed haplotypes are seen within a block (Gabriel *et al.* 2002). Haplotype tagging (ht)SNPs are defined as a minimal set of SNPs that allow all these common haplotypes to be distinguished (Johnson *et al.* 2001). A goal of the HapMap project is to characterize patterns of haplotype structure and LD across the human genome (The International HapMap Consortium 2003). Utilizing this information could possibly lead to ~3-fold average genotyping savings in western European populations (Goldstein *et al.* 2003; Ke *et al.* 2004). On the other hand, the DNA samples for the HapMap project have come from a relatively modest number of individuals (N=270) belonging to four populations (U.S. residents with northern and western European ancestry, Japanese, Han Chinese, and Yorubans from Nigeria) (The International HapMap Consortium 2003) and there is only limited evidence of the usefulness of this approach in other populations.

2.2 Identification of genes underlying complex diseases

2.2.1 Challenges of complex disease studies

Human disorders can be thought of as forming a continuum ranging from diseases that are primarily genetic (such as diseases of the Finnish disease heritage) to those that are primarily environmental (breaking a bone due to an accident). Diseases with complex inheritance are located somewhere in the middle of this continuum. 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) can complicate the analysis of both monogenic and complex diseases. Furthermore, in polygenic inheritance, multiple genetic factors contribute to the phenotype (Sheffield *et al.* 1998; Thornton-Wells *et al.* 2004). A further subdivision can be made to situations where multiple alleles have a true additional effect (the phenotype is seen when a disease threshold is reached) and to those where a single major gene and additional modifier genes produce the phenotype. This interaction between genes, where the effect of one gene is altered or masked by another gene, is called epistasis and dealing with it in statistical analysis can be very demanding (Cordell 2002). Furthermore, based on twin studies, the contribution of genetic factors to susceptibility to complex diseases is typically below 60% (Boomsma *et al.* 2002). Thus environmental factors evidently have a significant role, which increases the complexity even further.

Other factors that complicate the search for complex disease genes include phenocopies and the variability of disease phenotype within families (Thornton-Wells *et al.* 2004). Phenocopies are individuals who have the disease phenotype but different underlying genetic susceptibility factors. This can be a major problem especially when studying migraine or some other highly prevalent disease. Furthermore, migraine is a good example of a disease with phenotypic variability. The two common forms of migraine, migraine with and without aura, are both commonly present in the same families and the same patient can have both types of attacks (Blau 1995; Kallela *et al.* 2001b).

The main difficulty in identifying genetic susceptibility factors for complex diseases is, however, the fact that an individual variant will in most cases have only a small effect on disease risk. To overcome this problem, many different study designs have been developed but the suitability and success of these depends dramatically on the assumptions that have been made. Thus, to be able to choose the most successful design, we should know what we are looking for. Key parameters are the number of genes involved, the frequency of the risk

alleles and the magnitude of the effect these alleles have on risk. There is continuous debate over the allelic structure of common diseases (Reich and Lander 2001; Pritchard and Cox 2002; Smith and Lusis 2002). The common disease – common variant (CD-CV) hypothesis suggests that relatively common alleles with weak effect size could predispose to a disease and that each underlying locus contains only one or a few disease alleles (Lander 1996; Cargill *et al.* 1999; Reich and Lander 2001). An alternative viewpoint is that the allelic structure of complex diseases resemble that seen in monogenic diseases, i.e. multiple rare alleles with moderate to large effect size (Terwilliger and Weiss 1998; Smith and Lusis 2002). Most of the confirmed alleles associated with common diseases tend to support the CD-CV hypothesis, but this could be due to the fact that such genes are easier to identify (Lohmueller *et al.* 2003). Prototypical examples include the APOE ε4 allele in Alzheimer's disease and the PPARγ Pro12Ala allele in type II diabetes (Corder *et al.* 1993; Altshuler *et al.* 2000).

Another open question is the type of sequence variations we should expect to find behind complex diseases. Previously, mainly variations located in the coding sequences of genes (cSNPs) have been sought but recent studies have identified causal or at least strongly disease-associated polymorphisms in the regulatory regions of genes (Enattah *et al.* 2002; Pajukanta *et al.* 2004). From the large number of SNPs localized within the non-transcribed genomic regions, those in evolutionarily conserved (and thus also potentially functionally important) areas are more likely to be associated with diseases (Dermitzakis *et al.* 2005). The information provided by comparative genomics methods can be used to select and prioritize these SNPs. It is also worth mentioning that most of the identified cSNPs associated with complex diseases were recently shown to affect amino acids that are far less conserved than those causing Mendelian diseases and thus likely to have milder effects on protein function (Thomas and Kejariwal 2004).

2.2.2 Study designs

2.2.2.1 Candidate gene strategy

Candidate gene studies are always hypothesis-driven. Genes are selected either on the basis of their location in a previously identified susceptibility region (positional candidates) or because of some evidence of their possible connection with the disease (functional candidates) (Tabor *et al.* 2002). The main drawback of this method is that in most cases the pathophysiology of the studied disease is still relatively poorly understood and thus selecting the most likely candidate among the 25,000 predicted human genes (International Human Genome

Sequencing Consortium 2004) can be extremely difficult. A population based (case-control) association study with multiple SNPs covering the selected gene(s) is the most common type of candidate gene study although family-based studies are also possible. Preferably, the gene should be resequenced in a group of cases and controls to find every variation within the gene. This is, however, quite expensive and still rarely done. Furthermore, in most cases only the coding sequence of the gene is sequenced (Hirschhorn and Daly 2005).

2.2.2.2 Genome-wide scanning

The aim of genome-wide scanning is to localize the disease gene by establishing statistically significant evidence of linkage or association. Since the whole genome is screened, no prior information about the location or the function of the gene is needed. The success of identifying complex disease genes by performing a genome-wide linkage analysis followed by traditional positional cloning has been limited. Signals are often weak and inconsistent: less than one third of the scans published until December 2000 showed significant evidence of linkage (Altmuller *et al.* 2001). However, this could mostly be due to the use of too sparse marker maps. In fact, it has been shown that dense re-genotyping of existing sample sets can add to the amount of information extracted considerably (Sawcer *et al.* 2004). Linkage analysis has been shown to be less powerful than association analysis for identifying common genetic variants with modest effects (<3-fold) on disease risk (Risch and Merikangas 1996; Risch 2000). Furthermore, the susceptibility regions identified by linkage analysis are often rather wide, typically exceeding 10 cM (Glazier *et al.* 2002). Once a susceptibility region has been identified, it must be restricted as much as possible by fine-mapping and looking for evidence of association (LD mapping). On the other hand, the advantage of linkage analysis is that allelic heterogeneity does not affect the results.

Genome-wide association studies performed thus far have mainly been limited to population isolates, in which LD can be detected over long distances using microsatellites (Ophoff *et al.* 2002). The proposal of using SNPs for genome-wide association screening in complex disease research was first made around a decade ago (Risch and Merikangas 1996; Collins *et al.* 1997) and the potential of this method has been evaluated in several recent reviews (Carlson *et al.* 2004; Hirschhorn and Daly 2005; Wang *et al.* 2005). The real breakthroughs have, however, thus far not occurred, but evidence of the efficacy of this method is now gradually mounting. As an example, a polymorphism causing age-related macular regeneration in a gene coding for complement factor H was recently identified by several groups, one of which performed a genome-wide association screen (Edwards *et al.* 2005;

Haines *et al.* 2005; Klein *et al.* 2005). On the other hand, this study design has also raised considerable controversy mainly because it is likely to succeed only if there is a single predominant disease allele at a given disease locus. This is because new mutations most likely arise on independent haplotypes and thus cancel out each other's signals (Terwilliger and Weiss 1998; Slager *et al.* 2000; Weiss and Terwilliger 2000).

Two approaches have been suggested for estimating the number of SNPs to be genotyped in a genome-wide association study. In direct association studies only functional variants including approximately 50,000 non-synonymous (resulting in amino acid change) SNPs plus an unknown number of regulatory SNPs would be tested (Risch and Merikangas 1996; Kruglyak and Nickerson 2001). Estimates of the htSNPs needed in an LD-based indirect approach vary from 170,000 to 500,000 in European populations (Gabriel *et al.* 2002; Goldstein *et al.* 2003; Carlson *et al.* 2004). The costs of large-scale genotyping are still a major limiting factor although pooling of samples has been suggested to be an effective way to reduce costs (Sham *et al.* 2002). However, the precise construction of the pools is demanding and requires multiple concentration determination steps, the detection power of pooled samples in the case of modest-risk alleles can be low and haplotype analysis of pooled data is complicated (Sham *et al.* 2002). Although genotyping of hundreds of thousands of SNPs might be feasible in the future, major challenges such as unrealistically large sample sizes needed due to the multiple testing problem are still faced (Risch and Merikangas 1996).

2.2.3 Burden of proof

Lack of reproducibility of published genetic associations has lately become a major concern. True variability between populations can explain some of this inconsistency and could be due to either different patterns of LD (when the causative variant is not directly studied), disease alleles that are specific to one ethnic group or different effect sizes because of modifying genetic or environmental factors (Colhoun *et al.* 2003). However, false positive and false negative studies are currently regarded as more likely explanations for these discrepancies (Colhoun *et al.* 2003). Multiple testing in modest sample sizes can easily produce associations significant at the 5% level by chance alone and preferential publication of positive results, the so-called publication bias, aggravates this effect. In addition, it has been shown that even if the association is true, the initial positive studies tend to overestimate the size of the genetic effect (Göring *et al.* 2001; Ioannidis *et al.* 2001). This winner's curse effect can lead to a situation where subsequent replication attempts are underpowered and the results thus false

negatives (Colhoun *et al.* 2003). Other reasons for false negative results are variations in study design, for example testing of a slightly different phenotype or a nearby genetic variant.

Replication of an association using an independent sample set is regarded as convincing evidence of a true finding, especially if the sample is large and population-based. Seeing an association both in a case-control setting and in a family based study increases the confidence further. However, associations can often be proven only by performing a meta-analysis where results of all published studies are combined (Ioannidis *et al.* 2001; Lohmueller *et al.* 2003). At the end, functional tests are needed to provide conclusive evidence of causality of the associated SNP in the disease pathogenesis. Using *in vitro* systems, the effect of SNPs on gene or protein expression and function can be tested. These effects can, however, be highly context dependent and seen only in certain cell types or experimental conditions and thus difficult to interpret. Producing transgenic animals with a similar phenotype as in humans is probably the most convincing evidence although these findings can also be hard to interpret (Glazier *et al.* 2002).

2.2.4 Solutions and success stories

Based on meta-analyses, the number of identified polymorphisms truly associated with complex diseases is below 50, but since this is a very rapidly moving target, the correct number is probably higher (Ioannidis *et al.* 2001; Lohmueller *et al.* 2003). The *NOD2* gene underlying Crohn's disease, a chronic inflammatory bowel disease, is commonly regarded as the first complex disease gene identified using positional cloning (Todd 2001). In 2001, five years after linkage to chromosome 16 had been established (Hugot *et al.* 1996), two independent groups showed an association of *NOD2* variants with this disease (Hugot *et al.* 2001; Ogura *et al.* 2001). *NOD2* is also a good example of a complex disease gene that has been identified in spite of moderate allelic heterogeneity. In **Table 3**, some examples of genetic variants associated with complex diseases are listed.

In spite of these successes, a typical situation in the complex disease field at the moment is that for any particular trait several whole-genome linkage analyses have been performed but most of the identified linkage signals are weak and found in only one study. Thus the number of consistently replicated loci is relatively small and in only a few of these have the gene and variant in question been conclusively identified. An excellent example is schizophrenia in which at least 18 susceptibility regions scattered over almost all chromosomes have been observed (O'Donovan *et al.* 2003). On the other hand, a recent meta-analysis of 20

Table 3. Examples of genetic variants associated with common forms of complex diseases. These variants are thought to be directly causal and not merely part of an associated haplotype.

Gene	Allele	Allele frequency	OR-Het	OR-Hom	Disease	Reference(s)
INS	small VNTR promoter alleles	0.85	NA	2.7	Type I diabetes	Bell *et al.* 1984
APOE	ε4 (C112R)	0.15	3	12	Alzheimer's disease	Corder *et al.* 1993
PPARγ	P12A	0.85 (P)	1.25*		Type II diabetes	Altshuler *et al.* 2000
NOD2	several coding variants	0.06 (combined)	2-4	~40	Crohn's disease	Hugot *et al.* 2001; Ogura *et al.* 2001
PTPN22	R620W	0.17	1.7#	3.4#	Type I diabetes + other autoimmune diseases	Bottini *et al.* 2004; Siminovitch 2004
CFH	Y402H	0.39	2.4	5.9	Age-related macular degeneration	Edwards *et al.* 2005; Haines *et al.* 2005; Klein *et al.* 2005

OR-Het = odds ratio for heterozygotes versus normal homozygotes; OR-Hom = odds ratio for variant homozygotes versus normal homozygotes; NA = not available; * allelic OR; # for type I diabetes

schizophrenia genome scans concluded that the linkage results are more consistent than has been previously recognized (Lewis *et al.* 2003). Furthermore, various association studies have also produced promising results since possible causative genes have been identified in six schizophrenia susceptibility loci (Hennah *et al.* 2004). The progress has, however, been very slow and many investigators feel that the results of complex disease genetics have thus far been rather disappointing. In the following sections some strategies that can improve the chances of identifying genetic factors for complex diseases are discussed.

2.2.4.1 Monogenic disease models

Much of the progress in understanding complex diseases has come from studying closely related rare monogenic forms of these diseases. These monogenic forms are usually more severe and/or have earlier onset than their complex counterparts. Examples of this kind of disease genes are genes coding for presenilin and amyloid precursor proteins in Alzheimer's disease (Nussbaum and Ellis 2003), MODY (maturity-onset diabetes of youth) genes in diabetes (Fajans *et al.* 2001), and *BRCA1&2* genes in breast and ovarian cancer (de la Chapelle and Peltomäki 1998). A popular hypothesis is that less severe variants in these genes might contribute to susceptibility to the more common subtypes of these diseases. Indeed, in two recent studies the resequencing approach has been successfully applied to candidate genes selected using this criterion: rare missense variants were identified in genes causing the Mendelian forms of colorectal cancer (Fearnhead *et al.* 2004) and low high-density lipoprotein cholesterol levels (Cohen *et al.* 2004) in individuals with multifactorial counterparts of these diseases.

2.2.4.2 Endophenotypes and animal models

One approach to decrease genetic complexity is to make use of intermediate or endophenotypes that are probably controlled by fewer susceptibility factors than the disease state itself (Bearden *et al.* 2004). Many of these intermediate phenotypes are quantitative traits, such as serum IgE and bronchial hyper-reactivity in asthma, plasma lipid concentrations and blood pressure in ischemic stroke, and cognitive traits in psychiatric disorders (Wright *et al.* 1999; Laitinen *et al.* 2004; Paunio *et al.* 2004). However, a typical problem in these kind of studies is the fact that the heritability of the studied traits has often not been established (Bearden *et al.* 2004). Alternatively, in association analysis 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. In some situations it may also be possible to decrease genetic complexity by using inbred animal strains for quantitative trait locus mapping (Cox and Brown 2003). After susceptibility loci or variants have been identified in an animal model, their role in the human disease phenotype can be examined. Naturally, animal models are of very limited use in diseases such as migraine where diagnoses are based on a clinical interview.

2.2.4.3 Isolated populations

The population history of Finland is quite unique. Based on Y chromosome haplotype variation data, the Finns are thought to descend from two different groups of settlers, Uralic speakers arriving from the east about 4,000 years ago and Indo-European speakers arriving from the south about 2,000 years ago (Kittles *et al.* 1998; Peltonen *et al.* 2000). During the 1500s and the 1600s, the population spread from the coasts (so-called early settlement regions) to the eastern and northern parts of the country thus creating regional subisolates (late settlement) (Peltonen *et al.* 2000; Varilo and Peltonen 2004). Until the beginning of the 1700s the Finnish population grew very slowly and several bottlenecks, such as the great famine of 1690s, further strengthened the effect of genetic drift. Population expansion from 250,000 to the present 5,200,000 inhabitants occurred during the past 250 years (Kere 2001). The Finnish disease heritage, a group of 35 mainly recessive diseases more prevalent in Finland than in other populations, has its origins in this population history (Norio *et al.* 1973; de la Chapelle 1993; Peltonen *et al.* 1999).

Isolated populations, such as Finns, Bedouin-Arabs, and the Amish, have proven to be ideal for the identification of genes causing rare monogenic diseases (Sheffield *et al.* 1998; Peltonen *et al.* 2000). An extreme example is the mapping of the infantile-onset spinocerebellar ataxia locus using only four affected Finnish individuals (Nikali *et al.* 1995). Founder populations have also been suggested to possess some advantages for mapping the genes underlying complex diseases (Wright *et al.* 1999). Reduced heterogeneity caused by a small number of founder individuals, subsequent bottlenecks, and genetic drift could have restricted the number of susceptibility alleles present in affected individuals. In addition, good genealogical records, a more uniform environment and lifestyle, as well as more easily standardized diagnostic criteria are obvious advantages of certain isolates (Peltonen *et al.* 2000). It has also been proven that LD often extends over wider intervals in founder populations than in general populations although this seems to apply only for young isolates (Eaves *et al.* 2000; Service *et al.* 2001; Varilo *et al.* 2003). Thus 10-20 generations old isolates such as Costa-Rica, Newfoundland, and internal isolates of Finland have been suggested to be especially powerful for the initial mapping phase (Wright *et al.* 1999). Indeed, several complex disease genes have been identified using positional cloning in the Finnish population (**Table 4**). Some of these studies have taken advantage of increased disease prevalence in certain areas of Finland due to a founder effect, examples being schizophrenia in the Kuusamo region and multiple sclerosis in western Finland (Hovatta *et al.* 1997; Tienari *et al.* 2004). On the other hand, it has been argued that disease alleles present in population isolates do not necessarily contribute to susceptibility in other populations. In a recent study, however, the genetic effects of risk variants were shown to usually be consistent although the allele frequencies can differ considerably across populations (Ioannidis *et al.* 2004).

Table 4. Complex disease genes identified using positional cloning in the Finnish population.

Phenotype	Locus	Subisolate	Gene	Reference(s)
Multiple sclerosis	17q22-q24	Botnia	*PRKCA*	Kuokkanen *et al.* 1997; Saarela *et al.* 2002
Familial combined hyperlipidemia	1q21-23	-	*USF1*	Pajukanta *et al.* 1998; Pajukanta *et al.* 2004
Schizophrenia*	1q42	Kuusamo ¤	*DISC1*	Ekelund *et al.* 2001; Hennah *et al.* 2003
Asthma	7p14-p15	Kainuu	*GPRA*	Laitinen *et al.* 2001; Laitinen *et al.* 2004
Dyslexia*	15q21	-	*DYX1C1*	Taipale *et al.* 2003
Psoriasis #	6p21.3	-	*HCR*	Asumalahti *et al.* 2002
Type II diabetes	20q12-q13	-	*HNF4A*	Ghosh *et al.* 1999; Silander *et al.* 2004
Obesity	Xq24	-	*SLC6A14*	Suviolahti *et al.* 2003

* locus was first identified using a translocation breakpoint; # samples from other populations were also included; ¤ (also whole Finland)

The deCODE project in Iceland is a unique example of identifying complex disease genes using genealogical tools. The parliament of Iceland gave permission for the deCODE company to anonymously link the genealogical records to health care records of all the 275,000 inhabitants of Iceland except for those who have asked to be excluded (Gulcher and Stefansson 2000). Thus deCODE has an exceptional opportunity to build large multigenerational families with several affected individuals. This strategy has proven to be very effective since, according to their website (http://www.decode.com), by August 2005 deCODE has identified 28 susceptibility loci and provided evidence of a causative gene in 12 of these. Some examples of identified genes are neuregulin 1 in schizophrenia, genes coding for 5-lipoxygenase activating protein and phophodiesterase 4D in myocardial infarction and stroke, and bone morphogenetic protein 2 gene in osteoporosis (Stefansson *et al.* 2002; Gretarsdottir *et al.* 2003; Styrkarsdottir *et al.* 2003; Helgadottir *et al.* 2004).

3 CLASSIFICATION OF HEADACHE DISORDERS

Since 1988, a generally accepted International Classification of Headache Disorders (ICHD), published by the International Headache Society (IHS), has been available (Headache Classification Committee of the International Headache Society 1988). During 2004 the second edition of this classification, ICHD-2, was published (Headache Classification Committee of the International Headache Society 2004). These criteria have been the scientific basis for diagnosing the heterogeneous group of headache disorders in most subsequent studies. According to the ICHD-2 criteria, headaches are divided into primary and secondary headaches. Headache is classified as secondary if it is attributed to some other disorder such as neck and/or head trauma, cranial or cervical vascular disorder (for example subarachnoid hemorrhage), and infections (for example bacterial meningitis) (Headache Classification Committee of the International Headache Society 2004). Primary headaches are classified into four subgroups: 1) migraine, 2) tension-type headache, 3) cluster headache, and 4) other trigeminal autonomic cephalalgias and other primary headaches (Headache Classification Committee of the International Headache Society 2004).

Tension-type headache is the most common headache disorder with a 1-year prevalence of approximately 60% (Ulrich *et al.* 2004). Environmental factors were recently shown to be of major importance in this disease and thus genetic factors seem to have only a minor role (Ulrich *et al.* 2004). The chronic form of tension-type headache has, however, a prevalence of only 2-3% and first-degree relatives have a 2.1 to 3.9-fold increased risk, but no genetic

factors predisposing to this disease have yet been identified (Russell *et al.* 1999; Ulrich *et al.* 2004). Cluster headache is regarded as the most severe primary headache. It is characterized by unilateral headache attacks lasting 15-180 minutes, occurring as frequent clusters and accompanied by ipsilateral autonomic features (Headache Classification Committee of the International Headache Society 2004). Prevalence of this disorder is between 1/500–1/1500 in the Caucasian population (Russell 2004). A 5-8 times increased risk for first-degree relatives has been estimated, suggesting the importance of genetic factors (Russell 2004). Thus far the only gene associated with this disease is *HCRTR2* that codes for a receptor for a newly discovered neuropeptid, hypocretin-2 (Rainero *et al.* 2004a).

4 MIGRAINE

4.1 Diagnosis

Since no diagnostic test for migraine or any other primary headache disorder exists, diagnosis is based on the patient's description of the attacks and exclusion of the other possible secondary causes of headache. To standardize migraine diagnosis, criteria published by the IHS is now widely accepted at least for scientific purposes (Headache Classification Committee of the International Headache Society 2004). **Table 5** presents the classification of

Table 5. Migraine sub-types according to the ICHD-2 (Headache Classification Committee of the International Headache Society 2004).

1.1	**Migraine without aura**
1.2	**Migraine with aura**
1.2.1	Typical aura with migraine headache
1.2.2	Typical aura with non-migraine headache
1.2.3	Typical aura without headache
1.2.4	Familial hemiplegic migraine
1.2.5	Sporadic hemiplegic migraine
1.2.6	Basilar-type migraine
1.3	**Childhood periodic syndromes that are commonly precursors of migraine**
1.3.1	Cyclical vomiting
1.3.2	Abdominal migraine
1.3.3	Benign paroxysmal vertigo of childhood
1.4	**Retinal migraine**
1.5	**Complications of migraine**
1.5.1	Chronic migraine
1.5.2	Status migrainosus
1.5.3	Persistent aura without infarction
1.5.4	Migrainous infarction
1.5.5	Migraine-triggered seizure
1.6	**Probable migraine**
1.6.1	Probable migraine without aura
1.6.2	Probable migraine with aura
1.6.5	Probable chronic migraine

migraine subtypes according to ICHD-2. Based on the presence or absence of the aura phase, migraine can be divided into two major subtypes, migraine without aura (MO) being more common and usually more disabling (Headache Classification Committee of the International Headache Society 2004). Migraine with aura (MA) can be further classified into several subtypes (**Table 5**). In **Table 6**, diagnostic criteria for migraine without aura (ICHD-1.1) and for typical aura with migraine headache (ICHD-1.2.1) are listed (Headache Classification Committee of the International Headache Society 2004).

Table 6. ICHD-2 diagnostic criteria for migraine with and without aura (Headache Classification Committee of the International Headache Society 2004).

1.1	**Migraine without aura**
	A) At least 5 attacks fulfilling criteria B-D
	B) Headache attacks lasting 4-72 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 (*eg*, walking or climbing stairs)
	D) During headache at least one of the following:
	1. Nausea and/or vomiting
	2. Photophobia and phonophobia
	E) Not attributed to another disorder
1.2.1	**Typical aura with migraine headache**
	A) At least 2 attacks fulfilling criteria B-D
	B) Aura consisting of fully reversible visual symptoms, sensory symptoms, and/or dysphasic speech disturbance, but no motor weakness
	C) At least two of the following:
	1. Homonymous visual symptoms and/or unilateral sensory symptoms
	2. At least one symptom develops gradually over ≥5 minutes and/or different aura symptoms occur in succession over ≥5 minutes
	3. Each symptom lasts 5-60 minutes
	D) Headache fulfilling criteria B-D for 1.1 begins during the aura or follows aura within 60 minutes
	E) Not attributed to another disorder

Of the MA subtypes, familial hemiplegic migraine (FHM) (ICHD-1.2.4) is known to be autosomal dominantly inherited and can be regarded as the monogenic disease model for the more common types of migraine. To be diagnosed as having FHM, a patient must have MA attacks including motor weakness and at least one first- or second-degree relative with similar attacks. Sporadic hemiplegic migraine (ICHD-1.2.5) patients have comparable attacks but no affected family members. Other important MA subtypes are the typical aura without headache (ICHD-1.2.3), in which patients simply have attacks with aura without the following

headache, and basilar-type migraine (ICHD-1.2.6), in which patients have MA symptoms originating from the brainstem such as dysarthria, vertigo, tinnitus, and simultaneous bilateral visual, sensory, or motor symptoms (Headache Classification Committee of the International Headache Society 2004).

4.2 Clinical features

4.2.1 Clinical features of MA and MO

The age of migraine onset is typically 10-40 years of age (Silberstein *et al.* 1998). Patients experience a median of 1 attack per month and 25% have at least two attacks per month (Launer *et al.* 1999). Migraine attack can be divided into four phases: premonitory symptoms, aura phase, headache phase, and resolution phase. Premonitory symptoms (prodrome) occur in one third of patients, hours to 1-2 days before the headache and include symptoms such as tiredness, yawning, neck stiffness, mood change, and gastrointestinal symptoms (Kelman 2004). One third of migraine patients have, at least occasionally, aura, a focal, gradually developing neurological symptom that typically precedes the headache and lasts for less than 60 minutes (Russell *et al.* 1995a; Launer *et al.* 1999). Patients often have multiple types of aura, the most common symptoms being visual disturbances (99% of patients) such as zig-zag lines and scotomas, followed by sensory (31%), aphasic (speech abnormalities; 18%) and motor (6%) symptoms (Russell and Olesen 1996).

The headache phase is the most debilitating part of the migraine attack. As the ICHD-2 criteria describe, typical migraine headache is unilateral, throbbing, moderate to marked in severity, and aggravated by physical activity. In adults, untreated headache attacks usually last 4-72 hours (Headache Classification Committee of the International Headache Society 2004). Headache is always accompanied by other symptoms, most typically nausea (90% of patients) (Silberstein 1995). Vomiting occurs in almost 70% of patients and sensitivity to noise (phonophobia) and light (photophobia) are also common (Silberstein 1995). Following the headache, in the so-called resolution phase, patients can feel exhausted, unusually refreshed or even euphoric (Silberstein *et al.* 1998).

4.2.2 Clinical features of hemiplegic migraine

FHM was first described in 1910 (Clarke 1910). FHM attacks are characterized by the presence of motor aura symptoms. This motor aura consists of gradually progressing unilateral motor weakness or paralysis and is almost invariably preceded by other aura symptoms (Thomsen *et al.* 2002a). The age of onset of this disease is typically 10-15 years

(Ducros *et al.* 2001). Some patients occasionally have severe attacks with impaired consciousness ranging from confusion to coma, fever, prolonged hemiplegia and/or seizures (Ducros *et al.* 2001). A mild head trauma is a typical trigger of such attacks (Ducros *et al.* 2001). In about 20% of FHM families the disease is associated with permanent neurological symptoms, mainly cerebellar ataxia and nystagmus (Ducros *et al.* 2001).

4.3 Epidemiology

As with other complex disorders, it is very important to make a distinction between population-based and clinic-based epidemiological studies of migraine. Those patients that seek medical help for their headaches naturally represent the most severely affected migraineurs and may differ significantly from unselected subjects. Thus this kind of study setting should not be used for epidemiological research. From the other point of view, medical diagnosis records should not be used for migraine prevalence estimations, since a significant proportion of migraine patients never consult a clinician because of headaches.

4.3.1 Prevalence

Prevalence is the proportion of the population with a disease during a particular period of time, typically either one year (active) or lifetime. The first prevalence study using ICHD criteria was performed in Denmark and reported a one-year migraine prevalence of 10% (6% in men, 15% in women) and a lifetime prevalence of 16% (8% in men, 25% in women) (Rasmussen *et al.* 1991). Since then, several other studies performed in western countries have confirmed the overall one-year prevalence of migraine to be 10-12% (Stewart *et al.* 1992; Russell *et al.* 1995a; Launer *et al.* 1999; Hagen *et al.* 2000; Lipton *et al.* 2001). These studies have also consistently shown that migraine has a male to female ratio of 1:2-3 (one-year prevalence of 6-8% among men and 15-18% among women). Although migraine is common in all races, it seems to be more prevalent in Caucasians than in Africans or Asians. This trend can be seen both in the admixed population of the United States (Stewart *et al.* 1996) and, even more clearly, in the recent population studies performed, for example, in Tanzania and Japan where the reported overall one-year migraine prevalence is 5-6% (Dent *et al.* 2004; Takeshima *et al.* 2004).

The only Finnish population-based epidemiological study of headache is based on a questionnaire mailed to twins in 1981 (Honkasalo *et al.* 1993). In this study, the overall prevalence of migraine was 10.1% among women and only 2.5% among men. Unfortunately,

this study has some limitations since the ICHD criteria were not available at the time of the study and the questionnaire was not designed for distinguishing between MA and MO.

Although the epidemiology of migraine in general has been thoroughly studied, population-based data on the prevalence of migraine subtypes are sparse. One-year prevalence of MA is estimated to be 4% and of MO 6% (Rasmussen 2001). Lifetime prevalences of MA and MO in Denmark are shown in **Figure 1** (Russell *et al.* 1995a). Since the various subtypes of MA are so rare, their epidemiology has not been thoroughly studied. As a rough estimate, approximately 100 FHM families and a similar number of patients with sporadic hemiplegic migraine have been described (Thomsen *et al.* 2002b). Recently, however, population-based prevalences of FHM and sporadic hemiplegic migraine have been studied in Denmark. Interestingly, from the entire Danish population of 5.2 million inhabitants, a population size very similar to Finland, 147 FHM patients from 44 different families were identified. The total prevalence of hemiplegic migraine was estimated to be 0.008-0.009 % and the familial and sporadic forms were found to be equally frequent (Thomsen *et al.* 2002b).

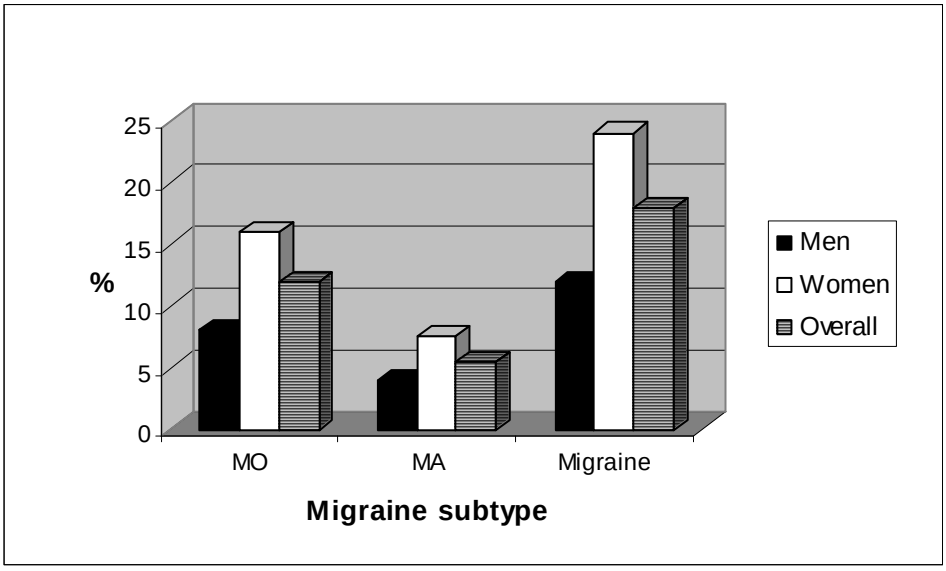

Figure 1. Lifetime prevalences of migraine with and without aura (Russell *et al.* 1995a).

4.3.2 Environmental and internal factors

Given the fact that only around 40-60% of the predisposition to migraine is believed to be due to genetic factors (Honkasalo *et al.* 1995; Larsson *et al.* 1995), environmental factors are clearly important. Twin studies have shown that individual environment is more important than the shared family environment (Ziegler *et al.* 1998; Svensson *et al.* 2003). However, no associations between MA and several socioeconomic and environmental factors including education, marital status, smoking and alcohol intake could be seen in the only published study using a discordant MZ twin pair approach (Ulrich *et al.* 2000). Furthermore, none of the

sociodemographic variables or factors related to lifestyle studied showed association with migraine in a population-based Danish epidemiological study, the only exception being sleep pattern (Rasmussen 1992, 1993). Because migraine is an episodic disorder, environmental triggering factors probably have an important role in precipitating attacks. Typically reported triggers include stress or the relief of stress, too much or too little sleep, weather changes, flickering lights or strong smells, missing a meal, and certain food triggers such as red wine and chocolate (Silberstein *et al.* 1998).

Before puberty, migraine is slightly more common in boys than in girls but in children aged over 12, a female preponderance is evident (Sillanpää 1983; Abu-Arefeh and Russell 1994). The increased prevalence of migraine in women is generally thought to reflect the additional trigger of fluctuating female hormones during the menstrual cycle. Although pure menstrual migraine (defined as attacks occurring exclusively between days -2 to +3 of menstruation) is rare, menstrually related attacks are very common and are believed to be due to falling concentrations of estrogen (Bousser 2004; MacGregor 2004). In a Swedish population-based study, 21% of women with MO and 4% of women with MA reported ≥ 75% of their attacks being menstrually related (Mattsson 2003). In a recent clinic-based study, migraine attacks were 1.7-2.5 times more likely to occur on days -2 to +3 of menstruation than at all other times and, furthermore, these menstrual attacks tended to be more severe and almost exclusively without aura (MacGregor and Hackshaw 2004). In addition, the effects of pregnancy, oral contraceptives, menopause and hormone replacement therapy on the course of migraine speak for the importance of hormonal factors in this disease (Bousser 2004).

4.4 Pathophysiology

The pathophysiology of migraine is still relatively poorly understood, although recent discoveries have increased our knowledge concerning the sequence of events leading to a migraine attack. Some phenomena are almost certainly essential, but the order and the cause-consequence relationship of these events are still debated. Both neuronal and vascular components seem to have a role in the pathophysiology of this disease and, in addition to the cerebral cortex, the brainstem and the trigeminovascular system are involved.

Wolff's classical view was that migraine aura is due to vasoconstriction and the throbbing headache is caused by dilated and pulsating blood vessels (Wolff 1963). Olesen and coworkers (1990) were able to prove this theory wrong by showing that a focally reduced regional cerebral blood flow (rCBF) is the first observable event during migraine attack, after

which the aura symptoms develop. Furthermore, they showed that headache begins before rCBF starts to gradually increase to an abnormally high level.

The causal connection of migraine aura and headache is still a matter of debate. An alternative view is that aura and headache are parallel rather than sequential events and that some as yet unidentified process could be responsible for both of these symptoms (Spierings 1988; Goadsby 2001). Evidence of the brainstem as the migraine generator exists given that during migraine attacks activated brain stem nuclei have been detected (Weiller *et al.* 1995; Bahra *et al.* 2001; Cao *et al.* 2002).

4.4.1 The interictal brain status – impaired cortical information processing

Recent electrophysiological studies performed during the interictal phase of migraine have demonstrated that migraine patients are characterized by a dysfunction in cortical information processing. In normal subjects, repetition of a stimulus produces amplitude reduction of the evoked cortical response, i.e. habituation. However, decreased habituation or even potentiation has been consistently observed in migraineurs (Ambrosini and Schoenen 2003). The lack of habituation could contribute to the enhanced susceptibility of migraine patients to many sensory stimuli, such as flashing lights and strong odors, which are well-known migraine attack precipitating factors. Interestingly, one study has also provided evidence of a genetic component in cortical-evoked potentials in 20 parent-child pairs affected by migraine (Sandor *et al.* 1999).

Although the lack of habituation in migraineurs has been proven, it is still debated whether it is due to an increased or decreased cortical preactivation level. A more widely accepted hypothesis states that the habituation deficit is due to a transient or persistent hyperexcitability of the cortex caused by either increased cortical excitability or decreased inhibition (Welch *et al.* 1990). According to the alternative hypothesis, decreased excitability of the cortex could be the underlying cause. In this “ceiling effect” model, habituation occurs only when cortical activation reaches the ceiling, i.e. slower if the cortical pre-activation level is low (Schoenen 1996).

4.4.2 Pathophysiology of the aura

More than 60 years ago, Lashley (1941) described the features of a typical visual aura – scintillation-scotoma that starts at the visual field center, then propagates to the peripheral parts within 10-15 minutes and finally returns to normal within another 15 minutes. These

characteristics were soon realized to strikingly resemble another phenomenon, cortical spreading depression (CSD) first described in animals by Leao (Leao 1944; Milner 1958). CSD is a slowly (3-5 mm/min) propagating neuronal and glial depolarization wave that spreads across the cortex and leads to a long-lasting suppression of neural activity along with dramatic changes in ion homeostasis (Lauritzen 1994). Modern neuroimaging techniques have enabled continuous recording during aura in humans. Several recent studies indicated a slowly spreading area of abnormal neuronal suppression during aura and provided clear evidence for support of the hypothesis that aura is evoked by an electrophysiological event similar to CSD and that vascular events develop due to these fluctuations in neuronal activity (Cutrer *et al.* 1998; Cao *et al.* 1999; Bowyer *et al.* 2001; Hadjikhani *et al.* 2001).

4.4.3 Pathophysiology of the headache

The meninges, layers that protect the central nervous system, are the only pain-sensitive part of the brain. Meningeal blood vessels are heavily innervated by trigeminal nerve fibers that serve to alert as well as defend brain tissue and headache can be regarded as a consequence of the activation of this alert system (Moskowitz and Macfarlane 1993). Trigeminal neurons are bipolar neurons whose cell bodies are located in the trigeminal ganglion and whose central projections make synaptic connections with the trigeminal nucleus caudalis in the brainstem. These fibers can be activated both by primary disturbances in the brain and other events occurring in the circulation or inside the vessel walls. Trigeminal axons contain vasoactive neuropeptides such as substance P, calcitonin gene-related peptide, and neurokinin A. In experimental animal models, release of these inflammatory mediators from activated trigeminal nerve endings initiates an inflammatory reaction ("neurogenic inflammation") characterized by vasodilation and plasma protein leakage within dura mater (Markowitz *et al.* 1987; Moskowitz and Macfarlane 1993). The headache in migraine is believed to result from similar activation of trigeminal neurons and the subsequent inflammatory reaction in the meninges (Moskowitz *et al.* 1979). Chemically-activated trigeminal afferents become sensitized and start to respond to normally innocuous mechanical stimuli. This explains how pulsating blood vessels can produce the throbbing pain of migraine and why coughing, bending over and other physical activities that increase intracranial pressure can worsen the pain (Strassman *et al.* 1996). Activated trigeminal neurons also transmit signals centrally to the trigeminal nucleus caudalis in the brainstem and to other brain structures involved in processing pain. On the other hand, defected descending antinociceptive activity of the

brainstem and subsequent decreased inhibition could make the trigeminovascular system hyperexcitable (Knight and Goadsby 2001).

4.4.4 Central sensitization and triptans

Using a rat model of intracranial pain Burstein and coworkers (2000) were able to show that after chemical simulation of the peripheral trigeminal neurons the central brainstem trigeminal neurons also become sensitized and start to respond to previously innocuous stimuli. These results could explain the fact that migraine is often associated with cutaneous allodynia, pain resulting from a non-noxious stimulus (for example heat, cold, or pressure) to normal skin. Cutaneous allodynia develops in 53-79% of patients and, interestingly, allodynamic patients have a longer history of migraine and experience attacks more frequently than non-allodynic patients (Burstein *et al.* 2000; Mathew *et al.* 2004).

Triptans, specific acute anti-migraine drugs, are serotonin (5-hydroxytryptamine; 5-HT) receptor agonists specific to receptor subtypes 5-$HT_{1B/1D}$. There are several explanations for their antimigraine effect including cranial vasoconstriction, inhibition of neurogenic inflammation, and inhibition of the excitability of trigeminovascular neurons by stimulating receptors in the brain stem (Ferrari 1998). Burstein's team has shown that migraine should be treated with triptans before the development of central sensitization (Burstein *et al.* 2004). If triptans are administrated late in the attack, patients are much less likely to be rendered pain-free although the throbbing of migraine pain is effectively terminated. Based on this finding and subsequent animal experiments, the authors suggested that the mechanism of action of triptans is to block synaptic transmission between peripheral and central trigeminovascular neurons (Levy *et al.* 2004). However, triptans have also been shown to activate antinociceptive descending pathways in the brainstem (Bartsch *et al.* 2004).

4.4.5 How aura can lead to headache

A recent study by Bolay and coworkers (2002) was the first to establish a pathophysiological connection between migraine aura and headache. They were able to demonstrate that CSD initiation in rats activates trigeminal afferents and evokes a series of meningeal and brainstem events that generate the headache. These events include meningeal vasodilation and plasma protein leakage within the dura mater. Furthermore, expression of c-fos, a marker for neuronal activity, was shown to be enhanced within the trigeminal nucleus caudalis, a brainstem region involved in the processing of nociceptive information. Trigeminal activation could be caused by CSD-induced changes in the composition of the extracellular fluid, such as increases in K^+,

H^+, neurotransmitter, and metabolite concentrations (Bolay *et al.* 2002). CSD has also been shown to induce changes in gene expression, especially expression of vasoactive peptides and L-type calcium channels (Choudhuri *et al.* 2002). On the other hand, the fact that the majority of migraine patients do not have aura is strong evidence against the causal relationship of migraine aura and headache. However, it has been suggested that in MO patients CSD could occur in a clinically silent area of the cerebral cortex (Woods *et al.* 1994; Cao *et al.* 1999).

4.5 The burden of migraine

4.5.1 Socio-economic impacts

Migraine and other headache disorders constitute an enormous public-health problem with an impact on both individual patients and society. Although migraine certainly affects the quality of life, the personal impact is difficult to measure. The health-related quality of life in migraineurs has been assessed using validated questionnaires in a general population (Lipton *et al.* 2000; Terwindt *et al.* 2000). In both of these studies migraineurs reported diminished mental and physical functioning and well-being. In addition to the suffering caused by the attacks, fear of the next attack restricts the lifestyle of many migraine patients. Migraine can have a major disturbing effect on normal family functioning, especially childcare and social activities. In a study performed in the United States, 61% of patients reported that their attacks had a significant effect on their families and in 5% were even the cause of divorce (Smith 1998). According to the Burden of Disease Statistics published yearly by the World Health Organization (WHO), 7666 (0.5% of total) disability-adjusted life-years (defined as one lost year of healthy life) were due to migraine in 2002 (www.who.int/whr/2004).

Socio-economic impacts of migraine are substantial and include both direct costs associated with utilization of health care services and indirect costs associated with lost workplace productivity. The indirect costs of migraine predominate (Lipton *et al.* 1997). The absence from work because of migraine was estimated at 270 days per year per 1000 employed in Denmark (Rasmussen *et al.* 1992). This is, however, an underestimation, since most of the work-loss because of migraine is due to reduced effectiveness (Schwartz *et al.* 1997). Severe migraine can also lead to unemployment (Lipton *et al.* 1997).

4.5.2 Comorbidity

Comorbidity, defined as the co-existence of two disorders with a frequency greater than chance, of migraine has been thoroughly studied. Diseases repeatedly associated with

migraine include neurological disorders such as epilepsy and stroke, psychiatric disorders such as depression, bipolar disorder and anxiety disorders as well as immunologic conditions such as asthma and allergy (Merikangas *et al.* 1990; Tzourio *et al.* 1993; Ottman and Lipton 1994; Lipton *et al.* 2000; Terwindt *et al.* 2000; Breslau and Rasmussen 2001; Radat and Swendsen 2005). Of these, the association between migraine and epilepsy is especially interesting since it has been speculated to be based on an underlying state of neuronal hyperexcitability that increases the risk of both of these disorders (Lipton *et al.* 1994). Migraine has also been shown to be a risk factor for ischemic stroke, this association being especially evident in young female MA patients (Tzourio *et al.* 1993; Tzourio *et al.* 1995; Chang *et al.* 1999; Etminan *et al.* 2005).

4.5.3 Migraine – a progressive disorder?

Two recent large studies, a population-based Dutch magnetic resonance imaging (MRI) study and a meta-analysis, have demonstrated an increased prevalence of subclinical white matter lesions in migraine patients (Kruit *et al.* 2004; Swartz and Kern 2004). Lesions in the cerebellum, the part of the brain vulnerable in FHM, were shown to be more common particularly in patients with MA and a high attack frequency (Kruit *et al.* 2004). However, more studies are needed to clarify whether these clinically silent brain lesions are a consequence of tissue damage caused by migraine attacks and whether migraine should actually be regarded as a progressive disorder (Lipton and Bigal 2005). Encouragingly, when comparing twins affected with migraine and their unaffected co-twins, migraine was not associated with deficits in cognitive functioning (Gaist *et al.* 2005).

5 GENETICS OF MIGRAINE

5.1 Genetic epidemiology

Familial occurrence of a disease is assumed to be either due to shared genes or shared environmental factors. The relative importance of genetic and environmental factors on the phenotype can be estimated using different family- and twin-study designs and statistical methods (Kaprio 2000).

5.1.1 Twin studies – the role of genetic and environmental factors

The most common and also the simplest twin study design is to compare the similarity (concordance) of monozygotic (MZ) and dizygotic (DZ) twins and thus assess the inherited component of a disease. If the concordance for the disease in MZ twins is greater than in DZ twins, genetic factors are believed to be important (Kaprio 2000). The structural equation modeling technique is a more sophisticated method for estimating the contribution of genetic factors in disease susceptibility. The aim of this method is to determine the combination of genetic and environmental liability factors that best fit the data. Heritability (h^2) is defined as the proportion of the overall phenotypic variance attributable to genetic factors (Kaprio 2000).

Several large population-based twin studies in migraine have been performed. The Finnish study published in 1995 produced a heritability estimate of 40-50% (Honkasalo *et al.* 1995). The recently established GenomEUtwin project (Peltonen 2003) includes almost 30,000 twin pairs affected by migraine from six countries (Mulder *et al.* 2003). In this patient material, the estimated genetic variability of migraine was shown to be 34-56% (**Table 7**). Based on structural equation modeling, additive genetic factors and non-shared environmental factors seemed to contribute significantly to the susceptibility to migraine while shared environmental factors did not seem to have any impact. Furthermore, two migraine studies comparing twins raised together and apart have been performed (Ziegler *et al.* 1998; Svensson *et al.* 2003). Neither of these studies provided any evidence of a role of shared rearing environment in migraine susceptibility.

Table 7. Heritability estimates for migraine in general according to the GenomEUtwin data (adapted from Mulder *et al.* 2003).

	Twin correlations*		h^2	Total pairs
Population	MZ	DZ	(ADE model)	(N)
Australia	0.34	0.15	0.34	2718
Denmark	0.56	0.21	0.56	1882
Finland, younger	0.41	0.12	0.41	1912
Finland, older	0.51	0.22	0.51	8187
Netherlands, younger	0.53	0.22	0.53	1139
Netherlands, older	0.52	0.28	0.52	330
Sweden	0.44	0.13	0.44	12121
UK	0.42	0.17	0.42	1428

* tetrachoric correlation of liability; MZ = monozygotic; DZ = dizygotic; h^2 = heritability; ADE = model including additive genetic (A), non-additive genetic (D) and non-shared environmental (E) influences

Although one of the GenomEUtwin project aims is to separate migraine subtypes in the future, studies based on the Danish Twin Registry are by far the only ones that have provided information on the specific concordance rates for MO and MA (Gervil *et al.* 1999a; Gervil *et al.* 1999b; Ulrich *et al.* 1999b; Ulrich *et al.* 1999a). Based on these results, the concordance rates for MO seem to be only a little lower than for MA. Furthermore, the heritability estimates for both migraine subtypes exceed 60% (**Table 8**).

Table 8. Heritability estimates and familial risks for migraine subtypes according to Danish studies (Russell and Olesen 1995; Gervil *et al.* 1999a; Gervil *et al.* 1999b; Ulrich *et al.* 1999b; Ulrich *et al.* 1999a).

	MO		MA	
Twin studies	MZ	DZ	MZ	DZ
Pairwise concordance rate*	0.28	0.18	0.34	0.12
Tetrachoric correlation of liability	0.62	0.41	0.68	0.22
Heritability #	0.61		0.65	
Family studies				
Population relative risk among first-degree relatives	1.9		3.8	

* the proportion of concordant pairs among all affected pairs (i.e. pairs in which at least one twin is affected); # estimated using structural equation model fitting

5.1.2 Family studies

Familial aggregation of migraine has been known for centuries but the high prevalence of the disease could cause this simply by chance (Merikangas 1996). Two population-based studies performed in the 1990's showed that the familial risk of migraine is indeed increased (Russell and Olesen 1995; Stewart *et al.* 1997). The American study found only modest evidence of increased migraine risk (~1.5-fold) in first-degree relatives but suggested familial aggregation to be associated with the severity of the disease (Stewart *et al.* 1997). The Danish study showed that first-degree relatives of probands with MO had a 1.9-fold increased risk of MO and 1.4 of MA while first-degree relatives of probands with MA had a 3.8-fold increased risk of MA and no increased risk of MO (**Table 8**) (Russell and Olesen 1995). In addition, they found that the spouses of MO probands had a 1.5-fold risk of MO and concluded that MO seems to be caused by a combination of genetic and environmental factors while MA is mainly determined by genetic factors (Russell and Olesen 1995).

5.1.3 Mode of inheritance

Complex segregation analysis performed in a population-based Danish sample indicated that the multifactorial inheritance model is most likely in both MO and MA (Russell *et al.* 1995b). A study analyzing a set of 31 high-risk MA families provided further support for this result

(Ulrich *et al.* 1997). However, the possibility of a subgroup of families having a Mendelian or mitochondrial pattern of inheritance still remains (Russell *et al.* 1995b).

5.2 Linkage studies

5.2.1 Genome-wide scans

It is somewhat encouraging that despite the known difficulties of complex disease linkage studies, seven successful whole-genome scans have been conducted for migraine (**Table 9**). Five of these have been performed in multiple migraine families, our study being the first that was published (Wessman *et al.* 2002). Both in this and a subsequent study by the Icelandic deCODE a susceptibility locus on chromosome 4q was identified. However, in our study significant linkage to the MA phenotype was observed, while the deCODE study used MO with a slightly relaxed diagnostic criteria as a phenotype (Wessman *et al.* 2002; Björnsson *et al.* 2003). The Canadian study showed that there is a susceptibility locus for MA also in chromosome 11q (Cader *et al.* 2003). Interestingly, this susceptibility region contains several ion channel genes. A recently published Australian genome-scan showed suggestive evidence of linkage to 18p11 using a "severe migraine" phenotype, determined with latent class analysis (LCA), as the affection criterion (Nyholt *et al.* 2004; Lea *et al.* 2005). Subsequently, this LCA phenotyping has been used in another Australian genome-scan involving twin pairs (Nyholt *et al.* 2005). This study produced evidence of a migraine susceptibility locus on chromosome 5q21 and associated this locus specifically with pulsating headache (Nyholt *et al.* 2005). The two other studies, each carried out with a single large family, identified susceptibility loci on 14q in an Italian MO family (Soragna *et al.* 2003) and on 6p in a Swedish family with both MA and MO patients (Carlsson *et al.* 2002).

Table 9. Genome-wide screens on migraine.

Identified locus	Migraine subtype	Family material	Patients (N)	LOD score	Reference
4q24	MA	50 Finnish	246	4.5	Wessman *et al.* 2002
6p12.2-p21.1	MA, MO*	1 Swedish	30	5.8	Carlsson *et al.* 2002
11q24	MA	43 Canadian	248	5.6	Cader *et al.* 2003
4q21	MO*	117 Icelandic	351	4.1#	Björnsson *et al.* 2003
14q21.2-q22.3	MO	1 Italian	22	5.3	Soragna *et al.* 2003
3q-tel 18p11	"LCA-severe"	92 Australian	380	2.3# 2.3#	Lea *et al.* 2005
5q21	"LCA-migraine"	790 Australian sib-pairs	556	3.7¤	Nyholt *et al.* 2005

MA = migraine with aura; MO = migraine without aura; LCA = latent class analysis; * slightly relaxed diagnostic criteria; # nonparametric LOD score; ¤ MERLIN-regress LOD score

5.2.2 Candidate locus studies

The contribution of several candidate chromosomal regions including 1q23, 1q31, 19p13, 15q11-q13, and the X-chromosome to migraine susceptibility has been investigated. A functional candidate locus on 15q11-q13 that contains several gamma-amino butyric acid (GABA) receptor genes has recently been shown to be linked to MA in 10 Italian families (Russo *et al.* 2005). Chromosomal areas 1q23, 1q31, and 19p13 have been studied because of their known or suspected involvement with FHM, the autosomal dominant form of migraine (Joutel *et al.* 1993; Ducros *et al.* 1997; Gardner *et al.* 1997). Most thoroughly studied is the 19p13 region that contains the *CACNA1A* gene, mutations of which cause FHM (Ophoff *et al.* 1996a). Results of these studies are summarized in **Table 10**. It has been thought that the same cellular pathways could underlie both common and rare migraine subtypes but the overall evidence for *CACNA1A* involvement in MA or MO is weak even though some studies have reported suggestive linkage (May *et al.* 1995; Nyholt *et al.* 1998a; Terwindt *et al.* 2001). Furthermore, *CACNA1A* screening was negative even in one Australian family showing linkage to 19p13 (Lea *et al.* 2001b). In addition, several other investigators have searched for

Table 10. Studies on the *CACNA1A* migraine susceptibility locus on chromosome 19p13.

Linkage analysis studies					
Families (N)	**Nationality**	**Methods**	**Results**	**Phenotype**	**Reference**
4	Finnish	Parametric linkage	negative	MA+MO	Hovatta *et al.* 1994
28	German	ASP	LOD 1.38	MA+MO	May *et al.* 1995
4	Australian	Parametric linkage, NPL	HLOD 3.59 NPL 6.64*	MA+MO	Nyholt *et al.* 1998a
82	Australian	NPL, association	negative	MA+MO	Lea *et al.* 2001a
36	Dutch	ASP	LOD 1.41	MA	Terwindt *et al.* 2001
64	Canadian	Linkage, TDT	negative	MA	Noble-Topham *et al.* 2002
72	Finnish	Parametric linkage, NPL	negative	MA	Kaunisto *et al.* 2005
Mutation screening studies					
Patients (N)	**Nationality**	**Methods**	**Results**	**Phenotype**	**Reference**
9	USA	SSCP	negative	migraine + vertigo	Kim *et al.* 1998
1 family linked to 19p13	Australian	Sequencing	negative	MA+MO	Lea *et al.* 2001a
32	Italian	SSCP	negative	MA	Brugnoni *et al.* 2002
143	German	Searching for known mutations	negative	MA+MO	Wieser *et al.* 2003
50	USA	dHPLC	negative	MA+MO	Jen *et al.* 2004b

ASP = affected sib-pair; LOD = logarithm of odds; HLOD = LOD score under heterogeneity; NPL = nonparametric linkage; TDT = transmission disequilibrium test; SSCP = single-strand conformation polymorphism; dHLPC = denaturing high-performance liquid chromatography; * GENEHUNTER Z score

CACNA1A mutations in migraine patients but the results have been consistently negative (Kim *et al.* 1998; Brugnoni *et al.* 2002; Wieser *et al.* 2003; Jen *et al.* 2004b).

Interestingly, another locus on 19p13 that is close to but separate from *CACNA1A* has been connected with migraine. Thirteen out of 16 MA families were shown to be linked to a region containing the insulin receptor gene (*INSR*), thus providing evidence of a major susceptibility locus for MA (Jones *et al.* 2001). This or the subsequently published association of MA to *INSR* polymorphisms (McCarthy *et al.* 2001) has, however, not yet been replicated.

Due to its more recent discovery, involvement of the FHM2 gene, *ATP1A2*, in common migraine has been studied considerably less than the contribution of *CACNA1A*. No *ATP1A2* mutations were found in 50 probands with migraine with or without aura (Jen *et al.* 2004b). On the other hand, in a very recent publication, rare *ATP1A2* variants were identified in two out of 45 migraine families and thus the gene was proposed to be involved also in the susceptibility to common migraine (Todt *et al.* 2005). The clinical details and functional results of this study are, however, somewhat confusing and thus no firm conclusions can be drawn at the moment. Nevertheless, it is worth mentioning that some evidence of linkage to chromosomal area 1q21-23 containing the *ATP1A2* gene has been found in two recent Australian migraine whole-genome scans (Lea *et al.* 2005; Nyholt *et al.* 2005).

Furthermore, the same Australian group has found some evidence of the presence of migraine susceptibility loci on 1q31 and Xq24-28 (Nyholt *et al.* 1998b; Nyholt *et al.* 2000; Lea *et al.* 2002). Two families showing linkage to this X-chromosomal region were identified and a candidate gene *HTR2C* coding for a serotonin receptor 2C was studied but no evidence of association or potential mutations were found (Johnson *et al.* 2003).

5.3 Association studies

Numerous association studies using mainly the case-control setting have been performed in migraine. Unfortunately, the impact of these studies is relatively modest since only one or two polymorphisms per candidate gene have been studied in a small number of patients. Furthermore, in most cases confirmatory replication studies have not been published. An exception is the C677T polymorphism of the methylenetetrahydrofolate reductase gene (*MTHFR*) that decreases the enzyme activity to less than half of that of the wild type and thus leads to a mild hyperhomocysteinemia. This functional SNP has been suggested to be associated with MA in Turkish, Spanish, Japanese, and Australian populations (Kowa *et al.*

2000; Kara *et al.* 2003; Lea *et al.* 2004; Oterino *et al.* 2004). Other frequently studied candidate genes code for hormone receptors, serotonergic and dopaminergic signaling components, nitric oxide synthases, and components of the endothelin system (Lea *et al.* 2001a; Tzourio *et al.* 2001; Palotie *et al.* 2002; Colson *et al.* 2005). To mention a few, migraine has been significantly associated with variants in both estrogen and progesterone receptors as well as promoter variants of the endothelin type A receptor, tumor necrosis factor α, and serotonin transporter genes (Tzourio *et al.* 2001; Colson *et al.* 2004; Rainero *et al.* 2004b; Colson *et al.* 2005; Marziniak *et al.* 2005).

6 CHANNELS AND CHANNELOPATHIES

6.1 Ion channels and pumps and their role in neuronal signaling

Ion channels are pore-forming membrane proteins through which specific ions can passively diffuse down an electrochemical gradient at a very high rate (~10^7 ions per second) (Ackerman and Clapham 1997). They are thus responsible for the selective ion permeability of the cell membrane and central to the function of excitable cells, but are also found in non-excitable cells and in a wide range of organisms from bacteria and fungi to mammals and plants (Anderson and Greenberg 2001). There are more than 400 channel genes in the human genome (Gargus 2003). Typically ion channels are highly selective for the type of ion that can pass through, and based on this selectivity channels are classified into potassium (K^+), sodium (Na^+), calcium (Ca^{2+}), chloride (Cl^-), and unspecific cation channels (Hübner and Jentsch 2002). Ion channels open and close in a tightly regulated way via a process called gating. Based on the gating-mechanism, channels can be grouped into channels gated by changes in the membrane potential (voltage-gated), by the binding of extracellular or intracellular ligands (ligand-gated), and by physical stimuli (swelling- or stretch-activated and heat sensitive channels) (Hübner and Jentsch 2002).

Most members of the superfamily of voltage-gated channels share a common molecular structure. The pore forming (α_1) subunits of the Na^+ and Ca^{2+} channels contain four sets of six hydrophobic transmembrane segments (S1-S6) and intracellular N- and C-terminal domains (see **Figure 2**, page 46). K^+ channels are the largest and most diverse class of voltage-gated channels. Most K^+ channels span the membrane only six times, thus four such subunits must aggregate to form a functional channel. However, some K^+ channels such as the inwardly rectifying K^+ channels, consist of only two transmembrane segments (2TM) resembling the S5 and S6 regions of the other channels (Armstrong and Hille 1998). It is likely that

analogous 2TM channels are the evolutionary starting point of the whole superfamily of voltage-gated ion channels since they are also found in prokaryotes (Armstrong and Hille 1998).

Ion pumps carry out the essential task of generating and maintaining concentration gradients by actively transporting specific ions against their gradients. Pumps can either use the electrochemical gradients of other ions (ion exchangers/antiporters) or directly acquire energy by hydrolyzing adenosine triphosphate (ATP) (ATPase pumps) (Purves *et al.* 1997). Na^+,K^+-ATPases found in the plasma membranes of virtually all animal cells are a classic example of such ion pumps. They are electrogenic E1-E2 type ion pumps and members of the P-type (phospho-intermediate type) ATPase family (Kaplan 2002). They transport three Na^+ ions out and two K^+ ions into the cell per every ATP-molecule hydrolyzed.

In neurons, as well as in all other animal cells, electrical transmembrane potential is based on the two factors described above: 1) selective ion permeability of the cell membrane that is largely achieved by ion channels and 2) ion concentration differences across the cell membranes created by energy-consuming ion pumps. Most importantly, the intracellular concentrations of K^+ and Na^+ ions in, for example, mammalian neurons are 140 and 5-15 mM while the extracellular concentrations are 5 and 145 mM (Purves *et al.* 1997). Certain K^+ channels that are open also in the resting cell and thus allow K^+ ions to leak out are mainly responsible for maintaining the negative resting membrane potential of -40 to -90 mV in neurons (Purves *et al.* 1997).

Neurons send information over long distances via action potential. Using squid giant axons, Alan Hodgkin and Andrew Huxley were able to describe the principles of the propagation of action potential more than 50 years ago (Hodgkin and Huxley 1952). When the membrane potential of a neuron depolarizes and reaches a threshold potential, an action potential occurs. Depolarization opens the voltage-gated Na^+ channels, which leads to a brief influx of Na^+ ions, further depolarization and the propagation of an action potential. A slower activation of voltage-gated K^+ channels leads to an efflux of K^+ ions and subsequent restoration of the resting-level membrane potential. When an action potential reaches a synapse, a specialized area that connects two interacting neurons, voltage-gated Ca^{2+} channels open. The increased internal Ca^{2+} concentration allows neurotransmitter-filled synaptic vesicles to fuse with the presynaptic membrane. Released neurotransmitters then diffuse throughout the synaptic cleft, bind to receptors in the plasma membrane of the postsynaptic neuron, and activate ligand-

gated ion channels (fast synaptic transmission) or other regulatory proteins (slow synaptic transmission).

6.2 Voltage-gated Ca^{2+} channels

Calcium regulates various neuronal processes. Basically, in all cells intracellular Ca^{2+} levels are held 10,000 fold lower than the extracellular levels and cells are activated when the intracellular Ca^{2+} concentration rises from the resting level of 100 nM to 1000 nM (Berridge *et al.* 2000). In neurons, the Ca^{2+} signal is generated mainly by allowing the entry of extracellular Ca^{2+} into the cell through activated voltage-gated or ligand-gated Ca^{2+} channels. This signal is then amplified using intracellular sources of Ca^{2+}, namely Ca^{2+} stored within the endoplasmic reticulum and released through Ca^{2+}-inducible inositol 1,4,5-triphosphate and ryanodine receptors (Berridge 1998).

Voltage-gated Ca^{2+} channels are composed of various subunits (**Figure 2**). α_1 subunits consist of intracellular C- and N-termini and four repeated domains (I to IV) each containing six

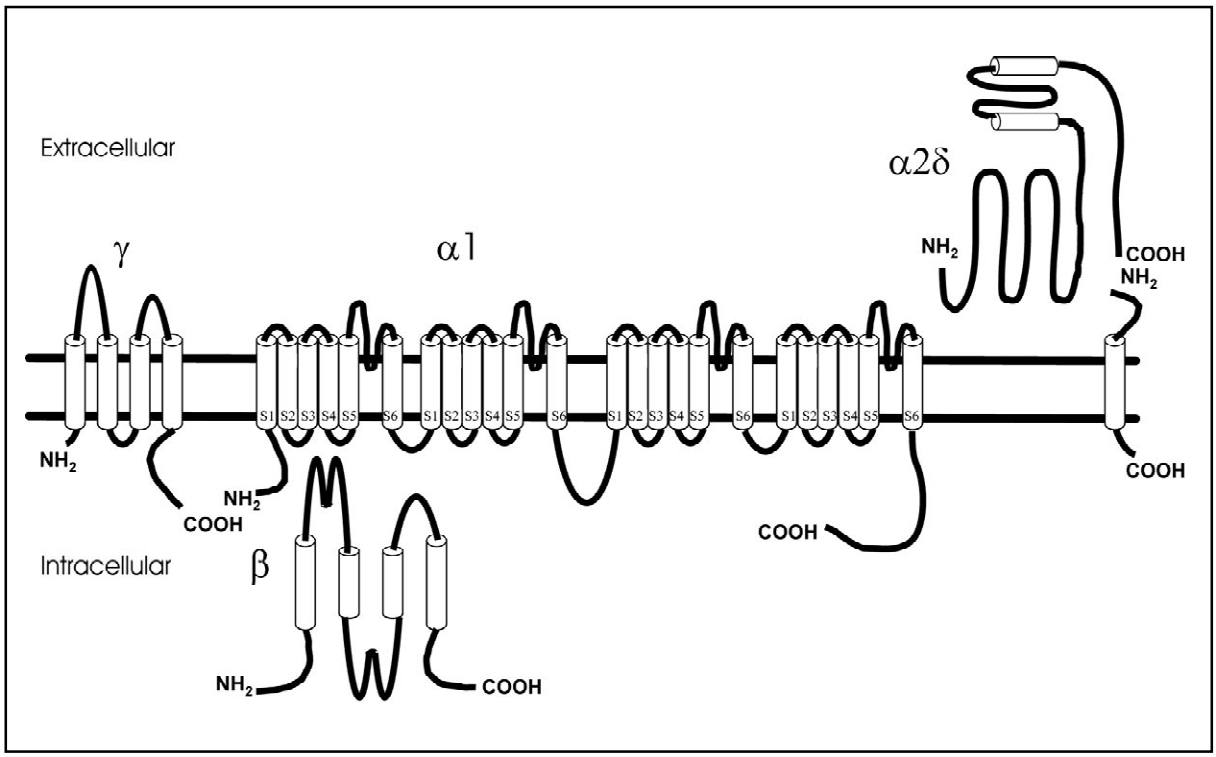

Figure 2. Voltage-gated calcium channel subunits (modified from Catterall 2000). α_1 subunit of 190-250 kDa is the main pore-forming channel subunit while three auxiliary subunits, $\alpha_2\delta$, β, and γ fine-tune channel function (Ertel *et al.* 2000). Intracellular β subunits have a large, isoform-specific effect on channel kinetics (Moreno *et al.* 1997). In addition, they are responsible for trafficking of the channel to the plasma membrane by masking the endoplasmic reticulum retention signal located in loop I-II (Bichet *et al.* 2000). The heavily glycosylated, mainly extracellular $\alpha_2\delta$ subunit and the γ subunit that contains four transmembrane regions also modify the electrophysiological properties of α_1 subunits (Catterall 2000).

membrane spanning segments (S1 to S6) and a membrane-associated loop between segments S5 and S6 (Tanabe *et al.* 1987). S4-S6 segments are the functionally most important channel parts. The S5 and S6 segments and the pore loops located between them form the pore lining responsible for the ion selectivity of voltage-gated ion channels. The 20 amino acids long pore loops narrow the pore of the channel and contain ion specificity-determining signature sequences (Armstrong and Hille 1998). Typically voltage-gated channels respond to membrane polarization by opening. The voltage sensor of the channel is formed by the S4 segments containing positively charged amino acids that move in response to voltage. This leads to a conformational change that opens the channel (Catterall 1995).

Ten genes coding for α_1 subunits have been identified while the auxiliary subunits are encoded by 3-8 genes each. Furthermore, at least α_1 and β subunits are subject to alternative splicing thereby adding to the complexity of possible channel subunit compositions (Pietrobon 2002). The ten α_1 subunits are grouped into three subfamilies (Ca_v1, Ca_v2 and Ca_v3) based on amino acid sequence similarities (**Table 11**) (Ertel *et al.* 2000). The amino acid sequences of α_1 subunits are more than 70% identical within a subfamily but less than 40% identical among subfamilies (Ertel *et al.* 2000). Furthermore, several types of electrophysiologically and pharmacologically distinct Ca^{2+} currents, named as L-, N-, P-, Q-,

Table 11. Voltage-gated calcium channel (Ca_v) α1 subunits.

α1 subunit	Gene	Location	Current type	Primary tissues	Related human diseases	Reference(s)
$Ca_v1.1$	*CACNA1S*	1q31-q32	L	skeletal muscle	hypoKPP, MHS	Ptacek *et al.* 1994; Monnier *et al.* 1997
$Ca_v1.2$	*CACNA1C*	12p13.3	L	heart, endocrine, neurons	Timothy syndrome	Splawski *et al.* 2004
$Ca_v1.3$	*CACNA1D*	3p14.3	L	endocrine, neurons	-	
$Ca_v1.4$	*CACNA1F*	Xp11.23	L	retina	CSNB2	Bech-Hansen *et al.* 1998
$Ca_v2.1$	*CACNA1A*	19p13.1-2	P/Q	neurons	FHM1, EA-2, SCA-6, IGE	Ophoff *et al.* 1996b; Zhuchenko *et al.* 1997; Chioza *et al.* 2001
$Ca_v2.2$	*CACNA1B*	9q34	N	neurons	-	
$Ca_v2.3$	*CACNA1E*	1q25-q31	R	neurons	-	
$Ca_v3.1$	*CACNA1G*	17q22	T	heart, skeletal muscle, neurons	-	
$Ca_v3.2$	*CACNA1H*	16p13.4	T	heart, neurons	absence epilepsy	Chen *et al.* 2003
$Ca_v3.3$	*CACNA1I*	22q2.3-q13.2	T	neurons	-	

hypoKPP = hypokalemic periodic paralysis; MHS = malignant hyperthermia susceptibility; CSNB2 = X-linked congenital stationary night blindness; FHM = familial hemiplegic migraine; EA = episodic ataxia; SCA = spinocerebellar ataxia; IGE = idiopathic generalized epilepsy

R-, and T-type currents, exist but the correlation with the different α_1 subunits is somewhat loose (**Table 11**) (Catterall 2000).

The Ca_v1 subfamily includes channels mediating L-type (long-lasting) currents while channels belonging to the Ca_v2 subfamily mediate primarily neuronal P/Q-, N-, and R-type currents (Ertel *et al.* 2000). T-type (transient) currents are mediated by channels belonging to the Ca_v3 subfamily. L-type currents are the main type of current in muscle and endocrine cells. This type of current, as well as N-, P/Q-, and R-type currents expressed mainly in neurons, requires a strong depolarization (~50 mV) for activation unlike T-type current which is activated at much more negative potentials and expressed in many cell types (Catterall 2000).

Different parts of the neurons contain specific Ca^{2+} channels that perform separate functions. Presynaptic membranes have N- and P/Q-type voltage-gated channels that trigger the release of neurotransmitters into the presynaptic cleft. The cell body and dendrites contain mainly L-type voltage-gated and ligand-gated Ca^{2+} channels that act in the generation of Ca^{2+} signals important in the modification of the strength of synaptic transmission. Furthermore, Ca^{2+} has been shown to regulate both long term potentiation and long term depression, processes known to be the basis of learning and memory (Augustine *et al.* 2003). These various effects of Ca^{2+} can only be achieved using an extensive network of signaling components and, indeed, numerous proteins such as calmodulin, troponin C, and synaptotagmin activate in response to increased Ca^{2+} concentration (Berridge *et al.* 2000).

6.3 Channelopathies

Channelopathies are a recently identified and rapidly expanding group of disorders defined as diseases caused by defective ion channels. The first channelopathies, characterized approximately 15 years ago, were a group of muscle disorders called periodic paralysis and nondystrophic myotonias (Ptacek *et al.* 1991; McClatchey *et al.* 1992). Since then, many different ion channel genes have been implicated in the pathophysiology of various disorders affecting the skeletal muscle, heart, peripheral nerves, and brain. In addition to the traditional genetic channelopathies, two other channelopathy forms have been identified. Autoimmune channelopathies include diseases such as Lambert-Eaton myasthenic syndrome in which channel function is disturbed due to antibodies raised against $Ca_v2.1$ channels (Lennon *et al.* 1995). Transcriptional channelopathies, on the other hand, are caused by dysregulated

transcription of non-mutated channel genes as a result of injury to neurons (Waxman 2001). An example of this kind of disease process is peripheral nerve injury, which can lead to altered Na^+ channel expression resulting in hyperexcitability, neuropathic pain and paresthesiae (Waxman 2001).

Practically all channelopathies are rare and episodic in nature and most have their onset in childhood. Furthermore, the attacks are precipitated by largely overlapping factors such as stress and fatigue and are treated with similar drugs, for instance carbonic anhydrase inhibitors (Ptacek and Fu 2004). The phenotype both within and between families is often highly variable. Locus heterogeneity is common since mutations in different ion channel genes can cause extremely similar phenotypes (Kullmann and Hanna 2002). A good example of this is the long QT syndrome caused by mutations in several different Na^+ and K^+ channel genes (Bulman 1997). On the other hand, different mutations in the same gene can lead to diverse phenotypes; for instance *CACNA1A* gene mutations cause both episodic ataxia and familial hemiplegic migraine (Ophoff *et al.* 1996a). Interestingly, channelopathies typically affect only one specific tissue type although the gene in question can be expressed in many other tissues. A recently described exception is a specific *CACNA1C* mutation that causes Timothy syndrome, a multisystem disorder with symptoms as diverse as arrhythmia, developmental abnormalities, and autism (Splawski *et al.* 2004). There are several possible mechanisms by which an ion channel gene mutation can cause a disease, including gain or loss of function of the channel, and dominant negative effects (Felix 2000). In order to understand the functional consequences of potential disease-causing mutations, functional *in vitro* tests, typically performed using patch clamp methods, are needed.

Although the best understood channelopathies are those affecting either cardiac contractility, such as long QT syndrome, or muscle-fiber excitability, for instance periodic paralysis and myotonias, many neuronal channelopathies have also been described. In **Table 12**, these central nervous system disorders are listed (Kullmann and Hanna 2002). Most of these diseases belong to a group of childhood idiopathic epilepsies. Several subtypes of this phenotype have been shown to be caused by mutations in both voltage-gated and ligand-gated ion channels and primary neuronal hyperexcitability is believed to be the underlying disease mechanism (Steinlein 2004). Other examples of neuronal channelopathies are autosomal dominant episodic ataxias, characterized by either brief (EA-1) or prolonged (EA-2) episodes of cerebellar incoordination (**Table 12**).

Table 12. Neurological channelopathies (excluding diseases associated with blindness or deafness).

Channel type	Gene	Phenotype(s)	Reference(s)
Voltage-gated			
K^+ channels	*KCNA1*	EA-1	Browne *et al.* 1994
	KCNQ2	BFNC	Singh *et al.* 1998
	KCNQ3	BFNC	Charlier *et al.* 1998
Na^+ channels	*SCN1A*	GEFS+ , SMEI, FHM	Escayg *et al.* 2000b; Dichgans *et al.* 2005
	SCN2A	GEFS+	Sugawara *et al.* 2001
	SCN1B	GEFS+	Wallace *et al.* 1998
Ca^{2+} channels	*CACNA1A*	FHM1, EA-2, SCA-6, IGE	Ophoff *et al.* 1996b; Zhuchenko *et al.* 1997; Chioza *et al.* 2001
	CACNA1C	Timothy syndrome	Splawski *et al.* 2004
	CACNA1H	Childhood absence epilepsy	Chen *et al.* 2003
	CACNB4	Juvenile myoclonic epilepsy	Escayg *et al.* 2000a
Cl^- channels	*CLCN2*	Absence epilepsy	Haug *et al.* 2003
Cyclic-nucleotide gated			
K^+ channels	*KCNMA1*	GEPD	Du *et al.* 2005
Ligand-gated			
Nicotinic acetylcholine receptors	*CHRNA4*	ADNFLE	Steinlein *et al.* 1995
	CHRNB2	ADNFLE	De Fusco *et al.* 2000
Glycine receptors	*GLRA1*	Familial hyperekplexia	Shiang *et al.* 1993
GABA-receptors	*GABRA1*	Juvenile myoclonic epilepsy	Cossette *et al.* 2002
	GABRG2	GEFS+	Wallace *et al.* 2001

EA = episodic ataxia; BFNC = benign familial neonatal convulsions; GEFS+ = generalized epilepsy with febrile seizures plus; SMEI = severe myoclonic epilepsy of infancy; FHM = familial hemiplegic migraine; SCA = spinocerebellar ataxia; IGE = idiopathic generalized epilepsy; GEPD = generalized epilepsy and paroxysmal dyskinesia; ADNFLE = autosomal dominant nocturnal frontal lobe epilepsy; GABA = gamma-amino butyric acid

6.4 Diseases caused by *CACNA1A* mutations

CACNA1A codes for the α_1 subunit of $Ca_v2.1$ channels. The complementary DNA (cDNA) for this gene was first cloned and sequenced from rabbit and rat brain in the beginning of 1990s (Mori *et al.* 1991; Starr *et al.* 1991). The human ortholog (previously called *CACNL1A4*) is located on chromosome 19p13 (Diriong *et al.* 1995). The gene was shown to cover more than 300 kb and consist of 47 exons that encode a protein of 2261 amino acids (Ophoff *et al.* 1996a). Several alternatively spliced exons have since been identified (Soong *et al.* 2002). The $Ca_v2.1$ channels are widely expressed in the presynaptic terminals, cell bodies and dendrites of cerebellar, hippocampal and most other neurons, expression being most prominent in the Purkinje cells (Westenbroek *et al.* 1995). Expression at the neuromuscular junction is also abundant (Protti *et al.* 1996). The $Ca_v2.1$ channels have a predominant role in the regulation of neurotransmitter release by directly interacting with proteins of the synaptic vesicle docking/fusion machinery (Catterall 1998). The postsynaptic $Ca_v2.1$ channels have

other important functions especially in the cerebellum, including shaping neuronal excitability, activity-dependent gene expression, as well as neuronal survival and differentiation (Pineda *et al.* 1998; Sutton *et al.* 1999; Miyazaki *et al.* 2004). $Ca_v2.1$ channels are tightly regulated and fine-tuned by numerous mechanisms as could be expected because of their diverse roles (De Waard *et al.* 1997; Lee *et al.* 1999; Zhong *et al.* 1999).

CACNA1A mutations have been shown to cause at least three neurological diseases, i.e. EA-2, FHM, and spinocerebellar ataxia type 6 (SCA-6). In addition, there is evidence of association to idiopathic generalized epilepsy (Kors *et al.* 2004b).

6.4.1 Episodic ataxia type 2

6.4.1.1 Clinical features

Patients with periodic ataxia were first described in 1946 (Parker 1946). Episodic ataxia type 2 (EA-2; previously also called hereditary paroxysmal cerebellar ataxia) 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 (Vahedi *et al.* 1995). Attacks are often associated with vertigo, nausea, and migraine headaches (Baloh *et al.* 1997). Interictal findings include mainly gaze-evoked nystagmus and mild permanent cerebellar ataxia although in some patients ataxia can also be slowly progressive (Vahedi *et al.* 1995). In rare cases, the clinical phenotype also includes mental retardation and/or epilepsy (Denier *et al.* 1999; Jen *et al.* 2004a). In many ways, this disease can be regarded as a prototype of an ion channel disorder. It is of episodic nature, the phenotype both between and within families is very variable and the attacks can be precipitated by several factors such as emotional stress, physical exercise, alcohol, caffeine, and heat (e.g. sauna). Age of onset is typically in childhood, before the age of 20 (Baloh *et al.* 1997). Acetazolamide, a carbonic anhydrase inhibitor, prevents attacks in most patients (Griggs *et al.* 1978). Although the typical clinical course of EA-2 can be regarded as being relatively benign, fear of the next attack can be stressful and disturb a patients social life similarly to severe migraine.

6.4.1.2 Genetics

EA-2 is inherited as an autosomal dominant trait with incomplete penetrance. Some sporadic cases with *de novo* mutations have also been described (Jen *et al.* 2004a). Significant evidence of linkage to chromosome 19p13 was obtained by several groups during 1995 (Kramer *et al.* 1995; Teh *et al.* 1995; Vahedi *et al.* 1995; von Brederlow *et al.* 1995). A year later, mutations in *CACNA1A* were identified and at the same time the disease was shown to

be allelic to FHM (Ophoff *et al.* 1996a). At least 46 EA-2-causing mutations in the *CACNA1A* gene have been described. Twenty-one of these are nonsense mutations that lead to premature STOP-codons either directly or by deleting or inserting 1, 2 or 4 nucleotides and thus producing a frameshift (**Table 13**). Furthermore, nine splice site mutations (all but one of them affecting the donor splice site) have been identified (**Table 13**). Interestingly, two of these mutations do not involve the invariant GT donor splice site nucleotides, but still cause aberrant splicing (Wan *et al.* 2005a). In addition to the mutations described above, small

Table 13. *CACNA1A* nonsense mutations causing EA-2 (in parenthesis the nomenclature used in the original publication).

Nucleotide and protein change (ref. sequence X99897)	**Location**	**Domain**	**Reference(s)**
IVS6+1G>A→ aberrant splicing	intron 6	I S5-S6	Subramony *et al.* 2003
IVS11+1G>A→ aberrant splicing	intron 11	II S1-S2	Denier *et al.* 1999
del2145-48 TTCA→ FS + stop at aa 658	exon 14	II S5	van den Maagdenberg *et al.* 2002
del2317-8 AG→ FS + stop at aa 780	exon 16	II S5-S6	Denier *et al.* 1999; van den Maagdenberg *et al.* 2002; Jen *et al.* 2004a
”del2259-60 AG→ FS + stop at aa 780”	exon 16	II S6	Denier *et al.* 1999
ins 3091G→ FS + stop at aa 1067	exon 19	II-III loop	Scoggan *et al.* 2001; Rucker *et al.* 2005
ins3689C→ FS + stop at aa 1144	exon 20	II-III loop	Matsuyama *et al.* 2003
del3765C (3772) → FS + stop at aa 1186	exon 20	II-III loop	Spacey *et al.* 2005
IVS21+1G>A→ aberrant splicing	intron 21	II-III loop	Eunson *et al.* 2005
“del3797C→ FS + stop at aa 1293”	exon 22	III S1	Denier *et al.* 1999
del4073C→ FS + stop at aa 1294	exon 22	III S1	Ophoff *et al.* 1996b
C4110T→ R1279X	exon 23	III S1-S2	Yue *et al.* 1998
IVS24+1G>A→ aberrant splicing	intron 24	III S3	Ophoff *et al.* 1996b
IVS24+3insT→ aberrant splicing	intron 24	III S3	Wan *et al.* 2005a
del4451C→ FS + stop at 1430	exon 26	III S5	van den Maagdenberg *et al.* 2002
IVS26+1G>A→ aberrant splicing	intron 26	III S5-S6	Denier *et al.* 1999
C4607G→ Y1444X (1443)	exon 27	III S5-S6	Denier *et al.* 1999
G4621A→ W1449X (1451)	exon 27	III S5-S6	Jen *et al.* 2004a
IVS27+1G>T→ aberrant splicing	intron 27	III S5-S6	Jen *et al.* 2004a
C4914T→ R1547X (1546)	exon 29	III-IV loop	Denier *et al.* 1999; Jen *et al.* 1999
C4959T→ Q1562X (1561)	exon 29	III-IV loop	Spacey *et al.* 2005
del5123G→ FS + stop at aa 1624	exon 30	IV S2	Scoggan *et al.* 2001
C5616T→ R1781X (1785)	exon 35	IV S5-S6	Jen *et al.* 2004a
C5733T→ R1820X*	exon 36	C-terminus	Jouvenceau *et al.* 2001
IVS36-2A>G→ aberrant splicing	intron 36	C-terminus	Kaunisto *et al.* 2004a
del5837-38CA→Y1854X	exon 37A	C-terminus	Kors 2004
C5837G→Y1854X	exon 37A	C-terminus	Kors 2004
del IVS41+3-6→ aberrant splicing	intron 41	C-terminus	Wan *et al.* 2005a; Eunson *et al.* 2005
C6378T→ Q2035X (2039)	exon 42	C-terminus	Jen *et al.* 2004a
del6431C→ FS + stop at aa 2123 (2126)	exon 42	C-terminus	Jen *et al.* 2004a

FS = frameshift; del = deletion; ins = insertion; aa = amino acid; * epilepsy phenotype. If the mutation is inside quotation marks, the reference sequence used in the original publication is not known.

expansions of the CAG-repeat normally causing the SCA-6 phenotype have been identified in two EA-2 families (Jodice *et al.* 1997).

In the thus far largest EA-2 study, 18 families and nine sporadic patients were studied (Jen *et al.* 2004a). Approximately 60% of the EA-2 families were shown to be linked to 19p13, thus it seems that there has to be other EA-2 susceptibility loci but these have not yet been identified. Mutations in *CACNA1A* were found in nine of 11 linked families and in four of nine sporadic patients. Four of these 13 mutations were missense mutations. Other studies have also shown that non-truncating EA-2 mutations are not as rare as previously thought. Thus far 16 different missense mutations have been identified in EA-2 patients (**Figure 3**, **Table 14**). Interestingly, they seem to be clustered in the highly conserved S5-S6 linkers of the channel (Jen *et al.* 2004a; Mantuano *et al.* 2004). It is possible that these parts of the channel are particularly vulnerable and that missense mutations in these areas are more likely to produce the EA-2 phenotype (Mantuano *et al.* 2004). Furthermore, is has been suggested that the clinical phenotype of the patients carrying missense EA-2 mutations is possibly somewhat milder (later age of onset, no mental retardation) than that of patients with truncating mutations (Mantuano *et al.* 2004).

Table 14. Missense mutations and small in-frame deletions that cause EA-2 (in parenthesis the nomenclature used in the original publication).

Nucleotide and protein change (ref. sequence X99897)	**Location**	**Domain**	**Reference**
714G>A → E147K*	exon 3	I S2	Imbrici *et al.* 2004
1032C>T→ H253Y	exon 5	I S5-S6	van den Maagdenberg *et al.* 2002
1041T>C→ C256R	exon 5	I S5-S6	Mantuano *et al.* 2004
1135G>A→ C287Y	exon 6	I S5-S6	Jen *et al.* 2004a
1152G>A→ G293R#	exon 6	I S5-S6	Yue *et al.* 1997
2272C>T→ T666M¤	exon 16	II S5-S6	Jen *et al.* 2004a
4486T>G→F1404C (F1406C)	exon 26	III S5-S6	Jen *et al.* 2001
4722G>A→ G1483R	exon 28	III S6	Mantuano *et al.* 2004
del4739-44GTCCAT→ del MS 1488-9	exon 28	III S6	Mantuano *et al.* 2004
4747T>C→ F1491S	exon 28	III S6	Guida *et al.* 2001
4755G>A→ V1494I	exon 28	III S6	Mantuano *et al.* 2004
del5056-58CTT→ delY1595, A1594D (delY1594, A1593D)	exon 30	IV S2	Denier *et al.* 1999
5260G>A→ R1662H (R1666H)	exon 32	IV S4	Friend *et al.* 1999
5485A>T→H1737L (H1736L)	exon 34	IV S5-S6	Spacey *et al.* 2004
5544G>A→ E1757K	exon 35	IV S5-S6	Denier *et al.* 2001
6681C>T→R2136C	exon 45	C-terminus	Mantuano *et al.* 2004

Additional phenotypic features: * epilepsy, # severe progressive ataxia, ¤ hemiplegic migraine

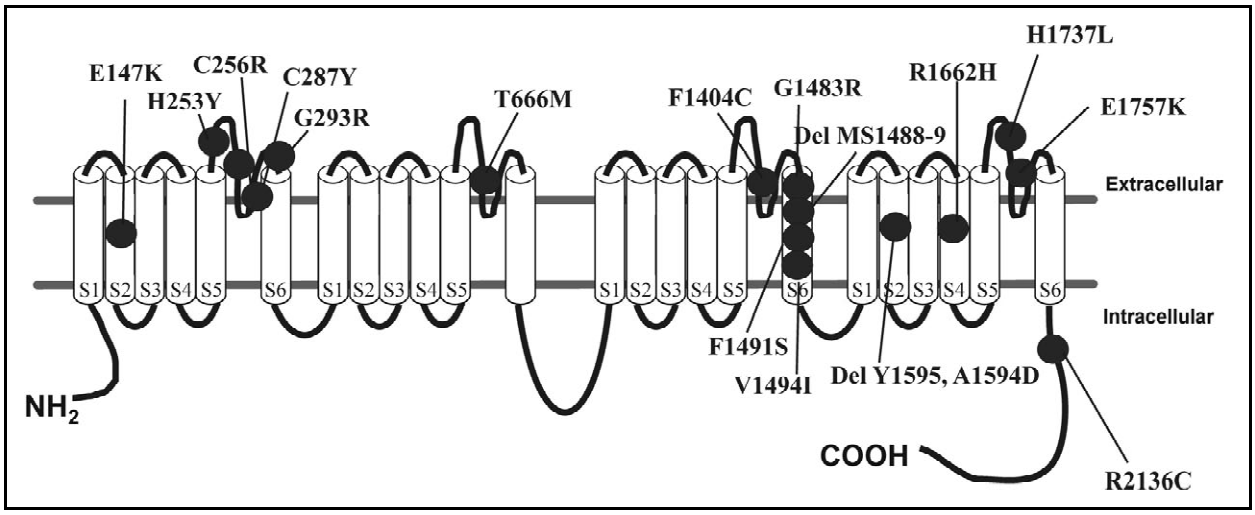

Figure 3. Identified missense EA-2 mutations shown on the $Ca_v2.1$ channel topology.

In summary, a wide range of *CACNA1A* mutations have been shown to cause EA-2. These mutations are located all over the gene and nearly all have been found in only one family or patient. Thus no common mutations that would make genetic testing easier have been identified (Jen *et al.* 2004a).

6.4.1.3 Functional consequences of EA-2 mutations

The pathogenetic mechanisms of EA-2 are still incompletely understood. In principle, the *CACNA1A* mutations resulting in truncated channel proteins could completely or partially prevent the translation process due to nonsense-mediated mRNA decay (Baker and Parker 2004). If truncated gene products are present, they might be unstable and degrade quickly or might not be targeted to the cell membrane. Alternatively, these truncated gene products could be targeted normally, but severely disrupt the channel function.

Electrophysiological studies have consistently shown that EA-2 mutations cause a complete or pronounced loss of function of the $Ca_v2.1$ channel (Guida *et al.* 2001; Jen *et al.* 2001; Wappl *et al.* 2002; Imbrici *et al.* 2004; Spacey *et al.* 2004; Wan *et al.* 2005b). This loss of function is seen in both missense and nonsense mutations and is either due to decreased single channel conductance or to reduced current density or both, depending on the mutation. Furthermore, in contrast to most published results, two missense EA-2 mutations were recently shown to cause abnormal channel trafficking in addition to altered channel kinetics (Wan *et al.* 2005b). It is still under debate whether simple haploinsufficiency could explain the EA-2 symptoms or if a dominant negative effect, e.g. competition for auxiliary subunits, is the underlying mechanism. Recently, a dominant negative effect was reported in a study of a construct mimicking one of the EA-2 truncating mutations (Page *et al.* 2004). In co-

expression experiments this two-domain mutant construct strongly inhibited $Ca_v2.1$ currents. Misfolding of the full-length channels due to interaction with the truncated channels and subsequent activation of an endoplasmic reticulum resident ribonucleic acid (RNA)-dependent kinase, a component of the unfolded protein response, was suggested to be the mechanism of this dominant-negative suppression (Page *et al.* 2004).

Since EA-2 patients are typically very responsive to acetazolamide, the effects of this drug on $Ca_v2.1$ channels have also been investigated. However, no effect on the electrophysiological properties of either wild type or mutated channels was seen and thus the mechanism of action of this drug is currently unknown (Spacey *et al.* 2004)

6.4.1.4 Relevant mouse models

Several spontaneous mutations in the mouse orthologue of *CACNA1A* have been identified. Tottering (*Cacna1a*^{tg}), rocker (*Cacna1a*^{rkr}) and rolling Nagoya (*Cacna1a*^{rol}) phenotypes are caused by missense mutations and characterized by relatively mild ataxia and, except for rolling Nagoya, intermittent seizures that resemble human absence epilepsy (Fletcher *et al.* 1996; Mori *et al.* 2000; Zwingman *et al.* 2001). A splice site mutation in the 3' end of *Cacna1a* is the cause of the leaner phenotype (*Cacna1a*$^{tg\text{-}la}$) characterized by more severe ataxia, degeneration of cerebellar neurons and a shortened lifespan (Fletcher *et al.* 1996). Electrophysiological studies have shown that the main effect of the tottering, leaner and rolling Nagoya mutations is, similar to EA-2 mutations, marked reduction in Ca^{2+} current density (Wakamori *et al.* 1998; Mori *et al.* 2000). In addition, tottering and leaner mice show defective neurotransmitter release and, interestingly, elevated thresholds for cortical spreading depression (Caddick *et al.* 1999; Ayata *et al.* 2000; Plomp *et al.* 2000).

Two different *Cacna1a* knock-out strains have been developed (Jun *et al.* 1999; Fletcher *et al.* 2001). Since N-and L-type channel currents are elevated in these knock-out mice and thus compensate for the lack of P/Q-type currents, they are initially viable but develop a rapidly progressive neurological deficit with ataxia and dystonia and die at the age of 3-4 weeks. Similar to the spontaneous mutant mice, heterozygous knock-out animals are healthy even though the P/Q-type current density is 50% reduced (Fletcher *et al.* 2001).

6.4.2 Spinocerebellar ataxia type 6

Spinocerebellar ataxias (SCAs) comprise a large and heterogeneous group of dominantly inherited, slowly progressive neurodegenerative disorders. Spinocerebellar ataxia type 6

(SCA-6) is characterized by atrophy of cerebellar Purkinje cells, which leads to a late-onset cerebellar ataxia. The overall prevalence of SCAs is estimated to be 0.003% and SCA-6 patients comprise around 15% of all SCA cases (Schöls *et al.* 2004). Like several other SCAs, SCA-6 is a polyglutamine disorder since small expansions of the CAG repeat located in the 3'-end of *CACNA1A* are the cause of this disease (Zhuchenko *et al.* 1997; Frontali 2001). The pathogenic mechanism of polyglutamine disorders is thought to involve cytotoxic aggregation of the polyglutamine tract-containing proteins within neurons (Schöls *et al.* 2004). However, SCA-6 differs in many ways from the other trinucleotide repeat diseases. In SCA-6 the CAG repeat expansion is exceptionally small (21-33 copies) and relatively stable (Zhuchenko *et al.* 1997; Frontali 2001). Furthermore, anticipation is quite rare (Frontali 2001). Based on these features and recent electrophysiological evidence it has been suggested that dysfunctioning $Ca_v2.1$ channels cause the SCA-6 phenotype (Toru *et al.* 2000; Matsuyama *et al.* 2004). Thus this disease could also be regarded as a channelopathy rather than a polyglutamine disorder (Frontali 2001).

6.4.3 Idiopathic generalized epilepsy

In one sporadic patient and one family, the *CACNA1A* mutations have been shown to cause a complex phenotype with both ataxia and epilepsy (Jouvenceau *et al.* 2001; Imbrici *et al.* 2004). In addition, *CACNA1A* SNPs located within and around exon 8 have been shown to be associated with idiopathic generalized epilepsy (Chioza *et al.* 2001; Chioza *et al.* 2002). This is, however, still controversial since a replication study found no evidence of association (Sander *et al.* 2002).

6.4.4 Familial hemiplegic migraine type 1

6.4.4.1 Genetics

Like EA-2, familial hemiplegic migraine is inherited as an autosomal dominant trait with incomplete penetrance (Joutel *et al.* 1993). The penetrance has been estimated to be about 0.9 and, interestingly, environmental factors also seem to have a role since MZ twins discordant for FHM have been reported (Ducros *et al.* 1995). The first locus linked to FHM was mapped to chromosome 19p13 using two large pedigrees and a candidate locus strategy in 1993 (Joutel *et al.* 1993). After demonstrating genetic heterogeneity of this disease, narrowing the critical chromosomal region and excluding the protein-kinase C substrate heavy-chain (*PRKCSH*) gene, a Dutch group identified causative mutations in *CACNA1A* (Ophoff *et al.* 1994; Ophoff *et al.* 1996b; Ophoff *et al.* 1996a). Mutations in this gene are the cause of FHM

in at least 50% of families including practically all of the families with progressive cerebellar symptoms (Ducros *et al.* 2001). By August 2005, 17 different *CACNA1A* mutations resulting in FHM1 have been described in 40 families and 6 sporadic cases (**Figure 4**) (Ophoff *et al.* 1996a; Battistini *et al.* 1999; Carrera *et al.* 1999; Ducros *et al.* 1999; Friend *et al.* 1999; Gardner *et al.* 1999; Vahedi *et al.* 2000; Ducros *et al.* 2001; Kors *et al.* 2001; Takahashi *et al.* 2002; Terwindt *et al.* 2002; Wada *et al.* 2002; Alonso *et al.* 2003; Kors *et al.* 2003; Alonso *et al.* 2004; Beauvais *et al.* 2004; Kors *et al.* 2004a). However, only a small percentage of patients with sporadic hemiplegic migraine seem to carry *CACNA1A* mutations (Carrera *et al.* 1999; Vahedi *et al.* 2000; Ducros *et al.* 2001; Terwindt *et al.* 2002; Beauvais *et al.* 2004).

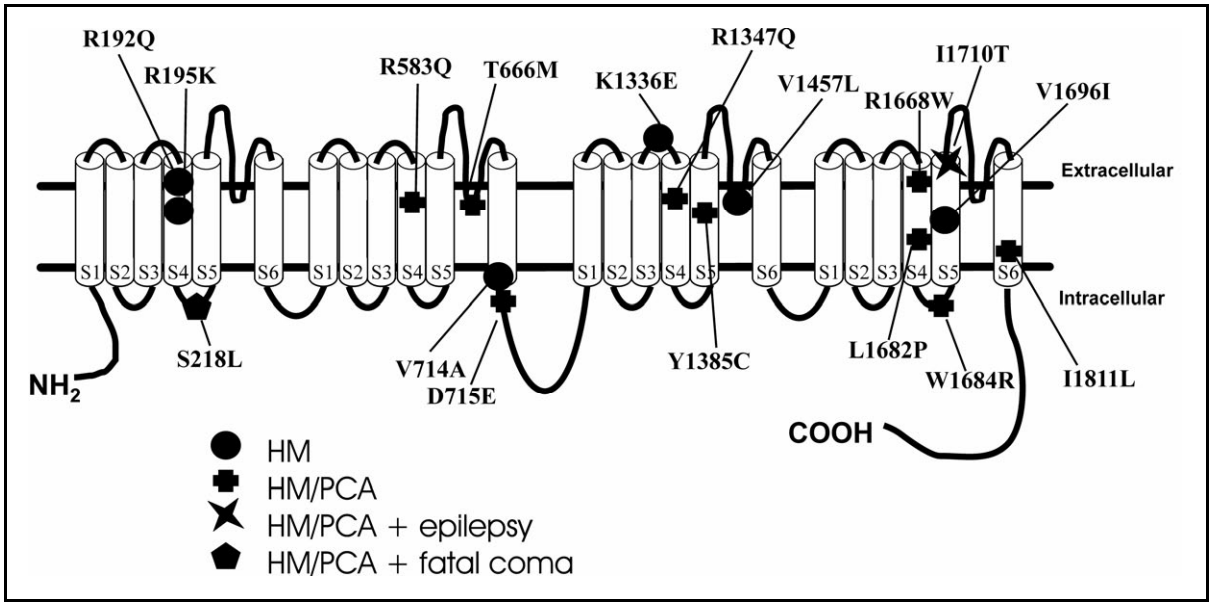

Figure 4. Identified FHM1 mutations. HM =hemiplegic migraine; PCA = progressive cerebellar ataxia.

The FHM1-associated mutations in *CACNA1A* are exclusively missense mutations and are clearly aggregated in the functionally most significant parts (transmembrane domains S4-S6 and the P-loop) of the channel. The unquestionably most common mutation is T666M, identified in 18 families and 3 sporadic cases (Ophoff *et al.* 1996a; Ducros *et al.* 1999; Friend *et al.* 1999; Takahashi *et al.* 2002; Terwindt *et al.* 2002; Wada *et al.* 2002; Kors *et al.* 2003; Jen *et al.* 2004b). Typically, this mutation causes a phenotype of hemiplegic migraine associated with cerebellar signs, although a strict genotype-phenotype relationship does not seem to exist (Wada *et al.* 2002; Kors *et al.* 2003). The R583Q mutation is also recurrent (described in 5 families and 1 sporadic patient) and, similar to T666M, is associated with the phenotype of permanent neurological signs (Battistini *et al.* 1999; Ducros *et al.* 2001; Terwindt *et al.* 2002; Alonso *et al.* 2003). All other FHM1 mutations have only been

identified once or twice. Of these, mutations S218L and I1710T are associated with distinctive FHM phenotypes. In addition to FHM and progressive ataxia, I1710T causes epilepsy while S218L can lead to a delayed severe cerebral edema (swelling caused by an abnormal accumulation of fluid) and coma after a minor head trauma (Kors *et al.* 2001; Beauvais *et al.* 2004; Kors *et al.* 2004a).

In the thus far largest FHM1 genotype-phenotype correlation study, 89% of the *CACNA1A* mutation carriers were shown to be affected and one third of these at least occasionally had severe attacks with either coma or prolonged hemiplegia (Ducros *et al.* 2001). Of the patients that carry mutations associated with progressive neurological symptoms, only approximately 80% actually demonstrate these signs (Ducros *et al.* 2001). Reasons for the incomplete penetrance and phenotype variability are unknown, although modifier genes are one possible explanation (Buchner *et al.* 2003).

6.4.4.2 Electrophysiological studies and the knock-in mice model

Electrophysiological techniques have been used to investigate the pathophysiological consequences of most known FHM1 mutations (Kraus *et al.* 1998; Hans *et al.* 1999; Kraus *et al.* 2000; Tottene *et al.* 2002; Melliti *et al.* 2003; Müllner *et al.* 2004; Tottene *et al.* 2005). These mutations have been shown to affect the biophysical properties of individual channels, especially channel gating and the density of functional channels on the cell surface, but uncovering an overall mechanism common to all FHM1 mutations has not been straightforward. Using cerebellar neurons from *Cacna1a* knock-out mice, Tottene and coworkers (2002) were able to show that the functional deficiency relevant for FHM pathogenesis is most likely a defect in gating that allows the mutant channel to open in response to smaller depolarizations than in the wild type channel. This enhanced channel activity at negative potentials leads to an increased local Ca^{2+} influx (gain of function at the single-channel level), which could contribute to the neuronal hyperexcitability, a possible underlying factor in migraine susceptibility. Consistent with this hypothesis, the gain of function effect of the S218L mutation, producing the most severe FHM phenotype, was recently shown to be exceptionally large (Tottene *et al.* 2005). The effect of the mutations has also been shown to vary depending on the type of β-subunit present and thus to be cell-type specific (Müllner *et al.* 2004).

A recently developed *Cacna1a* knock-in mouse homozygous for the human FHM R192Q mutation shows enhanced neurotransmission at the neuromuscular junction and also, most interestingly, increased susceptibility to cortical spreading depression (van den Maagdenberg *et al.* 2004). Electrophysiological studies performed using cerebellar granule cells of this mouse model have also provided further evidence of the gain of function mechanism in FHM. However, contradictory studies suggesting a loss of function/dominant negative effect for FHM mutations also exist (Cao *et al.* 2004; Barrett *et al.* 2005a; Cao and Tsien 2005).

6.5 *ATP1A2* – the second FHM gene

Soon after identifying the FHM1 locus on chromosome 19p13 it was realized that all FHM families are not linked to this chromosomal region (Joutel *et al.* 1994; Ophoff *et al.* 1994). The second FHM-locus on chromosome 1q was discovered in 1997 by the research group of Elisabeth Tournier-Lasserve (Ducros *et al.* 1997). They conducted a genome-wide linkage scan in one large French family and, after identifying the 1q21-q23 locus, showed that two more families were also linked to this region (Ducros *et al.* 1997). Evidence of linkage to 1q was also seen in a large German-Native American family (Gardner *et al.* 1997). A large Italian FHM family made refining the 1q21-23 locus possible (Marconi *et al.* 2003). By identifying mutations in this and another FHM family, the same group showed that *ATP1A2* encoding the Na^+,K^+-ATPase α2 subunit is the underlying gene (De Fusco *et al.* 2003). In addition to being a particularly interesting finding for migraine researchers, this was also the first report of a disease caused by mutations in a Na^+, K^+-ATPase. After that, mutations in the *ATP1A3* gene encoding the Na^+,K^+-ATPase α3 subunit have been shown to cause rapid-onset dystonia-parkinsonism, an autosomal dominant movement disorder (de Carvalho Aguiar *et al.* 2004).

Na^+,K^+-ATPases consist of at least two subunits, a catalytic α subunit and a modulatory β subunit, while a γ subunit with unknown function has also been discovered (Mercer *et al.* 1993). Four different tissue-specific and developmentally regulated Na^+,K^+-ATPase α subunits have been identified and all but the testis-specific α4 are expressed in the brain (Moseley *et al.* 2003). α1 is ubiquitously expressed and functions as a housekeeping enzyme. Expression of the α3 isoform is restricted to neurons and heart while expression of α2 is most abundant in skeletal muscle, heart and brain, especially in glia (astrocytes) (Orlowski and Lingrel 1988; Watts *et al.* 1991; He *et al.* 2001). However, at least in mice the α2 isoform is

highly expressed in neurons during development and at the time of birth (Moseley *et al.* 2003).

ATP1A2 consists of 23 exons that span approximately 25 kb (Shull *et al.* 1989). In **Figure 5**, the secondary structure of the Na^+,K^+-ATPase α2 subunit is depicted. The N-terminal region of the protein contains four membrane-spanning domains (M1-4) followed by a large intracellular loop and six more transmembrane domains (M5-10) (Kaplan 2002). Altogether 23 FHM2 mutations have been identified thus far (**Figure 5**) (De Fusco *et al.* 2003; Vanmolkot *et al.* 2003; Gardner *et al.* 2004; Jurkat-Rott *et al.* 2004; Kaunisto *et al.* 2004b; Spadaro *et al.* 2004; Vanmolkot *et al.* 2004; Riant *et al.* 2005). Most of them localize within the large intracellular M4-5 loop of the protein. This loop is critical for the correct function of the channel since it harbors hydrolase and ATP-binding domains and undergoes major conformational changes during the enzymatic cycle (Kaplan 2002). All but two of the published FHM2 mutations are missense mutations. Of these, the X1021R mutation changes the STOP-codon to an amino acid coding one and thus extends the protein by 27 residues (Jurkat-Rott *et al.* 2004) and the other, a 2 nucleotide deletion, leads to a frameshift and a

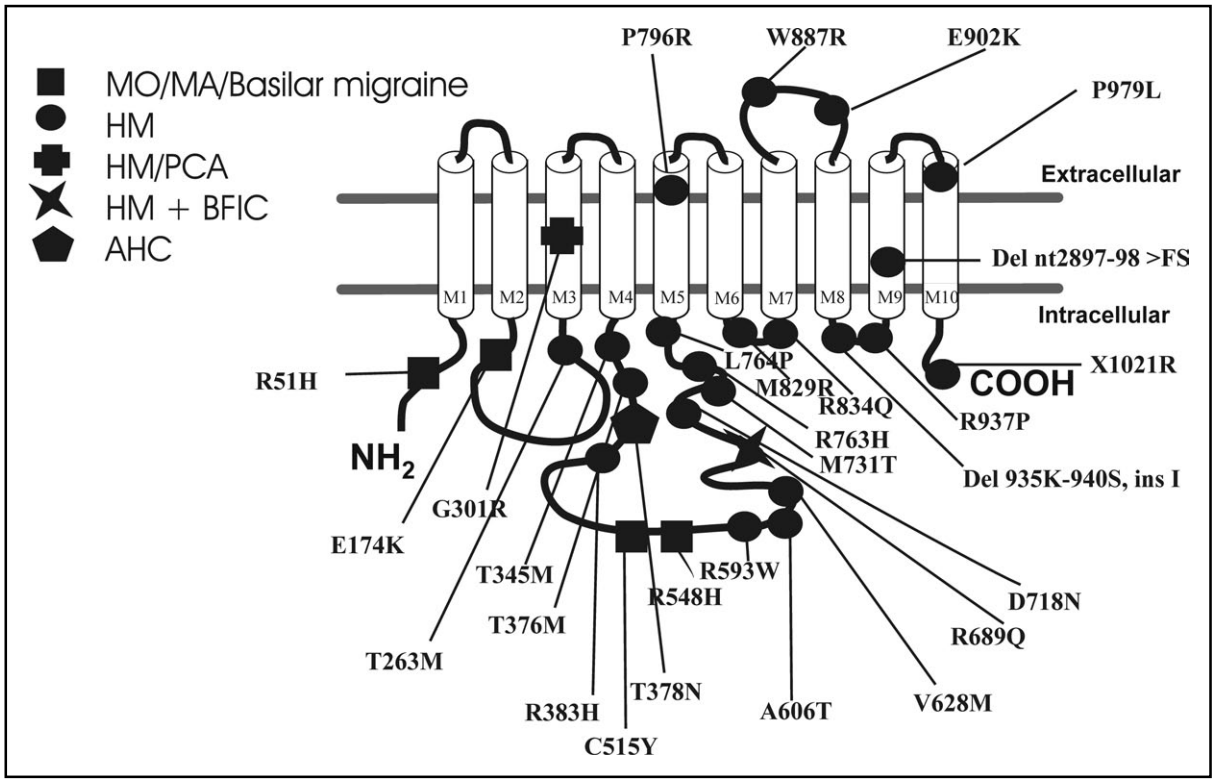

Figure 5. Schematic structure of the Na^+, K^+-ATPase and the identified *ATP1A2* mutations. MO = migraine without aura; HM =hemiplegic migraine; PCA = progressive cerebellar ataxia; BFIC = benign familial infantile convulsions; AHC = alternating hemiplegia of childhood; FS = frameshift

premature STOP-codon within the second last exon of *ATP1A2* (Riant *et al.* 2005). Some FHM2 mutations have been investigated by transfecting cells with mutant *ATP1A2* constructs. Both complete abolishment of Na^+,K^+-ATPase activity and subtle kinetic alterations leading to reduced activity, depending on the mutation, have been observed (De Fusco *et al.* 2003; Segall *et al.* 2004; Segall *et al.* 2005).

In addition to the FHM2 mutations listed above, four other *ATP1A2* missense mutations have been described in families with common forms of migraine (**Figure 5**) (Castro *et al.* 2004; D'Onofrio *et al.* 2004; Todt *et al.* 2005). More functional studies are, however, needed to prove their causality. Furthermore, the T378N mutation in *ATP1A2* has been identified in a family with alternating hemiplegia of childhood (AHC), a phenotype that is clinically different but overlapping to FHM (Bassi *et al.* 2004). The T378N mutation affects the highly conserved ATPase phosphorylation site located in the hydrolase domain. However, no mutations have been identified in the other familial or sporadic AHC patients studied (Bassi *et al.* 2004; Kors *et al.* 2004c).

There are no definitive phenotypic differences between FHM1 and FHM2. The penetrance is possibly somewhat lower for FHM2 (Ducros *et al.* 1997). Cerebellar signs have been almost exclusively associated with FHM1 families. However, subtle cerebellar signs have also been reported in some of affected family members of two FHM2 families (Cevoli *et al.* 2002; Spadaro *et al.* 2004). The *ATP1A2* R689Q mutation was described in a family segregating both FHM and benign familial infantile convulsion (BFIC) (Vanmolkot *et al.* 2003). In light of this finding, it has been noted that a history of seizures is much more common among FHM2 patients than FHM1 patients (Ducros *et al.* 1997; Marconi *et al.* 2003; Jurkat-Rott *et al.* 2004).

Mice carrying spontaneous mutations in the *Atp1a2* gene have not been identified but *Atp1a2* knock-out mice strains are available. *Atp1a2*$^{-/-}$ mice die after birth as they are unable to breath, possibly due to altered neuronal activity in respiratory center neurons (Moseley *et al.* 2003). Enhanced neuronal activity as a result of impaired neurotransmitter uptake causes extensive neuronal cell loss in the amyglada and piriform cortex of these mice (Ikeda *et al.* 2003). *Atp1a2*$^{+/-}$ mice appear healthy, and show an increase in the force of contraction of both cardiac and skeletal muscle (Lingrel *et al.* 2003). However, the heterozygous mice have enhanced fear-anxiety behaviors and enhanced neuronal activity in the amyglada and piriform cortex after conditioned fear stimuli (Ikeda *et al.* 2003). Knock-in mice with FHM2 mutations

would certainly increase our knowledge of the pathophysiology of FHM but these mice have not yet been developed.

6.6 Latest advances in FHM genetics

Very recently, a third locus and gene for FHM, *SCN1A* on chromosome 2q24, was identified (Dichgans *et al.* 2005). This gene codes for a neuronal voltage-gated sodium channel and mutations in it have previously been associated with different epilepsy phenotypes. Only one FHM mutation, Q1489K, has been published thus far. The finding seems, however, to be reliable since the same mutation was found in three German families and was shown to accelerate the recovery of a homologous channel from fast inactivation and thus probably leads to excessive firing of neurons expressing the mutant channel (Dichgans *et al.* 2005).

Furthermore, identification of a *de novo* mutation in the *SLC1A3* gene coding for a glutamate transporter (excitatory amino acid transporter; EAAT1) of a single patient with episodic ataxia, seizures, migraine, and alternating hemiplegia has raised a possibility of a fourth FHM gene (Jen *et al.* 2005). This gene was studied based on a hypothesis that defected transmission of glutamate, the most abundant excitatory neurotransmitter, might be the underlying pathological mechanism of FHM (Moskowitz *et al.* 2004). The activity of the mutant EAAT1 transporter was markedly reduced thus supporting the role of this gene in the disease phenotype described (Jen *et al.* 2005) but more studies are clearly needed to clarify whether glutamate transporters contribute to the FHM phenotype more generally.

6.7 Migraine as a channelopathy

The main symptoms of FHM are undoubtedly very similar to those of MA. Cortical hyperexcitability and the following increased susceptibility to cortical spreading depression have been suggested as the underlying shared pathophysiological mechanism (Moskowitz *et al.* 2004). Since $Ca_v2.1$ channels play a major role in the release of the excitatory neurotransmitter glutamate from cortical neurons, increased Ca^{2+} influx through mutant channels could lead to hyperexcitability (Turner *et al.* 1992; Moskowitz *et al.* 2004). In accordance with this hypothesis, the *Cacna1a* R192Q knock-in mice show a decreased threshold for CSD (van den Maagdenberg *et al.* 2004). $Ca_v2.1$ channels have also been demonstrated to have a role in modulating trigeminal nociception (Knight *et al.* 2002; Akerman *et al.* 2003).

Although no convincing evidence of association of *CACNA1A* or *ATP1A2* with MA or MO exists, certain clinical features (especially the episodic occurrence of symptoms and the existence of triggering factors) definitely support the hypothesis of the common forms of migraine also being channelopathies (Ptacek 1998). Theoretically, several polymorphisms in different ion channel and receptor genes that contribute to maintaining the delicate balance of excitatory and inhibitory influences could together lead to increased neuronal excitability (Ptacek 1998).

AIMS OF THE PRESENT STUDY

The main aim of this work was to search for genetic loci and variants predisposing to migraine with aura – a common complex trait – and its rare Mendelian subtype, familial hemiplegic migraine. Two research strategies, a genome-wide linkage approach and a candidate locus/gene strategy, were applied.

The specific aims were to:

1. Search for genetic loci predisposing to MA using a genome-wide linkage strategy.

2. Study the role of a migraine susceptibility locus on chromosome 19p13 and the role of *MTHFR* and *ESR1* candidate genes in a large family/case-control sample.

3. Establish linkage and identify the causative mutation in a Finnish FHM family.

4. Identify the mutation underlying EA-2, a phenotype allelic to FHM, in a Finnish family.

PATIENTS AND METHODS

1 STUDY SUBJECTS

Informed consent was obtained from all study subjects. The ethics committee of the Department of Neurology, Helsinki University Central Hospital has approved these studies.

1.1 MA families and patients

Since 1996, we have recruited migraine families mainly from headache clinics in Helsinki, Turku, Jyväskylä, and Kemi. Furthermore, we have advertised in the newsletter of the Finnish Migraine Society. More than 700 families with at least four affected family members (most of them having MA) are taking part in this migraine project. Participants have been asked to provide a blood sample and to complete the validated Finnish Migraine Specific Questionnaire for Family Studies (Kallela *et al.* 2001a). The index cases and other family members have been diagnosed according to the IHS criteria on the basis of this questionnaire, which contains 37 detailed questions about headache and other aspects of migraine, especially the characteristics of aura. The diagnoses of individuals with incomplete or contradictory answers in questionnaires have been confirmed by telephone interviews.

In study I, 50 multigenerational families with a seemingly autosomal dominant mode of inheritance were selected for the genome-wide analysis. An additional 22 families were included when the contribution of the chromosome 19p locus in MA was studied (study II). **Tables 15** and **16** describe the diagnosis distribution and the pedigree structure of these families.

In study III, 898 unrelated migraine patients were compared to 900 healthy control individuals. The patient group consisted mainly of the index cases of our migraine family cohort. However, several genetically unrelated individuals from each pedigree were selected

Table 15. Diagnosis distribution of the genotyped subjects.

Diagnosis (IHS classification)	Subjects (N)	
	Genome-scan (study I)	19p13 locus (study II)
Typical aura with migraine headache (1.2.1)	246	417
Typical aura without headache (1.2.3)	6	8
Migraine without aura (1.1)	53	91
Headaches other than migraine	10	23
No headache	91	170
Missing Diagnosis	24	48
Total	430	757

Table 16. Pedigree structure of the studied MA families.

Number of	Genome-scan (study I)	19p13 locus (study II)
Pedigrees	50	72
Generations/pedigree		
1	5	3
2	19	27
3	24	41
4	2	1
Subjects in pedigrees	646	978
Genotyped subjects	430	757
Genotyped subjects/pedigree (mean)	8.6	10.5
Genotyped MA (MA+ MO) patients/ pedigree (mean)	4.9 (5.9)	5.8 (7.1)

MA = migraine with aura; MO = migraine without aura

when applicable. In addition, 285 patients originated from a Finnish cohort of like-sexed twin pairs born before 1958 (Kaprio *et al.* 1978). All patients had a family-history of migraine. Of these, 718 (80.0%) were women and all had headache fulfilling the IHS criteria for migraine as well as aura symptoms before the headache. However, the aura symptoms did not fulfil the IHS criteria for MA in 114 (12.7%) patients.

1.2 Control samples

For association analysis (study III), the age- and sex-matched control sample comprised 900 unrelated individuals from a Finnish twin-cohort of opposite-sex pairs born 1939-1957. Of the control individuals 76% were women and all self-reportedly did not have migraine. Furthermore, to minimize the presence of genetic migraine susceptibility factors, only individuals reporting that their co-twin and other first-degree family members do not have migraine were included.

In studies IV and V, the presence of the identified mutations in 132 healthy control individuals was excluded. This same control sample consisting of anonymous Finnish blood donors was also used in study IV when the frequencies of the identified polymorphisms were determined in pooled DNA samples. For this purpose, a Coriell Cell Repositories human variation DNA pool NA16129 consisting of 24 individuals belonging to different Asian, American Indian and Pygmy populations was also analyzed.

1.3 FHM2 family

In study IV, a Finnish FHM family ascertained through Helsinki University Children's Hospital with 11 affected family members was studied (**Figure 14**, page 94). Diagnoses were

made according to the IHS criteria. **Table 17** describes the typical clinical characteristics of migraine in these patients. Interictal nystagmus or ataxia was not present in any of the affected individuals and none of them had seizures.

Table 17. Characteristics of the FHM-patients.

Patient	II:2	II:4	II:6	II:10	II:11	III:6	III:7	III:9	III:11	III:14	III:15
Sex	F	F	F	F	M	F	M	F	F	F	F
Age at onset of migraine	31	8	9	13	7	11	10	5	12	6	5
Migraine frequency (times/year)	24	12	2	6	NA*	12	NA*	24	1	1	24
Visual aura	-	+	+	+	+	+	-	+	-	+	+
Hemisensoric/hemiplegic aura	+/+	+/+	+/+	+/+	+/+	+/+	-/+	+/+	+/+	+/+	+/+
Confusion/coma	-/-	+/-	-/-	-/-	+/+	-/-	+/+	+/+	-/-	-/-	+/+
Dysphasia	-	+	+	+	+	+	+	+	+	-	+
Fever	-	-	-	-	+	-	+	+	-	-	+
Head trauma as a trigger	-	-	+	-	+	-	+	+	-	-	+

F = female, M = male, NA = not applicable; * ceased at the age of 14-15

1.4 EA-2 family

In study V, clinical information and DNA samples were obtained from 47 family members belonging to a four-generation Finnish EA-2 family (**Figure 15**, page 98). After identification of the disease-causing mutation, seven additional more distant relatives (family of the affected grandfather's sister) were studied. For the linkage analysis, 13 individuals were diagnosed as affected. The most common attack symptoms included vertigo, nausea, vomiting, ataxia, impaired focusing ability, dysarthria, and fatigue. In addition, one individual was regarded as possibly affected since her only symptom was an exquisite sensitivity to alcohol. The attacks started at the age of 4-35 years (mean age of onset 10 years). The attacks, typical duration of which was two hours, were commonly triggered by exercise, emotional stress, coffee, bright sunshine, and heat. The interictal neurological status of most patients was either normal or only slightly impaired (for example gaze-evoked nystagmus) but three elderly individuals had pronounced cerebellar ataxia. Furthermore, eight family members were diagnosed as having MO.

2 METHODS

2.1 DNA extraction

Genomic DNA was extracted from peripheral blood either using the standard phenol-chloroform extraction procedure (Blin and Stafford 1976) or the Autopure LS automated DNA purification instrument (Gentra Systems, Minneapolis, USA).

2.2 Genotyping

2.2.1 Microsatellite genotyping

In studies I, II, IV, and V subjects were genotyped using polymorphic microsatellite markers and fluorescence-based detection methods. In the genome-wide scan, 350 microsatellite markers spaced approximately 11 cM apart were amplified by polymerase chain reaction (PCR) using multiplex PCR assays with fluorescent primers in microtiter 96-well plates. The markers were mainly from the Human MapPairs Genome-Wide Screening Set (LI-COR Biosciences, Lincoln, NA, USA), a modification of the ninth version of the Weber lab screening set (Broman *et al.* 1998). The genotyping was performed using a LI-COR DNA 4200 Genetic Analyzer (LI-COR) and the Saga1.0 software package (University of Washington and LI-COR). In study II, eight microsatellite markers (D19S247, D19S427, D19S592, D19S391, D19S394, D19S221, D19S1150, and D19S226) surrounding the *INSR* and the *CACNA1A* genes (study II, Figure 1) were analyzed on an ABI3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA) using the GeneScan and Genotyper Software (Applied Biosystems). In both cases, the genotyping was performed at the University of California, Los Angeles (UCLA).

In studies IV and V, three microsatellite markers (D19S221, D19S1150, and D19S226) flanking the previously identified FHM1/EA-2 susceptibility locus on 19p13 (*CACNA1A*) were genotyped. In study IV, 20 additional markers covering the chromosomal area 1q21-32 (FHM2 locus) were genotyped. Primer sequences for the markers were obtained from the GDB Human Genome Database (http://www.gdb.org). In both studies, Cy5-labeled PCR fragments were separated using denaturing polyacrylamide gels on an Automated Laser Fluorescence (ALF) DNA sequencer (Amersham Biosciences, Buckinghamshire, UK) and the genotypes assigned with the AlleleLinks software (Amersham Biosciences).

The order and distances between markers were determined from The Marshfield Medical Research Foundation Genome Database (http://research.marshfieldclinic.org/genetics/) and from the USCS Genome Browser (http://genome.ucsc.edu/). Allele sizes were standardized to known Centre d'Etudes du Polymorphisme Humain (CEPH) control individuals (http://www.cephb.fr/). All genotypes were verified by human inspection and Mendelian inheritance confirmed by the PedCheck program (O'Connell and Weeks 1998). In addition, the mistyping option of SimWalk2 program (Sobel and Lange 1996) was used to detect genotyping errors.

2.2.2 SNP genotyping

In studies III, IV, and V, SNPs were genotyped either to detect variants associated with MA (study III) or to study the frequency of potential mutations and other sequence variants identified by sequencing (studies IV and V). The SNPs that were genotyped in study III were selected from the public dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/).

2.2.2.1 Minisequencing

After the potential mutations had been identified in studies IV and V, family members and healthy controls were screened for the presence of the variation in question using solid-phase minisequencing (study IV) or cycle minisequencing (study V), a modification of the minisequencing method (Syvänen *et al.* 1990; Järveläinen *et al.* 2001). Cycle minisequencing was also used to estimate the population frequencies of the *CACNA1A* polymorphisms identified (study V). The principle of solid-phase minisequencing is shown in **Figure 6** with the primers used in study IV. Shortly, two aliquots of biotinylated PCR

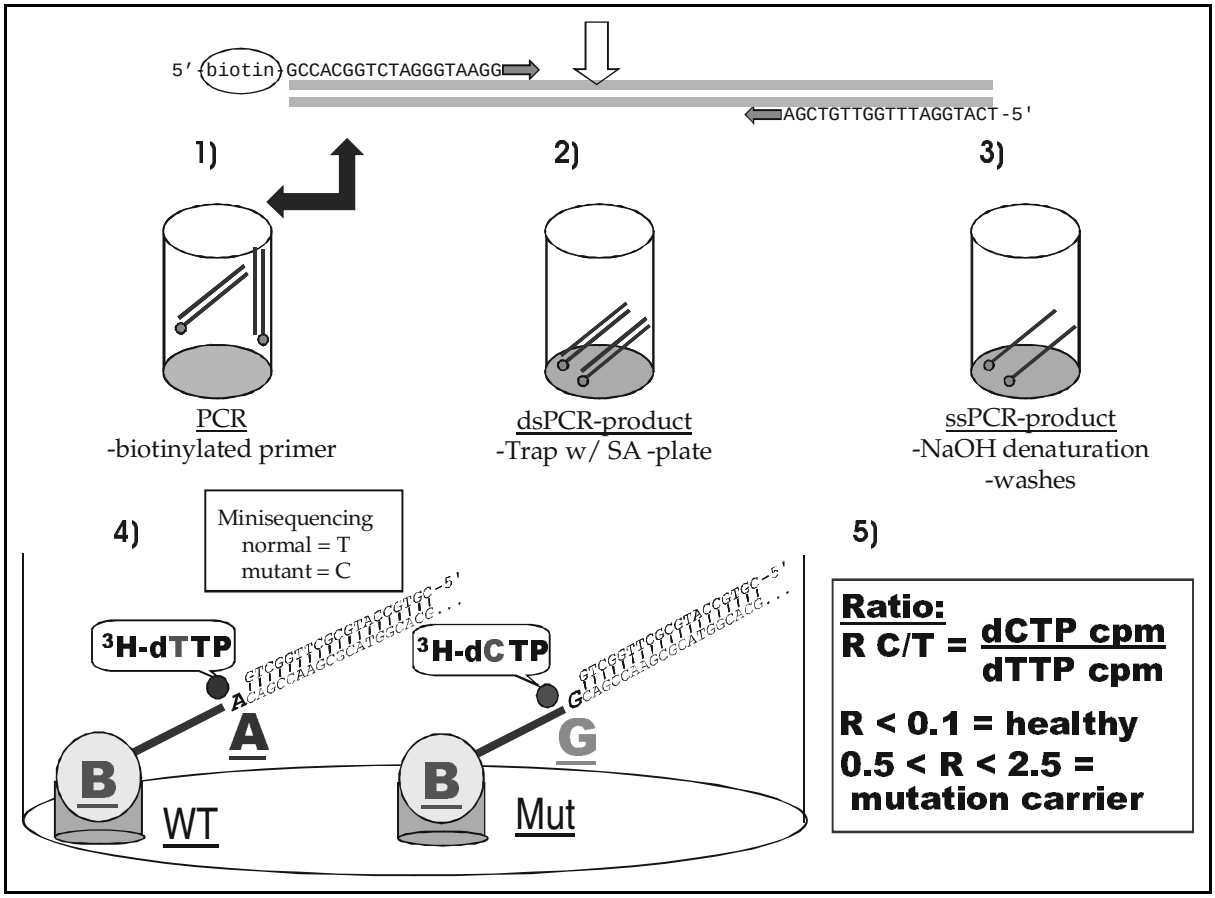

Figure 6. Principle of the solid-phase minisequencing method. The figure is a kind gift from docent Arto Orpana.

product were immobilized on streptavidin-coated microtiter wells (Wallac/PerkinElmer, Wellesley, USA) and denatured with NaOH to remove the unbiotinylated complementary DNA strand. Next a detection primer was added and elongated by a single ^{3}H-labeled nucleotide (normal or mutant) using a DNA polymerase. The genotypes were then defined by calculating the ratio of the radioactivity of the incorporated nucleotides measured with a scintillation counter (Microbeta/PerkinElmer). The cycle minisequencing procedure differed from standard minisequencing in two main ways: normal PCR-primers were used while the extension primer was biotinylated and the primer extension reaction was performed by cycling at 96°C and at 59°C for 50 cycles using a PCR-machine.

2.2.2.2 Sequenom MassARRAY system

In study III, over 53,000 genotypes were produced using the Homogenous MassExtend MassARRAY system (Sequenom, San Diego, CA, USA). Similar to minisequencing, this method is based on primer extension but here the extension products are analyzed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. In this method, the genotypes are assigned based on the differences in mass between the two primer extension products (**Figure 7**). The assays were designed using the AssayDesign software (Sequenom) so that the extension products were 1-3 bases longer than the extension primer and multiplexing of the SNPs was possible. The multiplex PCR and primer extension reactions were performed according to the manufacturer's instructions except that more DNA (9 ng) was used as a template. SpectroTYPER software (Sequenom) was used for determining the genotypes, although verified by human inspection.

In study IV, the same MassARRAY system together with the Allelotyping (Sequenom) and the MassARRAY Typer (Sequenom) software were used for estimating allele frequencies of the identified *ATP1A2* polymorphisms in pooled DNA samples. Previous studies have shown that this method can be used to estimate allele frequencies with high precision (Mohlke *et al.* 2002). For each SNP, 12 replicate PCRs and extension reactions were performed on a pool consisting of equimolar amounts of DNA from 132 Finnish individuals. Furthermore, four replicates on the Coriell NA16129 DNA pool sample consisting of 24 individuals were performed for each SNP. When pooled samples are used, it must be taken into account that the peak heights and areas of the two alleles are usually unequal. Thus we also genotyped 15 individual samples, calculated the ratio of the peak areas of the two alleles in heterozygous

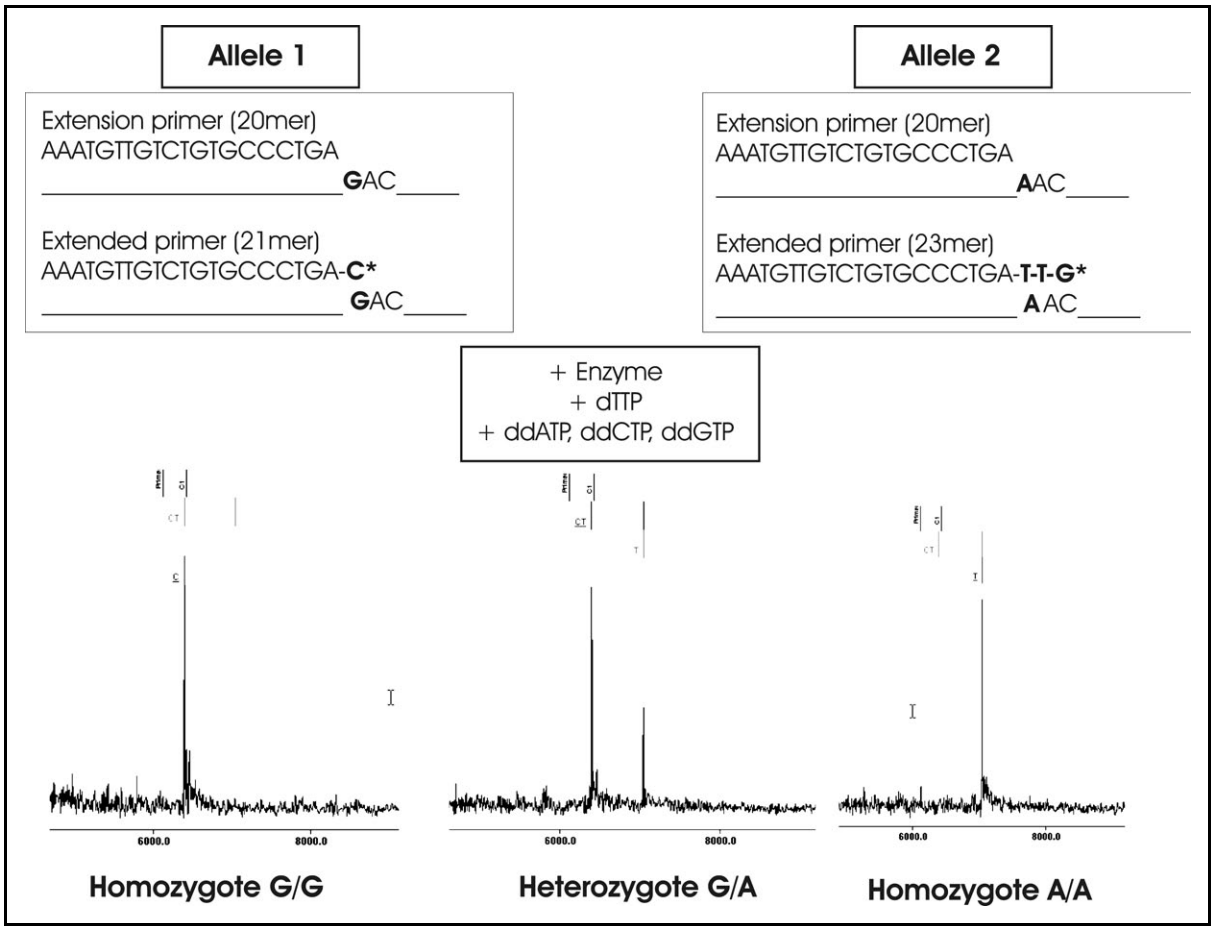

Figure 7. Principle of the Sequenom MassARRAY SNP genotyping system.

individuals and used this information to correct the allele frequency estimations in the DNA pools.

2.3 Sequencing

PCR was performed using intronic primers under conditions optimized for each reaction. PCR-products were then purified enzymatically and sequenced in both sense and antisense directions using BigDye terminator chemistry (Applied Biosystems), a modification of the dideoxy chain termination method of Sanger (Sanger *et al.* 1977). After a sodium acetate/ethanol precipitation, sequencing reactions were run on an automated sequencer (ABI377 or ABI3700; Applied Biosystems).

2.4 RNA analysis

After identifying the *CACNA1A* splice site mutation in study V, we wanted to investigate the effect of this mutation on mRNA splicing. The normal and mutated *CACNA1A* sequences were analyzed using Splice Site Predictions, a neural network-based program

(http://www.fruitfly.org/seq-tools/splice.html). Furthermore, we aimed at studying the effect of this mutation using laboratory methods. Although *CACNA1A* is known to be expressed almost exclusively in the brain, we hypothesized that a minimal background expression in lymphocytes could make it possible to reverse transcribe and amplify the mRNA in question. We tested this using total RNA extracted from blood samples of healthy control individuals with the RNeasy kit (Qiagen, Hilden, Germany). RNA extracted from human fetal second trimester brain tissue samples (cerebellum/cerebrum) was also used to ensure the functioning of the reverse-transcriptase PCR (RT-PCR) procedure. The quality of the RNA samples was monitored using β-actin specific primers. RNA samples were transcribed to cDNA using the Titan One tube RT-PCR system (Roche, Basel, Switzerland), RNAquard RNase inhibitor (Amersham Biosciences) and a reverse primer annealing to exon 40 of *CACNA1A*. PCR was performed using the cDNA as a template with primers annealing to exons 35 and 39, and subsequently (nested-PCR), with inner primers using the first PCR product as a template. The RT-PCR and nested PCR products were evaluated by agarose gel electrophoresis, purified with the MinElute Gel Extraction Kit (Qiagen) and sequenced.

2.5 Statistical methods

2.5.1 Simulations and power calculations

In studies II and V, it was essential to evaluate the informativeness of the sample size used. Simulations were performed to estimate whether the 72 pedigrees genotyped in study II were sufficient to detect linkage. The SLINK program was used to generate 500 data replicates (a marker with 5 alleles completely linked to the disease locus) that were then analyzed with the programs MSIM and ELODHET (Ott 1989, 1991; Weeks *et al.* 1994). The expected maximum LOD score (EMLOD), which is the average of the maximum LOD scores for each replicate, and power, the probability of rejecting the null hypothesis of no linkage when it is false, were evaluated. Power was defined as the proportion of replicates where the LOD score was equal to or greater than a given threshold, 3.3, and was calculated with variable proportions of unlinked families.

In study III, the power of the case-control sample to replicate the association of MA to SNPs in candidate genes *ESR1* and *MTHFR* was evaluated with the Genetic Power Calculator program (Purcell *et al.* 2003). The disease prevalence of MA was estimated to be 5%, the allele frequencies were as found in our material and the odds ratios (OR) for the at-risk

genotypes were assumed to be somewhat smaller than previously reported (Colson *et al.* 2004; Lea *et al.* 2004).

2.5.2 Linkage analysis

Both in study I and II, parametric two-point linkage analysis (MLINK option of the LINKAGE package), nonparametric affected sib pair (ASP) analysis, family-based association analysis (TDT, HRR) and homogeneity testing (HOMOG) were performed for each marker with the help of the ANALYZE package (Penrose 1953; Lathrop and Lalouel 1984; Ott 1991; Terwilliger 1995; Kuokkanen *et al.* 1996). Furthermore, another helper program, AUTOSCAN, enabling the analyses of all autosomal chromosomes in a single run was used (Hiekkalinna and Peltonen 1999). The parametric analyses were performed using a dominant mode of inheritance, a disease gene frequency of 0.001, penetrance of 0.9, and a phenocopy frequency of 0.024 (Hovatta *et al.* 1994). An affecteds-only strategy was used to circumvent problems of incomplete penetrance by coding only individuals with MA (stage I) and MA or MO (stage II) as affected. All other individuals were classified as unknown. The allele frequencies were estimated from all genotyped individuals using the DOWNFREQ program. For the region showing evidence of linkage in the genome-wide scan, multipoint parametric and nonparametric analyses were performed using the GENEHUNTER program (version 2.1) (Kruglyak *et al.* 1996). To enable the comparison of different results, the two-point LOD scores were also converted to p-values (Chiano and Yates 1995; Nyholt 2000). In study II, nonparametric linkage (NPL) analysis was performed using statistics B (the maximum number of alleles among the affecteds descended from any one founder-allele; most powerful for dominant traits) and E (NPL_all statistic as implemented in GENEHUNTER) of the SimWalk2 program (version 2.82) (Sobel and Lange 1996).

In studies IV and V, two-point LOD scores were calculated using the MLINK option of the LINKAGE package assuming autosomal dominant inheritance with incomplete penetrance (0.8) and a disease gene frequency of 0.0001. No phenocopies were allowed. Individuals with MO or headache other than migraine were considered unaffected while an unknown status was given to asymptomatic family members under the age of 20.

2.5.3 Association analysis

In study III, association of several *MTHFR* and *ESR1* SNPs with migraine was studied. The pairwise LD of the genotyped markers and the haploblock structure of the genes in our study sample was determined with the Haploview program (Barrett *et al.* 2005b). The allele and

genotype frequencies of the SNPs were compared between cases and controls using standard χ^2-analysis. Furthermore, logistic regression analysis was applied for five *ESR1* SNPs showing nominal evidence of association to calculate the odds ratios for individuals with one and two copies of the minor allele (compared to those having none). Alternatively, subjects carrying two copies of the minor allele were compared with all the other subjects. These analyses were performed with the SPSS program.

For the five *ESR1* SNPs showing nominal evidence of association, haplotypes were estimated using both the Haploview and the PHASE programs and association analysis of these reconstructed haplotypes performed with the Haploview and the Haplo-assoc software (Stephens and Donnelly 2003; Barrett *et al.* 2005b).

RESULTS AND DISCUSSION

1 GENOME-WIDE SCAN WITH 50 FINNISH MA FAMILIES

In study I, we genotyped 350 microsatellite markers in 430 individuals belonging to 50 multigenerational MA families with seemingly autosomal dominant inheritance. In two-point linkage analysis, 18 markers produced LOD or HLOD (LOD score under heterogeneity) scores above 1 (**Table 18**). The HLOD scores were always at least as high as the LOD scores assuming locus homogeneity but when converted to p-values, the LOD results at some markers were more significant than the HLOD results because the p-values of HLOD scores require more correction (Nyholt 2000). Results of this genome-wide scan are presented in a graphical form in **Figure 8**.

Results of the nonparametric ASP analysis were almost invariably less significant than the results of parametric linkage analysis. The only markers showing LOD scores >1.0 in the ASP analysis but not in the parametric analysis were D14S1426, D17S1830, and D18S535 producing LODs of 1.13, 1.44, and 1.41, respectively.

Table 18. The markers with two-point LOD scores ≥ 1.0 in the genome-wide scan (study I).

Marker (Location)	Position (cM)	Under locus homogeneity			Under locus heterogeneity		
		LOD Score	Pointwise p-value	θ	HLOD Score	Pointwise p-value	θ
D1S552 (1p36.13)	45	0.36	NS	0.28	1.08	0.020	0.00
D1S3462 (1q42.2)	247	0.70	0.036	0.30	1.66	0.0046	0.34
D4S1517 (4q13.3)	82	1.66	0.0029	0.26	1.66	0.0046	0.26
D4S3243 (4q21.2)	88	1.01	0.016	0.26	1.01	0.023	0.26
D4S2409 (4q22.1)	96	1.85	0.0018	0.20	1.89	0.0026	0.16
D4S2380 (4q22.3)	101	1.75	0.0023	0.22	2.26	0.0011	0.08
D4S1647 (4q24)	**105**	**4.20**	**0.000006**	**0.18**	**4.20**	**0.000011**	**0.18**
D4S3240 (4q25)	114	0.76	0.031	0.30	1.01	0.023	0.16
D4S2394 (4q28.2)	130	0.98	0.017	0.28	1.05	0.021	0.22
D4S1520 (4q31.1)	141	1.55	0.0038	0.20	1.55	0.0061	0.20
D5S2500 (5q12.1)	69	1.11	0.012	0.44	1.16	0.016	0.20
D15S659 (15q21.1)	43	0.05	NS	0.42	1.09	0.019	0.06
D15S655 (15q25.3)	83	0.70	0.036	0.24	1.38	0.0092	0.04
D16S753 (16p12.3)	58	0.004	NS	0.40	1.22	0.014	0.00
D17S945 (17p13.1)	21	0.96	0.018	0.30	1.01	0.023	0.24
D18S877 (18q12.1)	54	0.18	NS	0.40	1.00	0.024	0.00
D19S427 (19p13.2)	21	1.70	0.0026	0.22	1.70	0.0042	0.22
DXS9896 (Xp)	31	1.08	0.013	0.30	1.08	0.020	0.30

θ = recombination fraction at which maximum LOD was found

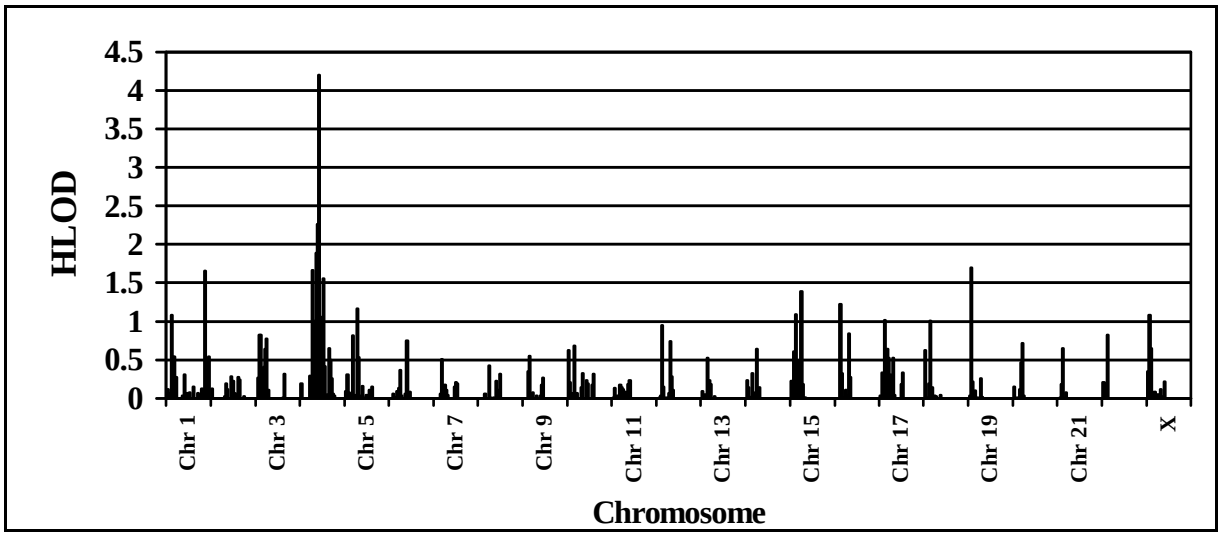

Figure 8. Plot of LOD scores from the genome-wide scan in 50 Finnish MA families (HLOD = LOD score under heterogeneity).

1.1 Chromosome 4q21-24 locus

Significant evidence of linkage was seen between MA and chromosome 4q. Marker D4S1647 gave the largest LOD score, 4.20 (under homogeneity p = 0.000006; under heterogeneity p = 0.000011), at θ = 0.18. Seven other adjacent markers within a 59 cM region on chromosome 4q showed two-point maximum LOD scores ranging from 1.01 to 2.26 (**Table 18**). For the region showing the best two-point LOD scores, multipoint parametric and nonparametric analyses were performed with the GENEHUNTER program. The results are shown in **Figure 9** and **Table 19**. The highest HLOD, 4.45, was observed between markers D4S2409 and D4S2380 with a parametric test. At this location, the fraction of linked families (α) was estimated to be 50%. The maximum NPL score, obtained at the same position as the best parametric multipoint LOD, was 3.43. The 4q region showing evidence of linkage was at least 30 cM wide. Although problematic in the restriction phase, this increased the reliability of our results even further since it has been suggested that true positive linkage peaks are generally longer than false positive peaks (Terwilliger *et al.* 1997).

Figure 9. Multipoint linkage analyses of the 4q region (Wessman *et al.* 2002).

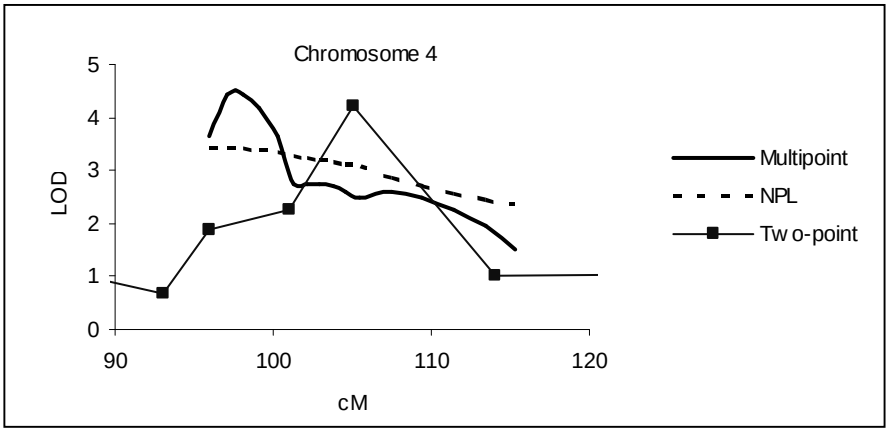

Table 19. Multipoint linkage analysis results along the linked region on chromosome 4.

Marker	Map location (cM)	HLOD	α	NPL-all	p-value	Information content
D4S2409	96.16	3.66	0.45	3.41	0.0008	0.70
	97.08	4.43	0.50	3.43	0.0007	0.65
	98.00	4.45	0.50	3.43	0.0007	0.64
	98.91	4.17	0.47	3.40	0.0008	0.66
	99.80	3.65	0.42	3.35	0.0009	0.71
D4S2380	100.75	2.73	0.34	3.27	0.001	0.81
	101.59	2.75	0.34	3.23	0.001	0.76
	102.43	2.74	0.34	3.19	0.001	0.74
	103.26	2.69	0.34	3.15	0.002	0.74
	104.10	2.61	0.33	3.12	0.002	0.75
D4S1647	104.94	2.49	0.31	3.09	0.002	0.78
	106.76	2.59	0.34	2.87	0.003	0.67
	108.58	2.47	0.33	2.69	0.005	0.62
	110.40	2.26	0.31	2.55	0.007	0.60
	112.22	1.96	0.28	2.44	0.009	0.62
D4S3240	114.04	1.50	0.23	2.36	0.01	0.68

α = Fraction of linked pedigrees at maximum HLOD

Even more encouragingly, significant evidence of linkage to a very nearby chromosomal area was seen in a genome-wide scan performed by the Icelandic company deCODE Genetics (Björnsson *et al.* 2003). The company used their unique genealogical strategy for identifying 117 families with altogether 351 affected individuals. In contrast to our strategy, they concentrated mainly on MO patients. Furthermore, slightly relaxed diagnostic criteria for MO were used to maximize the number of affected individuals. With this strategy, they observed linkage (LOD 2.87) to markers on chromosome 4q21. When only affected females were included in the analysis, marker D4S2409 produced a significant LOD score (4.08; p = 0.0000072). This marker is located approximately 10 cM centromeric to our peak marker (D4S1647) and even closer to our multipoint peak. Since it has been shown that the variation of location estimates in complex disease linkage studies is substantial, with 95% confidence intervals covering tens of cM, it is likely that these 4q findings resulted from the same susceptibility locus (Roberts *et al.* 1999).

It is rather surprising that these results providing a replication of our finding were obtained using a different phenotype, MO (including also patients not fulfilling all of the IHS criteria), and that the results were more significant when only females were analyzed. In our genome-wide study, the results were also analyzed with both MA and MO patients as affected. However, when the 53 MO patients were included, the 4q area showed only suggestive evidence of linkage with a maximum LOD score of 2.48 for the D4S1647 marker (see **Figure**

11, page 81). In conclusion, it seems possible that this locus contributes to both MA and MO susceptibility although in our study this region is only linked to the MA phenotype.

After conducting the genome-wide scan, our group has concentrated on restricting and fine-mapping the 4q21-24 locus (unpublished data). Sixteen of the 50 genotyped families were chosen for further genotyping based on location scores (directly comparable with multipoint LOD scores) of individual families calculated with SimWalk2 (criterion: location score >0.27). Furthermore, another set of 30 families has been studied for linkage to 4q. The maximum combined heterogeneity location score of SimWalk2 was, however, only 0.49 (with $\alpha = 0.15$, nearest marker D4S2409) with this set of families. Although it was thus evident that most of these families are not linked to chromosome 4q, we were able to select 11 families for further studies using a similar criterion as above. It is possible that the heterogeneity of results is due to slightly different family selection criteria since the majority of the patients belonging to the 30 additional families had migraine with a very typical (as described in the literature) aura phase with spreading symptoms. Because of this, another whole-genome scan has been performed in this family set but the results are not yet available.

Sixty microsatellite markers covering an approximately 30 cM region between markers D4S1517 (82.1 cM) and D4S3240 (114.0 cM) have been genotyped in the combined subset of 27 families showing evidence of linkage to 4q21-24. Furthermore, 149 SNPs (located between microsatellite markers D4S2409 and D4S3240) have been genotyped in these same families. Both linkage and association analyses have been conducted. Using the subset of 16 linked families selected from the 50 initially genotyped, we have been able to highlight a 6 Mb region between markers D4S1578 (104.8 cM) and D4S1572 (108.0 cM) as the most likely location for variant(s) predisposing to MA (**Figure 10a**). Our two-point top marker D4S1647 (104.9 cM) is located within this susceptibility region. Interestingly, the peak region of this multipoint curve is located more telomeric than our initial multi-point peak and thus further away from the area detected by deCODE (their top marker D4S2409; 96.2 cM).

The chromosomal area covered by SNPs contains 32 known genes, some of which are shown in **Figure 10b** and **Table 20**. Some of the SNPs have shown nominal evidence of association with MA (**Figure 10c-d**). However, the mean distance between SNPs is ~110 kb and denser SNP coverage is clearly needed before the actual susceptibility variant can be identified.

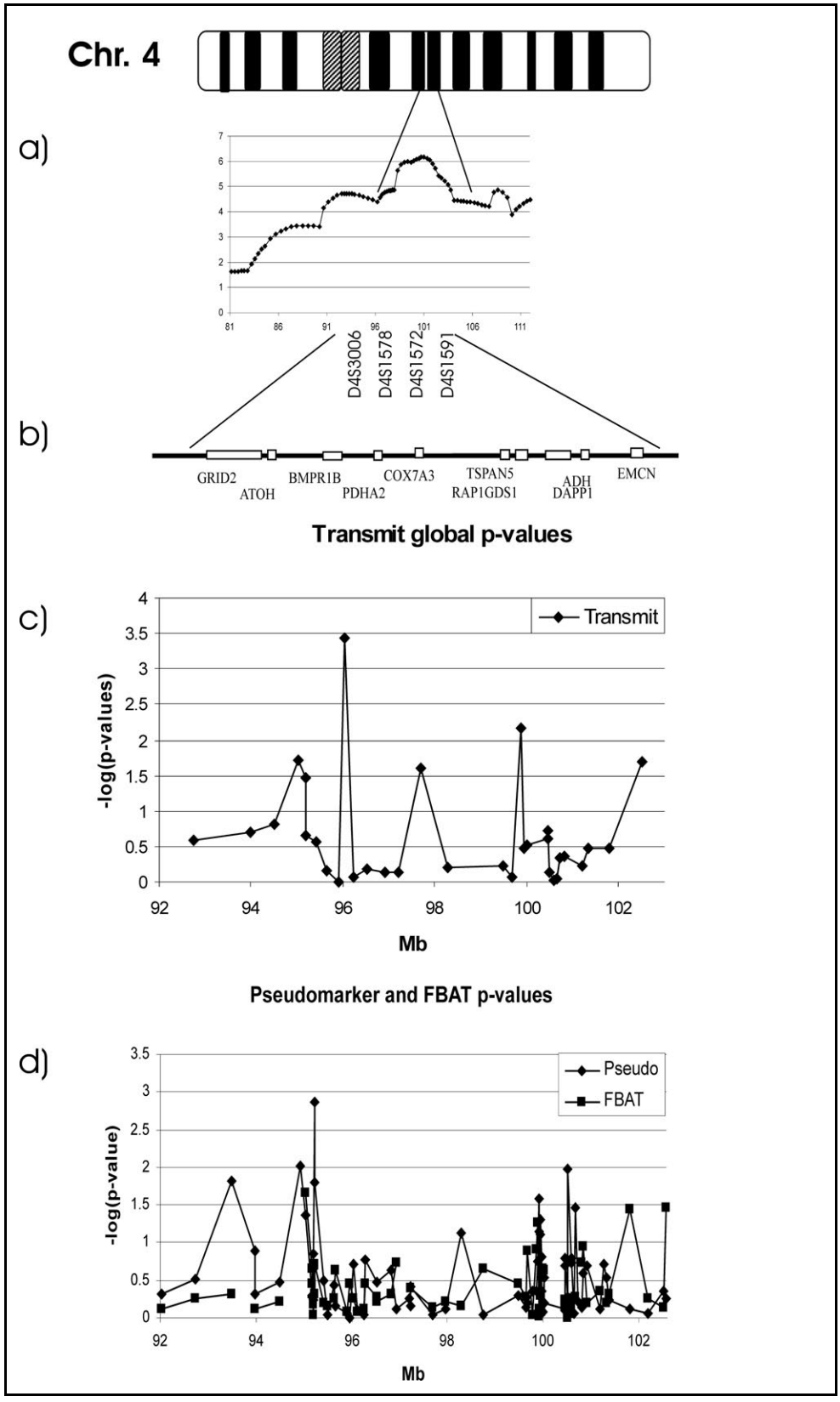

Figure 10. A) "Conditional" parametric multipoint linkage analysis results. B) Examples of candidate genes located within the susceptibility area. C) Results of the haplotype analysis performed using the Transmit program. D) Model-based Pseudomarker results (LD given linkage) and global FBAT analysis results.

Table 20. Some interesting candidate genes in the chromosome 4q21-24 migraine susceptibility area.

Gene symbol	Name	Function	Expression
GRID2	Glutamate receptor, ionotropic, delta 2	Ionotropic glutamate receptor	Cerebellar Purkinje cells
ATOH1	Atonal homolog 1 (Drosophila)	Involved in the development of the nervous system	NA
UNC5C	Unc-5 homolog C (*C. elegans*)	Transmembrane member of the immunoglobulin superfamily, involved in cell migration during cerebellum development	Ubiquitous
RAP1GDS1	RAP1, GTP-GDP dissociation stimulator 1	Stimulatory GDP/GTP exchange protein with GTPase activity	Ubiquitous
TM4SF9	Tetraspanin 5	Cell-surface protein, signal transduction	Ubiquitous
ADH cluster (7 genes)	Alcohol dehydrogenase	Catalyzes the conversion of alcohols to aldehydes	Some ubiquitous, some liver specific
PPP3A	Protein phosphatase-3, catalytic subunit, alpha isoform (calcineurin A)	Calmodulin dependent serine/threonine phosphatase	Ubiquitous

NA = information not available

1.2 Other regions of interest

Statistically significant or suggestive linkage was not observed in any other chromosomal region although markers in nine other regions (1p, 1q, 5p, 15q, 16p, 17p, 18q, 19p, Xp) produced LODs >1.0 (**Table 18**, page 75). Of these, 1q, 19p, and the X-chromosome were especially interesting since these regions have previously been connected either to FHM or the common forms of migraine. The marker on chromosome 1q with a maximum HLOD score of 1.66 was, however, located on 1q42 while the FHM2 gene *ATP1A2* localizes to 1q21-23 and the other migraine susceptibility region to 1q31 (Ducros *et al.* 1997; Lea *et al.* 2002). Similarly, the DXS9896 marker with a LOD of 1.08 is on Xp and no evidence of linkage to Xq24-28, the location of the susceptibility region in two Australian families, was observed in our study (Nyholt *et al.* 2000). The results concerning the chromosome 19p locus are discussed in more detail in the following chapter. When MO patients were also classified as affected, marker D18S877 on 18q showed suggestive evidence of linkage (LOD 2.32 versus LOD 1.00 with MA affection criteria). Another region showing higher LODs with the broader phenotype was 3p25, with a maximum LOD of 1.83 (**Figure 11**). Interestingly, evidence of linkage to a locus on 3p21 was recently found in a large Dutch family with hereditary vascular retinopathy associated with migraine and Raynaud's phenomenon (Ophoff *et al.* 2001). The distance between these two chromosome 3p loci is, however, approximately 30 cM.

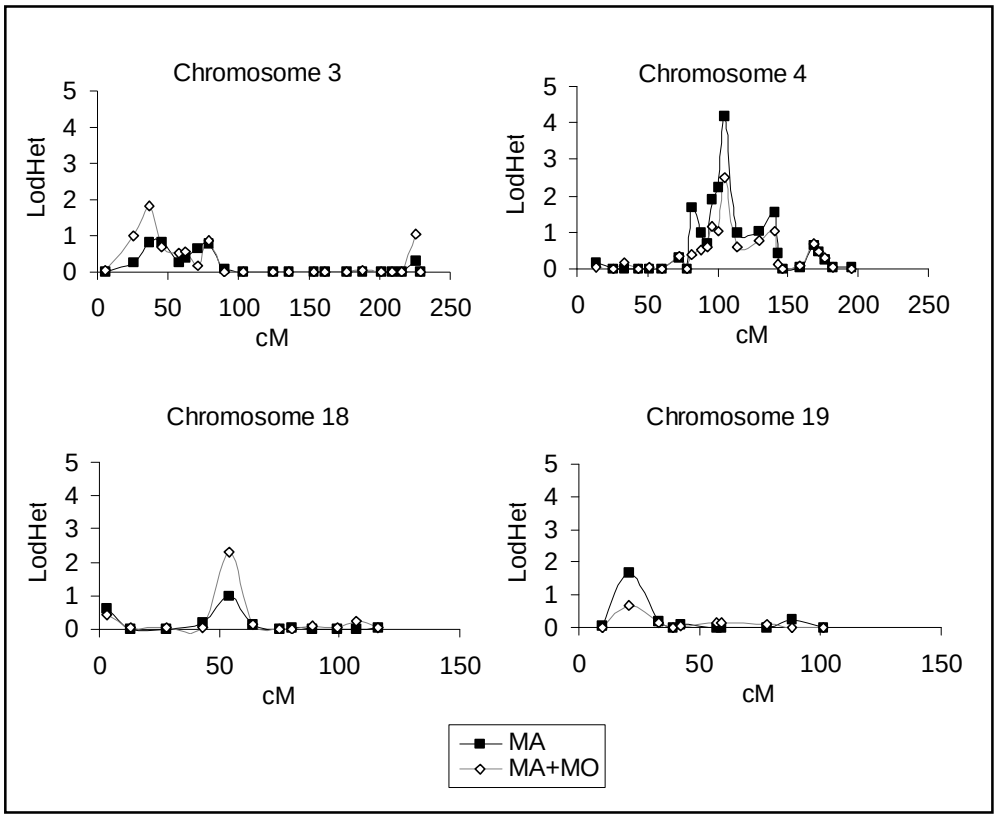

Figure 11. Two-point LOD scores for those chromosomes where changing the affection criteria (only MA versus MA+MO patients affected) had the largest effect.

After conducting this study, several other genome-wide scans and candidate locus studies on migraine have been performed. Loci on chromosomes 5q21, 6p12.2-21.1, 11q24, 14q21.2-q22.3, and 15q11-q13 have been significantly linked to migraine (Carlsson *et al.* 2002; Cader *et al.* 2003; Soragna *et al.* 2003; Nyholt *et al.* 2005; Russo *et al.* 2005). Furthermore, in a recent study by the Australian migraine group suggestive evidence of linkage to loci on 18p11 and 3q was found (Lea *et al.* 2005). The loci on 6p and 14q have been identified in a single multigenerational pedigree with mainly MO patients (Carlsson *et al.* 2002; Soragna *et al.* 2003) while the Canadian and the Italian studies producing evidence of susceptibility loci on 11q and 15q have both used a study design similar to ours and genotyped MA families (N= 43 and N=10, respectively) chosen for an apparent autosomal dominant transmission pattern (Cader *et al.* 2003; Russo *et al.* 2005). Nevertheless, none of these other scans showed any evidence of linkage to 4q21-24. On the other hand, not even nominal evidence of linkage to any of these other susceptibility areas was seen in our study. Even the two markers on chromosome 15 producing LODs >1.0 in our sample are located on 15q21 (43 cM) and 15q25 (83 cM), more than 30 and 70 cM away from the identified 15q11-q13 susceptibility locus (Russo *et al.* 2005).

It is, however, worth mentioning that two of the reported less significant loci overlap with our findings (**Table 21**): in the Canadian study a LOD score of 2.22 was seen on 16p12 with

marker D16S769 (51 cM) (Cader *et al.* 2003) while we obtained a HLOD of 1.22 with marker D16S753 (58 cM). Furthermore, LODs ≥ 1.0 were obtained with marker D18S877 on 18q both in the deCODE study (Björnsson *et al.* 2003) and in our study (Wessman *et al.* 2002). Similarly, in the Australian study (Lea *et al.* 2005), a LOD >2 was obtained both on 14q22 (the Italian locus, Soragna *et al.* 2003) and on 18p11, which is one of the minor loci (LOD 1.6) observed by deCODE (Björnsson *et al.* 2003). Nevertheless, the susceptibility locus on 4q identified in our genome-wide scan remains as the only significantly replicated migraine locus (**Table 21**).

Table 21. Possible overlap in the migraine susceptibility loci identified using genome-wide scans.

Locus	Marker	Location (cM)*	LOD	Population	Phenotype	Reference
1q21-23	D1S2878	178	1.58	Australian	LCA-severe	Lea *et al.* 2005
	D1S1679	171	1.53	Australian	LCA-migraine	Nyholt *et al.* 2005
4q21-24	D4S1647	105	4.20	Finnish	MA	Wessman *et al.* 2003
	D4S2409	96	4.08	Icelandic	MO (women)	Björnsson *et al.* 2003
14q21-22	D14S978	53	3.70	Italian	MO	Soragna *et al.* 2003
	D14S258	76	2.06	Australian	LCA-severe	Lea *et al.* 2005
16p12	D16S753	58	1.22	Finnish	MA	Wessman *et al.* 2003
	D16S769	51	2.22	Canadian	MA	Cader *et al.* 2003
18p11	D18S453	43	1.57	Icelandic	MO	Björnsson *et al.* 2003
	D18S53	41	2.30	Australian	LCA-severe	Lea *et al.* 2005
18q12	D18S877	54	1.00	Finnish	MA	Wessman *et al.* 2003
	D18S877	54	1.50	Icelandic	MO	Björnsson *et al.* 2003

* according to the Marshfield map; data from UCSC genome browser (http://genome.ucsc.edu/)

1.3 Are MA and MO distinct traits?

In spite of replication of the linkage to 4q21-24 locus, the lack of consensus between migraine susceptibility loci identified in genome-wide scans is considerable and probably an indication of heterogeneity. This situation is, however, more a rule than an exception when studying complex traits with incomplete penetrance, phenocopies and locus heterogeneity (Altmuller *et al.* 2001). When designing our genome-wide scan, our aim was to diminish the genetic heterogeneity as much as possible. Given that all our migraine families are of Finnish origin, the advantages of using a founder population apply to our study although we did not concentrate on any specific subisolate (Varilo and Peltonen 2004). Even more importantly, we decided to concentrate on clinically well-defined families ascertained for MA since: 1) most MA patients have very typical visual aura symptoms that precede the headache and thus the questionnaire-based differential diagnosis of MA can be regarded as even more reliable than

the diagnosis of MO and 2) family-based data suggest that genetic factors are more important in susceptibility to MA than to MO (Russell and Olesen 1995).

However, it is still unknown whether MO and MA are genetically distinct disorders or if there are some genetic variants that predispose to migraine in general. A continuum-severity model of headache disorders, where tension-type headache represents the mild end and MA the severe end, has also been suggested (Featherstone 1985). Many clinicians support the view that MA and MO are variants of the same disorder, mainly based on frequent co-occurrence of both types of attacks in the same individual and on observations where migraine type has converted from one to another at some stage of life (Blau 1995). Some clinical differences between MA and MO exist, but since most of the attack features are similar (Russell *et al.* 1996; Kallela *et al.* 2001b) one would assume some liability genes to be shared. However, a Danish twin study showed that co-occurrence of MA and MO in twin pairs does not occur more frequently than expected by chance and thus suggested MA and MO to be distinct disorders (Russell *et al.* 2002). On the other hand, results of a recent Australian twin study utilizing LCA and identifying subgroups of migraine patients based on their patterns of symptoms do not support the distinct disorder hypothesis (Nyholt *et al.* 2004). Two successful Australian genome-wide scans have been performed based on this phenotype classification, thus providing further evidence of the usefulness of this approach (Lea *et al.* 2005; Nyholt *et al.* 2005).

1.4 Choosing the best model

Both parametric and NPL analysis methods were applied in this study. In parametric linkage analysis, the mode of inheritance must be specified while nonparametric methods examine the extent of marker allele sharing among affected family members without specifying the underlying genetic model. On the other hand, the drawback of nonparametric methods is their lower power compared to traditional linkage analysis (Abreu *et al.* 1999). The genome-wide scan was performed assuming an autosomal dominant mode of inheritance. This was reasonable, since in the families selected for this study migraine segregated as a seemingly autosomal dominant trait. In addition, a classic form of NPL analysis, affected sib-pair analysis, was performed for each marker. The ASP results were, however, almost invariably less significant than the parametric linkage analysis results. This is not surprising, since some power is inevitably lost when large pedigrees are split into nuclear families. After identifying the susceptibility locus on chromosome 4q, NPL analysis for this region was conducted with

GENEHUNTER but these results were also less significant than those produced by the parametric analysis. Thus it seems that, at least for this set of migraine families, the parametric method was more powerful than the nonparametric method.

Other parametric migraine linkage studies have also used a dominant inheritance model, while the other parameters have differed markedly between these studies. With current knowledge, it is impossible to judge which model is the most correct. It is likely that multiple genetic and/or environmental risk factors are needed before migraine becomes manifested and thus a significant proportion of individuals carrying any given susceptibility factor can be expected to be healthy. A method in which multiple liability classes are used to account for age-dependent variability in the penetrance has been applied in two of the genome-wide scans in migraine (Carlsson *et al.* 2002; Cader *et al.* 2003). Furthermore, since migraine is 2-3 times more common in women than in men, the disease penetrance of men can be assumed to be lower than that of women. Due to these complicating factors, we decided to use the affecteds-only approach and thus the penetrance model used had less significance. The disadvantage of this method is, however, that similar to NPL analysis, some power is lost because linkage information coming from the healthy family members is not used.

2 ANALYSING CANDIDATE LOCI AND GENES

Two different candidate gene approaches were applied in this thesis. In study II, the role of candidate locus 19p13 in MA susceptibility was investigated using a linkage approach while in study III, several SNPs in candidate genes *ESR1* and *MTHFR* and their association with MA were examined. Of these, the role of the 19p13 locus that surrounds the *CACNA1A* (FHM1) gene (**Table 10**, page 42) as well as the role of the *MTHFR* gene (**Table 23**, page 88) has been relatively extensively studied. The power of these previous studies has, however, been rather limited due to small sample sizes and thus the results have been inconclusive. Therefore the basic idea of studies II and III was to use a sample size large enough to be able to provide conclusive results of whether these loci/genes contain variants predisposing to MA.

2.1 Candidate locus on 19p13

The migraine candidate locus on 19p13 contains two genes associated with migraine: the *CACNA1A* (FHM1) gene and the nearby insulin receptor *INSR* gene (Ophoff *et al.* 1996a; Jones *et al.* 2001). In our genome-wide scan (study I), the D19S1150 marker, an intragenic marker of the *CACNA1A* gene, showed no evidence of linkage to migraine (HLOD 0.00).

However, marker D19S427, located approximately 20 cM distal to D19S1150 and near the *INSR* gene, produced a maximum LOD score of 1.70 (θ = 0.22). Thus we felt that this chromosomal region was worth studying further and genotyped eight polymorphic microsatellite markers surrounding the *INSR* and *CACNA1A* genes (Figure 1 of study II) for 757 individuals belonging to 72 families. This family sample was the largest used so far for studying chromosome 19. Furthermore, we performed simulation studies to confirm that with this set of families, it should be possible to detect linkage even if only a minority of them (~30%) are linked to this region (**Figure 12**).

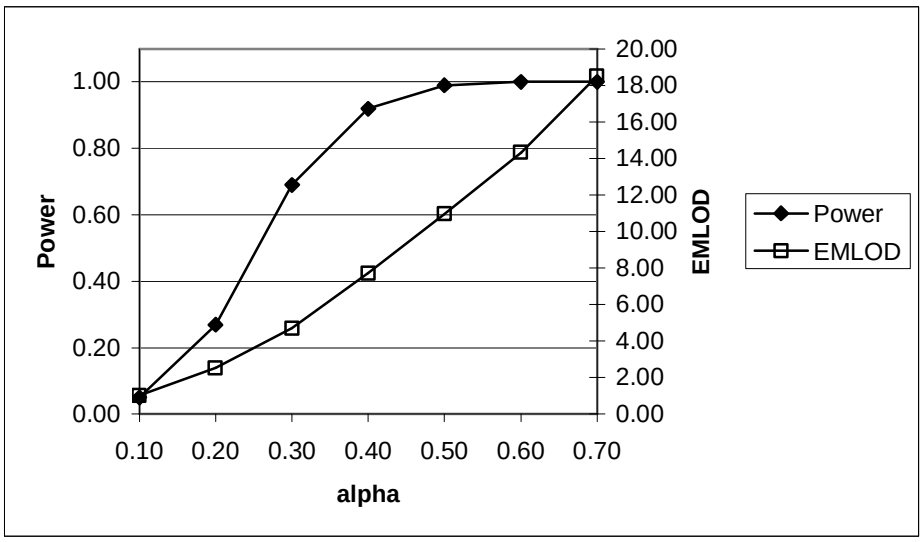

Figure 12. Simulation of the linkage in the 72 studied MA families. The x-axis shows the proportion of linked families (α) from 0.1 to 0.7. At α = 0.6, the power of 1 is reached.

Both parametric and NPL analyses with the affecteds-only model were performed. In summary, none of the studied markers covering a 34 cM region showed any evidence of linkage to MA either under locus homogeneity or heterogeneity (**Table 22**). Furthermore, neither the results of ASP analysis nor the results of NPL analysis performed using SimWalk2 supported linkage. Similarly, the results obtained using the broader affection criteria with MO patients also coded as affected were non-significant (maximum LOD and HLOD 0.13).

One of the main reasons why we were interested in conducting study II was the report published in 2001 of an MA susceptibility locus on 19p13 nearby but distinct from *CACNA1A* and the subsequently reported association of migraine with the *INSR* gene (Jones *et al.* 2001; McCarthy *et al.* 2001). We expected to find some evidence of linkage to the *INSR* region considering that the proportion of linked families was very high in the original study performed in 16 North American families of mainly western or northern European descent (Jones *et al.* 2001). Furthermore, the nominal LOD score of 1.70 obtained with marker

D19S427 (flanking the *INSR* gene) in our genome-wide screen suggested linkage (**Table 18**, page 75).

Table 22. Two-point LOD scores and NPL scores for the 19p13 markers and the location of the *INSR* and *CACNA1A* genes.

Marker	Position on chromosome 19		Under locus homogeneity			Under locus heterogeneity		NPL-all (SimWalk stat E)	
	cM*	Mb*	Max LOD	θ	LOD at θ = .00	Max HLOD	θ	-log (P)	p-value
D19S247	9.8	3.1	0.00	-	-45.24	.00	-	0.27	0.54
D19S427	20.8	6.1	0.00	-	-26.86	.07	.00	0.63	0.23
INSR gene		7.1 – 7.2							
D19S592	NA	7.3	0.00	-	-38.71	.00	-	0.71	0.19
D19S391	28.8	8.5	0.03	.46	-39.86	.03	.46	0.52	0.30
D19S394	34.3	10.6	0.00	-	-43.80	.16	.14	0.79	0.16
D19S221	36.2	12.6	0.00	-	-44.38	.00	-	0.36	0.44
CACNA1A gene		13.2-13.5							
D19S1150	NA	13.3	0.00	-	-44.65	.00	-	0.44	0.36
D19S226	42.3	14.5	0.00	-	-46.06	.00	-	0.39	0.41

NA = not available; * from p-tel

However, in study II, the HLODs of the two *INSR* flanking markers, D19S427 and D19S592, were practically zero (**Table 22**) thus producing no evidence of a migraine susceptibility locus in this region. It is likely that the LOD score obtained in the genome-wide scan was a false positive, as is often the case when only weak evidence of linkage is found. Although 50 of the 72 families were the same in both studies, the information provided by the 22 additional families and the fact that the marker D19S427 was genotyped again in all of the samples using another genotyping system (LI-COR versus ABI) are probable explanations for the observed LOD score drop (from 1.70 to 0.07). No other reports either replicating or excluding linkage or association to the *INSR* region have been published since. Furthermore, none of the published genome-wide scans has obtained a linkage peak in this area.

The *CACNA1A* area has been speculated to play a role in susceptibility to the common forms of migraine for more than ten years. Our group tested this hypothesis in 1994 using four Finnish MA/MO families and saw no evidence of linkage to 19p13 (Hovatta *et al.* 1994). However, after conducting that study, it has become evident that much larger family sets are required to detect complex disease loci. Thus, in addition to *INSR* markers, we wanted to genotype and test markers covering the *CACNA1A* region in our sample of 72 families. The linkage results for these *CACNA1A* markers were clearly negative and, most convincingly, the intragenic marker D19S1150 produced a HLOD score of 0.00 (**Table 22**). This was in accordance with the results of our genome-wide scan (study I). Many other research groups

have also studied the role of *CACNA1A* in migraine (**Table 10**, page 42). Some of these studies have reported suggestive linkage to this region, mainly based on ASP analysis (May *et al.* 1995; Nyholt *et al.* 1998a; Terwindt *et al.* 2001). However, results of all of the published linkage studies with larger sample sizes are negative and thus in accordance with ours (Lea *et al.* 2001b; Noble-Topham *et al.* 2002). Furthermore, none of the genome-wide scans have provided evidence of linkage to this region.

Although evidence against *CACNA1A* as a predisposing locus for common forms of migraine is now mounting, some families may still have high penetrance susceptibility alleles in this gene. Interestingly, sequencing of all *CACNA1A* exons in two Australian patients belonging to a MA family with suggestive linkage to this region showed no mutations (Lea *et al.* 2001b). This result is in accordance with our data (unpublished). We studied nine families with 2-5 patients having migraine attacks accompanied by hemiparetic or hemisensory symptoms for linkage to 19p13 (Kaunisto 1999). Three of these families showed LOD scores above 1 and the haplotype analysis showed that all MA patients with hemisensory or hemiparetic symptoms carried the same haplotype within the families. The pedigree structure and the 19p13 haplotypes of the most representative of these families are shown in **Figure 13**. Every *CACNA1A* exon was sequenced in six patients belonging to these three families but no mutations were found. Thus it seems that mutations in the coding regions of *CACNA1A* are not found even in those few migraine families showing linkage to the region. However, it is still possible that the variant(s) predisposing to common types of migraine reside in the promoter region, introns or still unidentified exons and are thus more difficult to detect.

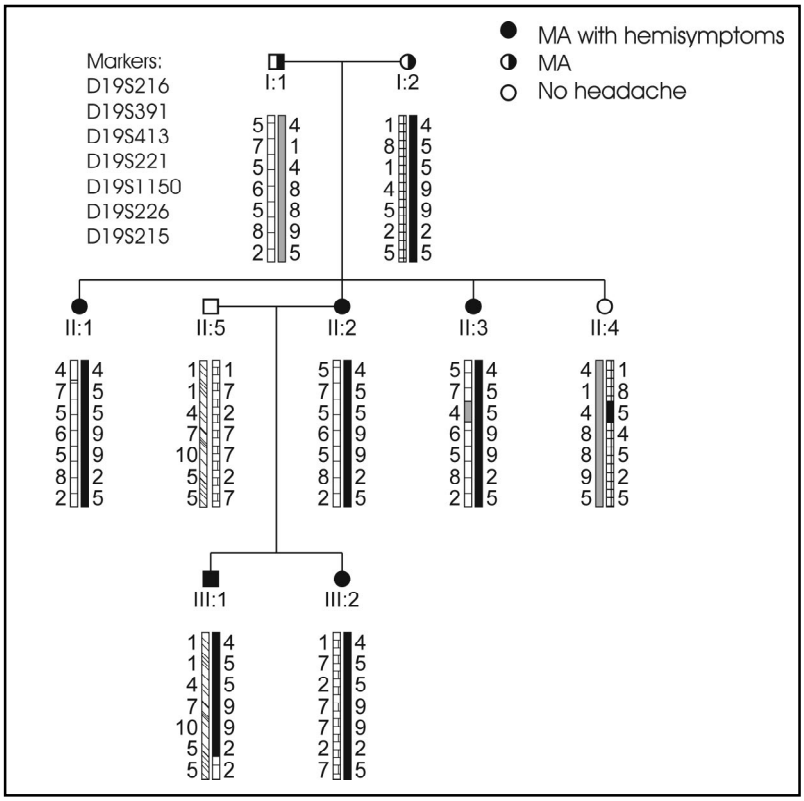

Figure 13. Pedigree and haplotypes of a Finnish MA family showing linkage to 19p13. The susceptibility haplotype cosegregating with FHM is shown by a black bar.

2.2 Candidate gene studies

2.2.1 The role of MTHFR and ESR1 genes in MA susceptibility

In study III, we aimed at selecting the most promising candidate genes previously reported to be associated with migraine and to evaluate their contribution in a substantially larger case-control sample set than used in the original studies. Although a considerable number of association studies on migraine have been published, only very few of the initial positive results have been replicated using an independent sample. Among these are polymorphisms in the methylenetetrahydrofolate reductase (*MTHFR*) and estrogen receptor (*ESR1*) genes located on chromosomal areas 1p36 and 6q25, respectively.

The association of a functional C677T variant of the *MTHRF* gene with migraine was originally described in Japanese patients and subsequently replicated in Turkish, Australian, and Spanish populations (Kowa *et al.* 2000; Kara *et al.* 2003; Lea *et al.* 2004; Oterino *et al.* 2004). These studies have consistently shown that the homozygous carriers of this variant have an increased risk for migraine, especially MA. However, the number of MA patients investigated in these studies has been very small, 22-170 (**Table 23**). Before our study, the G2014A variant of *ESR1* had only been studied in the Australian population (224 migraine patients) (Colson *et al.* 2004). The association of this variant with migraine susceptibility was,

Table 23. Results of association studies concerning the role of the *MTHFR* gene on migraine.

Population (reference)	**Japanese** (Kowa *et al.* 2000)	**Turkish** (Kara *et al.* 2003)	**Australian** (Lea *et al.* 2004)	**Spanish** (Oterino *et al.* 2004)	**Finnish** (study III)
Patients (N)	121	102	270	230	898
MA	22	23	170	78	898
MO	52	70	100	152	-
HA	47	9	-	-	-
Controls (N)	261	136	270	204	900
T637 allele frequency					
MA	0.64	0.26	0.40	0.42	0.24
MIG All	0.48	0.34	0.38	0.33 (MO: 0.29)	-
Controls	0.35	0.25	0.33	0.36	0.24
p-value	< 0.01	0.027*	0.017	0.006#	0.84
T677T genotype frequency					
MA	0.41	0.04	0.19	0.18	0.05
MIG All	0.20	0.08	0.15	0.12 (MO: 0.09)	-
Controls	0.10	0.02	0.09	0.13	0.06
p-value	<0.0001	0.015*	0.006	0.03#	0.83
OR¤	6.5	5.7*	2.5	2.3#	0.89

MA = migraine with aura; MO = migraine without aura; HA = headache; * all cases compared to controls; # MA cases compared to MO cases; ¤ for the TT genotype compared to others

however, replicated by the same group in an independent case-control sample of 260 patients (Colson *et al.* 2004). Since multiple subgroup testing and small sample sizes can easily produce associations significant at the 5% level by chance alone, there is a possibility that these results are false positives. Nevertheless, the *MTHFR* and *ESR1* genes are good candidate genes for migraine due to their function. Methylenetetrahydrofolate reductase catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate and reduced activity of this enzyme leads to mild hyperhomocysteinemia (Frosst *et al.* 1995). On the other hand, the role of hormone receptors in migraine susceptibility has been suggested because the female preponderance among migraine patients could be due to the additional trigger of fluctuating female hormone levels during the menstrual cycle (Bousser 2004).

Based on the information summarized above, we selected the *MTHFR* and *ESR1* genes to be genotyped in our candidate gene study. In the previous positive studies concerning these genes only 1-2 SNPs were tested. This type of study design is not, however, suitable for evaluating the potential role of the studied gene in migraine susceptibility. Although the polymorphisms tested have been assumed to be functional, the observed association signal could as well be due to some other variant within the gene that is in partial LD with the studied SNP. To avoid this problem, we aimed at covering the whole genes with SNPs. Genotyping results were obtained from 6 *MTHFR* SNPs (gene size 19 kb) and from 24 *ESR1* SNPs (gene size >200 kb) with a mean distance of 3.8 kb in *MTHFR* and 13.6 kb in *ESR1* (Table 2 of study III). The SNPs had a minor allele frequency of 10-45% in our population.

Since ~1800 samples were tested, more than 53,000 genotypes were produced with an average success rate of 98.6%. Based on the lack of discrepant genotypes among the duplicate samples the quality of our genotype data was considered to be high. The genotype distributions of all but two of the SNPs, rs746432 and rs3853248 located within the first exon and intron of the *ESR1* gene, were in Hardy-Weinberg equilibrium (HWE). Interestingly, the genotype distributions of these two SNPs violated HWE only among patient samples. Although a careful examination of the genotyping results revealed no genotyping errors, we noticed that due to weak allele signals several potentially heterozygous genotypes had been rejected, providing a possible explanation for the observed HWE violation. On the other hand, a small number of HWE failures can be due to chance alone. Most of the genotyped *MTHFR* SNPs were in high LD (Figure 1 of study III) and thus produced redundant information. Several blocks of high LD were also identified in the *ESR1* gene (Figure 1 of study III).

The main result of this study was that we were unable to replicate the previous association findings connecting the *MTHFR* C677T variant and the *ESR1* G2014A variant with migraine. The genotype and allele frequencies of these SNPs did not differ between cases and controls in our population (**Tables 23** and **24**). Similarly, no association with the other *MTHFR* SNPs was seen.

Table 24. Results of the *MTHFR* and *ESR1* SNPs previously associated with migraine.

SNP	MAF (%)		p-value	OR (confidence interval)	Genotype frequencies (%)						p-value
	controls	cases			controls			cases			
					11	*12*	*22*	*11*	*12*	*22*	
MTHFR C677T	24	24	0.84	0.98 (0.84-1.15)	58	36	6	58	37	5	0.830
ESR1 G2014A	19	20	0.50	1.06 (0.90-1.25)	66	30	4	63	33	4	0.548

On the other hand, the minor alleles of five *ESR1* SNPs located approximately 150-220 kb from the G2014A variant were slightly more common among cases than controls: the allele frequency comparison produced p-values of 0.008-0.067 (OR 1.15-1.21) and the genotype distribution comparison p-values of 0.007-0.034 (**Table 25**). Logistic regression analysis provided no evidence of an additive genetic model (OR for heterozygous individuals ~1) while the disease risk for individuals having two minor alleles was increased (**Table 25**). Haplotype association analysis was also performed and the overall haplotype distribution comparison with PHASE produced a p-value of 0.01. The haplotype formed by the minor alleles was found to be over-represented among cases while the haplotype formed by the major alleles was under-represented (Table 5 of study III). These results were, however, less significant than the single SNP association p-values.

Table 25. Results of the five *ESR1* SNPs showing nominal association with MA.

ESR1 SNP		MAF (%)	p-value	OR	Genotypes (N)			p-value	OR-Het*	OR-Hom#
					1 1	1 2	2 2			
rs6557170	cases	0.28	0.014	1.21	470	345	78	0.007	1.062	1.851
	controls	0.24			502	347	45			
rs2347867	cases	0.37	0.020	1.18	367	388	130	0.034	1.071	1.489
	controls	0.33			391	386	93			
rs6557171	cases	0.34	0.008	1.2	390	387	107	0.023	1.146	1.558
	controls	0.30			426	369	75			
rs4870062	cases	0.34	0.008	1.21	391	385	111	0.024	1.143	1.54
	controls	0.30			434	374	80			
rs1801132	cases	0.26	0.067	1.15	499	330	67	0.016	1.007	1.813
	controls	0.23			513	337	38			

* individuals with 1 2 genotype compared to those with 1 1 genotype;
\# individuals with 2 2 genotype compared to those with 1 1 genotype

Since polymorphisms in the estrogen receptor could be specifically associated with migraine susceptibility in females, the association analysis was also performed for females and males separately. These subgroup analyses had, however, only a negligible effect on the results. Furthermore, since 114 out of 898 migraine patients studied did not fulfil all the IHS criteria for MA, a subgroup analysis excluding these individuals was conducted but this had no effect on the results either.

2.2.2 Problems concerning candidate gene studies

The aim of the genome-wide linkage analysis method used in study I is to localize a disease susceptibility locus somewhere in the genome without prior assumption of its location. In studies II and III, a different approach i.e. candidate locus/gene strategy was applied. This type of study setting is based on a hypothesis about the possible role of a gene in the pathophysiology of the disease. The drawback of this method is that the disease mechanism is only rarely so well understood that selecting the correct gene from the estimated 25,000 in the human genome would be easy. In this thesis, the studied genes were selected because of previous evidence connecting them with migraine.

Other main issues concerning the experimental design of association studies are the selection of individuals and the SNPs to be genotyped. We used the distance between the SNPs as the selection criteria. An alternative approach could have been to use the so called htSNPs of the HapMap project (http://www.hapmap.org). However, there is only limited evidence of the usefulness of this approach in different populations.

A poorly matched control group can produce spurious associations. In this study, neither the cases nor the controls originated from a particular part of Finland but rather represented the Finnish population as a whole. Thus, population stratification should not be a problem in this case-control sample. Furthermore, a lot of effort was put into selecting control individuals that matched the cases for both age and sex. Although these variables should not affect the allele frequency distributions, they might have an effect on the disease penetrance. It is possible, for example, that a higher proportion of healthy males than females carries the risk allele assuming that the penetrance of the allele is higher in females because of hormonal or other factors. By selecting patients so that most had a strong family history of migraine and all had at least one affected family member, we aimed at increasing the amount of genetic susceptibility factors among cases. Furthermore, only controls without a family history of

migraine were included in the study. This selection criteria should be optimal for providing evidence of association.

3 *ATP1A2* MUTATION IN THE FHM2 FAMILY

3.1 Linkage analysis

In study IV, we identified a three-generation Finnish pedigree with 11 affected FHM family members. At the time this project was started, the second locus for FHM had been assigned to chromosome 1q but contradictory results existed as to whether the correct area is 1q21-23 or 1q25-31 (Ducros *et al.* 1997; Gardner *et al.* 1997). Furthermore, the FHM2 gene *ATP1A2* had not then been identified. Thus our first aim was to study this family for linkage to the known FHM susceptibility regions on 19p13 and 1q21-31 (Joutel *et al.* 1993; Ducros *et al.* 1997; Gardner *et al.* 1997). The two-point LOD scores for chromosome 19p13 markers were clearly negative, suggesting a lack of involvement of this locus, whereas significant evidence of linkage was found at the 1q21-23 locus. The maximum two-point LOD scores for chromosome 1q markers are shown in **Table 26**. The highest two-point LOD score, 3.37, was obtained at four nearby markers D1S2343, D1S2635, D1S1679, and D1S2844 at $\theta = 0.00$ using a model in which the two family members with MO were considered as unaffected. The

Table 26. Locations and two-point LOD scores for markers on 1q21-32.

Marker (location)	Position (cM)	LOD Score	θ
D1S514 (1p12)	152.45	1.27	0.00
D1S2343 (1q21.3)	155.89	3.37	0.00
D1S2346 (1q21.3)	158.75	3.15	0.00
D1S305 (1q21.3)	155.89	2.60	0.00
D1S2635 (1q23.2)	158.75	3.37	0.00
D1S2707 (1q23.2)	168.52	3.07	0.00
D1S2705 (1q23.3)	170.84	0.69	0.00
D1S1679 (1q23.3)	170.84	3.37	0.00
D1S2768 (1q23.3)	172.93	0.90	0.00
D1S2844 (1q23.3)	175.03	3.37	0.00
D1S426 (1q23.3)	177.86	1.09	0.10
D1S433 (1q24.2)	184.21	1.09	0.10
D1S2815 (1q24.3)	188.85	1.87	0.08
D1S215 (1q25.2)	194.89	1.09	0.10
D1S422 (1q31.2)	205.40	0.37	0.22
D1S2745 (1q31.3)	212.44	0.37	0.22
D1S2655 (1q32.1)	216.82	0.07	0.30
D1S249 (1q32.1)	220.65	0.61	0.20
D1S2782 (1q32.2)	222.84	0.07	0.30
D1S2703 (1q32.3)	231.67	0.14	0.30

markers covering the 1q31-32 locus produced only slightly positive LOD scores (**Table 26**). Unfortunately, the haplotype shared by all affected individuals of this pedigree extended from the most proximal marker studied, D1S514, to marker D1S2844 thus spanning at least 23 cM. As a result we were unable to refine the 1q21-23 FHM2 candidate region.

3.2 Mutation screening

The 1q21-23 area contained several potential candidate genes. We decided to screen one of them, *KCNN3*, coding for a small-conductance calcium-activated potassium channel SK3, for mutations. Small-conductance calcium-activated potassium channels (SK channels) modulate excitability in many cell types, including neurons, where they contribute to the after-hyperpolarizations that dynamically control the frequency of spontaneous rhythmic action potentials. Furthermore, these channels have been shown to be selectively coupled to the $Ca_v2.1$ channels, mutations of which cause FHM1 (Womack *et al.* 2004). Four mammalian SK channels (SK1-4, encoded by *KCNN1-4)* have been cloned, of which *KCNN3* is expressed in brain, striated muscle and lymphoid tissues (Kohler *et al.* 1996; Joiner *et al.* 1997). This gene consists of eight exons spanning over 163 kb and contains two intragenic N-terminal CAG-repeats of which the second is longer and highly variable in humans, similar in size and variability to that of the *CACNA1A* gene (Chandy *et al.* 1998; Sun *et al.* 2001). However, no potential mutations were detected in the promoter region or in the exons of this gene and only common, short alleles at the polymorphic CAG repeat were observed.

After the Italian migraine group observed that *ATP1A2* mutations are the cause of FHM2 (De Fusco *et al.* 2003), we screened this gene and identified a heterozygous single nucleotide change A1033G in exon 9 leading to a T345A missense mutation. None of the studied 132 Finnish control individuals had this A1033G change. In addition to this disease-associated mutation, 11 other polymorphisms, ten of which were intronic and one a synonymous exonic nucleotide change, were identified in the proband (Table 1 of study IV). Allele frequency information of only one of these sequence variants was available from the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP). Thus, to make certain that none of the other variants represented the actual disease-causing mutation, the allele frequencies of these polymorphisms were estimated using pooled DNA samples of the 132 control individuals. All the nucleotide changes were relatively common (minor allele frequencies 7-44%) and thus could not be regarded as potential mutations in a Mendelian trait.

3.3 Genotype-phenotype comparison

The T345A missense mutation was detected in 12 family members, ten of which had FHM attacks while two (aged 19 and 10) were asymptomatic at the time of this study (**Figure 14**). Recently, however, the younger of these two individuals has started having attacks.

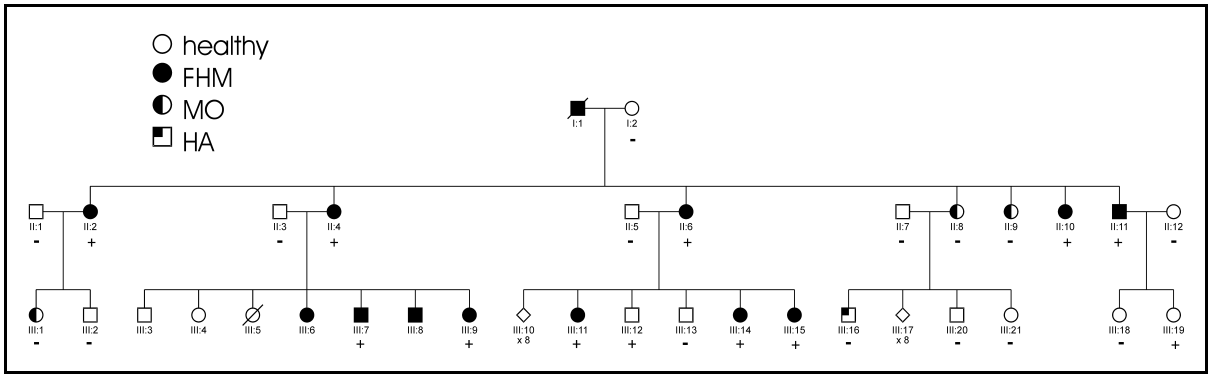

Figure 14. Pedigree of the FHM2 family. + = Presence of mutation; - = absence of mutation.

It has been suggested that FHM could be seen as a model for studying MA and MO although the FHM attacks are clearly more severe than the attacks of these more common forms of migraine (Ophoff *et al.* 1994). On the other hand, FHM families often include patients with MO and MA and FHM patients can also have attacks classified as non-hemiplegic migraine (Ophoff *et al.* 1994; Ophoff *et al.* 1996a). The occurrence of MA and MO in FHM probands and their first degree relatives has been investigated (Thomsen *et al.* 2003). The relative risk of MA was found to be about 7-fold both among FHM probands and their affected relatives and about 2-fold in non-FHM-affected relatives. No increased risk of MO was found and the authors concluded that sharing of genetic predisposing factors between FHM and MO is unlikely (Thomsen *et al.* 2003). In accordance with these results, three patients belonging to the FHM family investigated in study IV had MO but were not *ATP1A2* mutation carriers.

3.4 Pathophysiology of FHM

The structure of the Na^+,K^+-ATPase α2 subunit and the thus far identified mutations are presented in **Figure 5** (page 60). The T345A mutation, like most FHM2 mutations, is located in the large intracellular M4-5 loop that is critical for the correct function of the ATPase. At the cytosolic border of the transmembrane region M4 there is a characteristic amino acid sequence PEGL involved in energy transduction followed by a highly conserved 40 amino acids long sequence and a phosphorylation motif DKTGTLT (Figure 2 of study IV) (Moller *et*

al. 1996). The threonine 345 is situated in the middle of this conserved 40 amino acid connecting sequence.

The functional consequences of the first two published FHM2 mutations, L764P and W887R were evaluated using a robust *in vitro* cell growth phenotype test (De Fusco *et al.* 2003). Since Na^+,K^+-ATPase activity is essential for cell viability, blocking of the endogenous enzyme in cells transfected with the mutant ATPase leads to cell death if the mutant indeed abolishes the enzyme function. The L764P and W887R mutant enzymes did not support the growth of cultured cells and thus a haploinsufficiency effect caused by loss of function of the mutated Na^+,K^+-ATPase was suggested to be the disease mechanism of FHM2 (De Fusco *et al.* 2003).

We have studied the functional effects of the T345A mutation identified in this thesis in collaboration with a research group from the University of Montreal, Canada (Segall *et al.* 2004). Interestingly, the total loss of function suggested by the Italian group was not seen with this mutation. On the contrary, growth of the mutated cells was normal and the enzyme was shown to be fully active. However, detailed kinetic analysis revealed differences between the wild type and T345A mutation containing enzymes. Most importantly, the affinity of the mutant enzyme for extracellular K^+ was decreased ~2-fold (Segall *et al.* 2004). It has been shown that the N-terminal half of the M4-M5 loop has an important role in cation selectivity although the actual cation binding sites are located in the transmembrane regions (Blostein *et al.* 1999). The increased hydrophobicity created by the T345A mutation, located near the cytosolic border of the transmembrane region M4, affects the cation-binding pocket of the enzyme thus explaining the lowered affinity for extracellular K^+ (Segall *et al.* 2005).

Very recently, our collaborators published a kinetic analysis of two other FHM2 mutations (Segall *et al.* 2005). Similarly to the T345A mutation identified in this thesis, the mutations R689Q and M731T originally identified by the Dutch migraine group (Vanmolkot *et al.* 2003) were shown to lead to functional but kinetically altered Na^+,K^+-ATPase subunits. In the case of these mutations, the reduced enzyme activity was, however, due to decreased catalytic turnover of the Na^+,K^+-ATPase (Segall *et al.* 2005).

Since the first FHM2 mutations were identified, it has been speculated as to how mutations in the *CACNA1A* and *ATP1A2* genes, expressed in distinct cell types (neurons and astrocytes), can lead to practically identical FHM phenotypes. It is clear that increased Ca^{2+} influx through mutant $Ca_v2.1$ channels (FHM1) might lead to hyperexcitability due to increased release of

the excitatory neurotransmitter, glutamate (Turner *et al.* 1992; Moskowitz *et al.* 2004). On the other hand, astrocytes are known to have an important role in the termination of synaptic activity by removing glutamate and K^+ from the synaptic cleft with the help of the Na^+,K^+-ATPase (FHM2). It is likely that the *ATP1A2* mutations slow the removal of K^+ from the extracellular space. An increased extracellular K^+ concentration can either directly make the brain more susceptible to CSD or slow down the Na^+ gradient-driven glutamate transporter (Moskowitz *et al.* 2004; Segall *et al.* 2004). Indeed, blocking of Na^+,K^+-ATPases has been shown to induce spreading depression-like depolarization in rat hippocampus (Balestrino *et al.* 1999). The recently identified FHM3 gene, the neuronal voltage-gated sodium channel *SCN1A* (Dichgans *et al.* 2005), and the finding of a mutation in the glutamate transporter EAAT1 gene *SLC1A3* in a patient with a complex neurological phenotype (Jen *et al.* 2005) fit well into this idea of FHM pathophysiology. The *SCN1A* mutation identified was shown to be of gain of function type and thus lead to excessive firing of neurons while the *SLC1A3* mutation markedly decreased the glutamate uptake capacity.

Interestingly, Na^+,K^+-ATPase α2 isoforms are specifically localized in plasma membrane microdomains that overlie endoplasmic reticulum (Juhaszova and Blaustein 1997). Thus they seem to have a role in the regulation of local intracellular Ca^{2+} concentration via Na^+/Ca^{2+} exchangers that are also localized in these microdomains (Golovina *et al.* 2003). Consequently, reduced Na^+,K^+-ATPase α2 activity might elevate local Na^+ concentration, promote Ca^{2+} entry via Na^+/Ca^{2+} exchangers and thus amplify Ca^{2+} signalling. This alternative FHM2 disease mechanism is, however, less likely because *ATP1A2* expression within the nervous system seems to be astrocyte-specific in adults (Moseley *et al.* 2003).

4 THE *CACNA1A* MUTATION CAUSING EA-2 IN A FINNISH FAMILY

4.1 Identifying the *CACNA1A* mutation

In study V, we identified a multi-generation Finnish family with several individuals having symptoms characteristic of EA-2. All available family members were genotyped at three microsatellite markers flanking the *CACNA1A* region. As significant evidence of linkage to this area was obtained (maximum two-point LOD 4.48), *CACNA1A* was sequenced. We identified a heterozygous A to G nucleotide change at the 3' acceptor splice site of intron 36 (IVS36-2A>G substitution) in the proband and her affected mother and demonstrated that the mutation segregated with the EA-2 phenotype in the family (**Figure 15**). No other potential

disease-causing mutations were found. There were no carriers of this nucleotide change among 132 control individuals, further supporting its role as a pathogenic mutation.

We have subsequently identified three other Finnish EA-2 patients unrelated to the family segregating the IVS36-2A>G mutation. These patients are either sporadic or have only one affected family member, and are thus unsuitable for linkage analysis. These patients do not carry the IVS36-2A>G mutation and thus this mutation does not seem to represent a founder-mutation, as might be expected. Although no systematic study aimed at identifying all Finnish EA-2 patients has been carried out, the number of patients that we are aware of is smaller than could be assumed. This is a typical situation for many recessive traits frequent in Caucasian populations, although for dominant traits the situation is less evident.

4.2 Genotype-phenotype comparison

In total, 22 mutation carriers were identified in the extended family (**Figure 15**). Of these, 17 were affected, two had extreme alcohol sensitivity as their only symptom and three were asymptomatic at-risk subjects, aged 8, 15, and 65. The oldest of these mutation carriers was telephone interviewed for a second time but no history of any episodic or permanent neurologic dysfunction was disclosed. This is, however, not surprising since incomplete penetrance of EA-2 has been previously demonstrated (Denier *et al.* 1999). The other two healthy mutation carriers are still too young to draw any conclusions concerning their future affection status. Interestingly, among the more distant family members one described symptoms indistinguishable from the affected individuals but did not have the mutation. It is possible, although unlikely, that this phenocopy carries some other predisposing genetic variant. Alternatively, the social environment, i.e. witnessing the attacks of other family members, might somehow predispose her to imagining symptoms that mimic the disease.

The clinical features of patients with different types of *CACNA1A* mutations are widely overlapping. Both EA-2 and FHM patients may develop progressive ataxia and EA-2 patients often have concomitant symptoms that fulfill the diagnostic criteria for migraine. In accordance with this, six EA-2 patients of the family studied by us were diagnosed as having MO as well. However, in these individuals migraine headache was not part of the EA-2 attacks. One additional patient had symptoms fulfilling the criteria for basilar migraine during the EA-2 attack. Furthermore, two healthy family members were also diagnosed as having MO and thus migraine appeared to segregate independently of the *CACNA1A* mutation in this family.

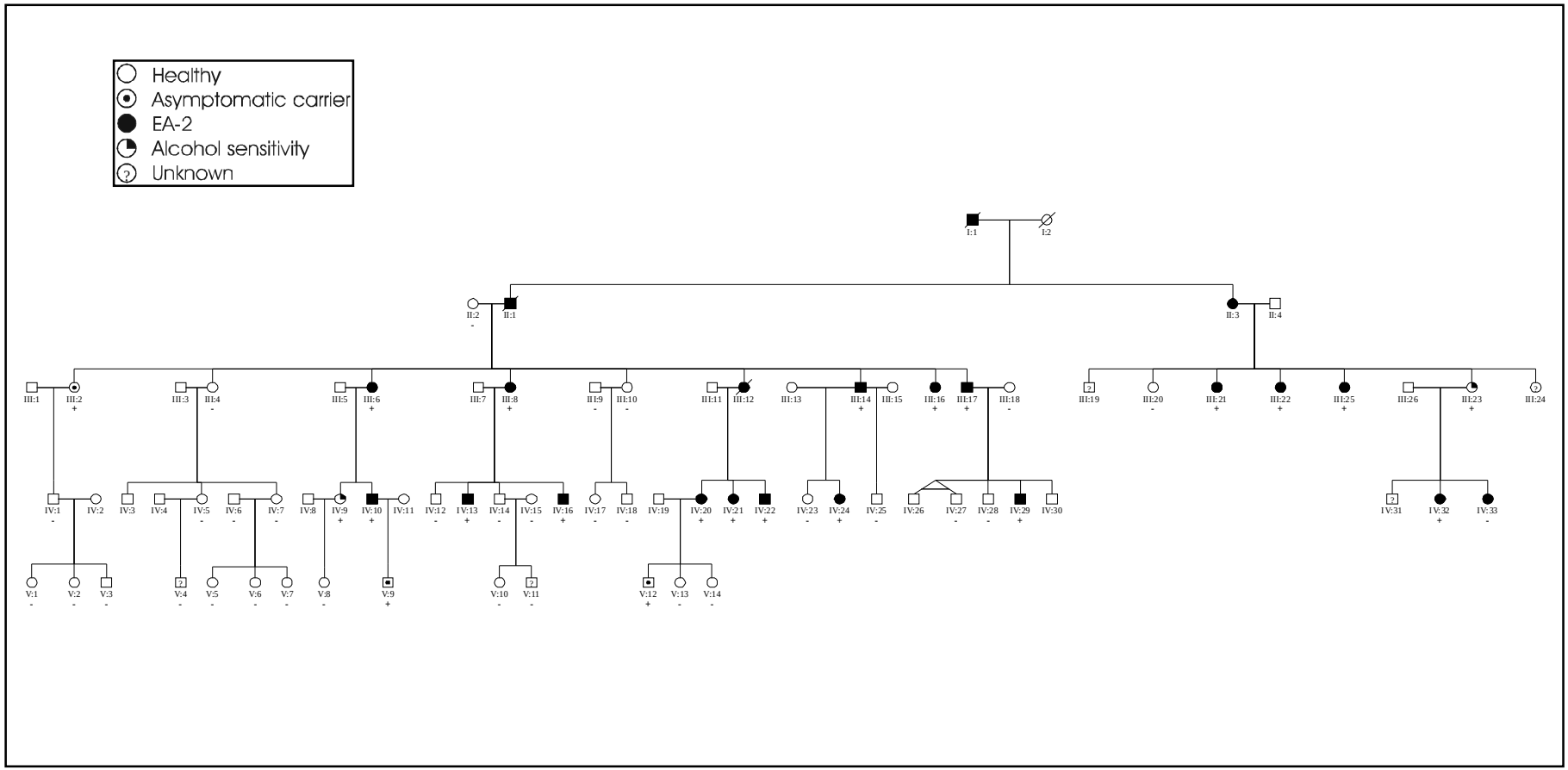

Figure 15. Pedigree of the EA-2 family. + = Presence of mutation; - = absence of mutation.

4.3 Disease mechanism

The IVS36-2A>G mutation destroys the highly conserved AG acceptor splice site consensus sequence of intron 36. It has been estimated that 99.24% of all mammalian splice site pairs are GT-AG and practically all of the other possible pairs also contain A in the -2 position (Burset *et al.* 2000). Several other *CACNA1A* splice site mutations have been published, but all of them affect the donor splice site (**Table 13**, page 52). In principle, splice site mutations most often lead either to skipping of an exon or to cryptic splice site usage (Krawczak 1992). Splice site prediction programs can be used to predict such effects. We utilized this approach to determine whether the DNA sequence around the IVS36-2A>G variant included any potential cryptic splice sites and, indeed, a cryptic acceptor splice site was found 65 bp downstream of the mutation site in exon 37 (**Figure 16**). This cryptic splice site produced a splice site score of 0.81, while the wild type has a score of 0.98 and the mutated site was not recognized at all. Based on these results, one likely consequence of the IVS36-2A>G mutation is the utilization of this cryptic splice site and a subsequent frameshift producing a premature STOP-codon at nucleotides 5891-5893 of *CACNA1A* (**Figure 16**).

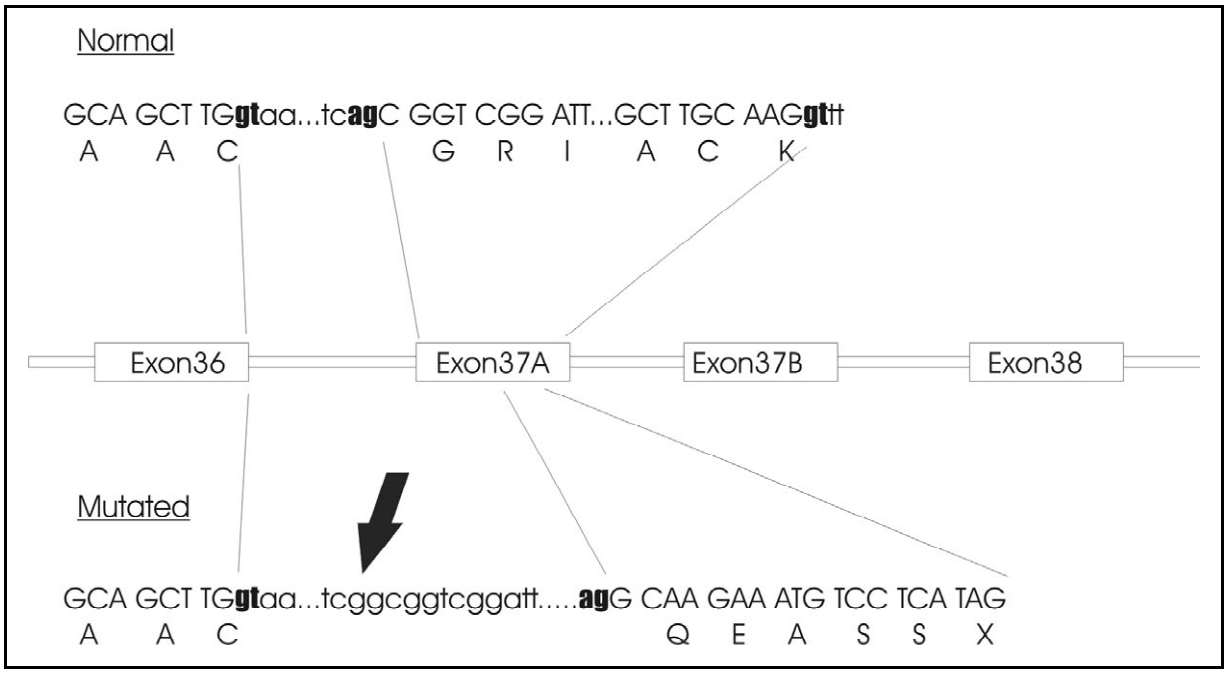

Figure 16. Predicted effect of the IVS36-2A>G mutation on *CACNA1A* mRNA splicing.

On the other hand, altered mRNA splicing should also be confirmed experimentally. Brain tissue samples of deceased patients would be ideal for this purpose, but since these are not available, we attempted to investigate the mutated *CACNA1A* mRNA using RNA isolated from lymphocytes. Although we were able to reverse transcribe and amplify the *CACNA1A* mRNA from peripheral blood lymphocytes of control individuals, the results were

inconsistent. The nested PCR amplification of the blood samples with inner primers specific for exons 35 and 39 resulted in several amplification products of different sizes that were verified by sequencing. In one product, all the exons 35 through 39 were present, in one product exon 37 was missing and in one product both exons 36 and 37 were missing. The number of splice variants varied between individuals and between samples from the same individual. This is likely to reflect stochastic differences in the amplification of a very low number of target molecules. Since the results of healthy individuals were so conflicting we concluded that comparing these with those of patients would not be reasonable. Thus the consequences of this as well as most of the other described EA-2 splice site mutations are currently unclear. However, after conducting this study, minigene constructs have been successfully used in determining the splicing defect from two intronic EA-2 mutations (Wan *et al.* 2005a). It would be interesting to apply this same method to the IVS36-2A>G mutation.

We can conclude that the IVS36-2A>G mutation most probably produces a truncated or aberrant $Ca_v2.1$ α_1 subunit, like the majority of previously identified EA-2 mutations. Based on electrophysiological studies it is likely that all EA-2 mutations, regardless of their type, lead to either complete or considerably reduced Ca^{2+} current density or channel activity (Guida *et al.* 2001; Jen *et al.* 2001; Wappl *et al.* 2002; Imbrici *et al.* 2004; Spacey *et al.* 2004). Thus a loss of function of $Ca_v2.1$ channels can be considered as the underlying disease-causing mechanism but the details of this are still unknown. In principle, either a simple haploinsufficiency of functional channels or a dominant-negative effect could be the primary cause. Since the pore-forming part of Ca^{2+} channels consist of only one protein subunit, the dominant negative effect is not as likely as in the case of K^+ channels, which are tetramers. Competition for auxiliary subunits or regulatory proteins could be hypothesized although recent results propose a mechanism mediated by misfolding of the full-length channel proteins due to interaction with the truncated channels (Page *et al.* 2004). However, this is still controversial since most of the other EA-2 mutations studied do not seem to affect transcription and translation processes or to cause degradation of the truncated proteins (Guida *et al.* 2001; Jen *et al.* 2001; Wappl *et al.* 2002). Thus, if haploinsufficiency is the underlying disease mechanism it is more likely to be due to either impaired channel function or inefficient targeting to the cell membrane than to reduced production of channels.

The IVS36-2A>G mutation is located in the part of the gene that codes for the intracellular C-terminus of the $Ca_v2.1$ channel while most of the previously published *CACNA1A* truncation mutations are predicted to prevent formation of at least one of the transmembrane regions of

the $Ca_v2.1$ subunit. Since no evidence of differences between the disease phenotype produced by these C-terminal truncations and the disease phenotype caused by more prematurely truncating mutations exists, the C-terminus must have an important role in the correct function of the $Ca_v2.1$ channel if the truncated proteins truly are stable and correctly targeted to the plasma membrane. The C-terminal part of the channel contains several important regulatory sites, including a β subunit interaction domain, a G-protein interaction site, an EF-hand-like domain, an IQ-like motif and a calmodulin binding domain, the three latter of which are involved in Ca^{2+}-dependent inactivation and facilitation of the channel (Catterall 2000; Lee *et al.* 2003; Chaudhuri *et al.* 2004).

Making things even more complicated, at least seven sites for alternative splicing have been identified in *CACNA1A* and one of them involves splicing of exon 37 (Soong *et al.* 2002). There are two mutually exclusive alternatives of this exon (exons 37A and 37B) coding for 72% homologous amino acid strands and thus producing two variants of the EF-hand motif (Trettel *et al.* 2000). These channel splice variants have different functional properties, the distribution of which is variable across brain regions (Chaudhuri *et al.* 2004). Furthermore, expression of these splice variants also seems to be developmentally regulated since >90% of the $Ca_v2.1$ channels in fetal brain contain the EF-B variant while the distribution is nearly equal in adulthood (Chaudhuri *et al.* 2004). It is possible that the IVS36-2A>G mutation does not lead to an aberrant splicing in the *CACNA1A* transcripts that contain exon 37B, which would mean that less than 50% of the channels produced are abnormal in mutation carriers. Interestingly, a similar situation is expected in two recently identified families with mutations in exon 37A producing a premature STOP-codon Y1845X within this exon (Kors 2004).

5 MIGRAINE PATIENTS AND THEIR CLASSIFICATION

A careful diagnosing and subclassification procedure is essential for genetic studies of complex diseases and can be especially demanding when no laboratory or other diagnostic test is available as is the case with migraine. This thesis work is part of the larger “Finnish Migraine Project” in which the main emphasis has been in collecting families segregating migraine with aura. Because of the large number of families and patients needed for complex disease studies, the cost-effectiveness of the recruitment and diagnosing procedures has been especially important. Most of the index cases have been recruited by neurologists working in clinics specialized for headache disorders. The diagnoses of the other family members have mainly been based on questionnaires and could thus be regarded as less reliable. However, the

questionnaire used has been validated by comparing the agreement between questionnaire-based and interview-based diagnoses of 200 individuals and was shown to have a sensitivity of 99% and a specificity of 96% for migraine in general (Kallela *et al.* 2001a). The response rate has also been good despite the extensiveness of the questionnaire. Since the recruitment of the index cases has been clinic-based, this patient sample does not represent the whole population of migraine patients, but rather the more severe end of the spectrum. Furthermore, we have concentrated on collecting families with several affected individuals. Although not very suitable for epidemiological studies, this is, however, exactly what is needed for genetic analyses.

The International Headache Society has provided a set of diagnostic criteria based on which diagnosing headache patients, at least for scientific purposes, is performed (Headache Classification Committee of the International Headache Society 2004). This has been shown to work well with most of our patients. However, in real life there are always patients that are difficult to classify. In the Finnish Migraine Project, the use of an additional category called "migraine with unclassified aura" has been found to be practical. This category comprises patients that, at least based on the questionnaire, do not completely fulfill the criteria for aura symptoms while definitely have migraine. According to the IHS criteria, these patients should be classified as having migraine without aura. This could, however, lead to potential wasting of useful genetic information if at least some of the underlying susceptibility loci are shared.

Another practical problem is the diagnosing of patients with hemiplegic migraine. The IHS criteria (2004) for hemiplegic migraine demand that the aura phase must include motor aura consisting of unilateral motor weakness or paralysis. It is relatively common that one or a few patients within large MA families fulfill these criteria and should thus be classified as having sporadic hemiplegic migraine. However, is has been consistently shown that these sporadic patients very rarely have mutations in the FHM genes *CACNA1A* and *ATP1A2* (Terwindt *et al.* 2002; Jen *et al.* 2004b). Similarly, we were unable to identify mutations when we sequenced the *CACNA1A* in index cases of families with hemisensory and/or motoric symptoms showing nominal evidence of linkage to chromosome 19. On the other hand, the phenotype of the FHM2 family studied in this thesis was clearly more severe with several patients that had been hospitalized during their attacks. Thus, for a geneticist, it seems that the FHM patients worth screening are those that belong to families where most of the migraine-affected individuals have hemiplegic symptoms. However, these kind of families seem to be very rare in Finland as well as in other countries.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Unraveling the genetic susceptibility factors of complex diseases has become the major goal of human genetic research but progress in this field has been relatively slow thus far. However, now it seems that convincing examples of polymorphisms implicated in the etiology of common diseases are starting to gradually mount. Compared with many other diseases with complex inheritance, the molecular genetics of migraine has been studied for a relatively short time. Furthermore, there are only a handful of research groups worldwide that are trying to tackle the genetics of this debilitating headache disorder. Therefore, it is not surprising that genetic susceptibility factors for the common forms of migraine have not yet been identified.

The main achievement of this thesis was the identification of the susceptibility locus for MA on chromosome 4q. The genome-wide scan we conducted was the first of its kind on migraine. Since then, several other whole genome linkage analyses have been performed, and, encouragingly, the 4q locus has been replicated in the Icelandic population. In spite of this replication and the truly significant evidence of linkage we saw, narrowing of the critical susceptibility region and identification of the causative gene variant(s) has turned out to be extremely challenging. Consequently, alternative approaches have been used in this thesis. One of the central ideas has been to utilize our exceptionally large migraine family and patient samples to investigate the role of susceptibility loci and genes previously associated with migraine. Using this approach it became obvious that the chromosome 19p13 locus important in FHM does not seem to be a major player in the susceptibility of common forms of migraine. This is a noteworthy finding, especially in view of the considerable previous controversy surrounding this subject.

Candidate gene studies have become popular in complex disease research. However, the presumably small increase in disease risk due to the at-risk genotype has to be taken account when planning these studies and thus large case-control sample sets are needed. In most of the candidate gene studies on migraine, only a couple of hundred patients have been studied. Thus planning and carrying out the case-control project comprising 1798 individuals can be regarded as a further achievement of this thesis, particularly since this sample will be used in the future for several other purposes.

It is possible that our limited understanding of the etiology of migraine is the main problem in these genetic studies. The patient is diagnosed as having migraine if an adequate number of

symptoms listed in the IHS criteria are present. Some of these criteria are, however, optional and only rarely do the patients fulfil all of them. For these reasons, we have hypothesized that it might be possible to define the phenotype by investigating these symptoms one at a time instead of using the end-point migraine diagnosis as the affection status. This trait component analysis using the genotypes of our original genome-wide scan is currently in progress and it seems that new susceptibility loci can be identified using this approach.

The pathophysiology of migraine is still very poorly understood. Identification of even one susceptibility gene might open up totally new perspectives on the cellular processes involved in this complex disease and thus facilitate the development of more effective treatments and more precise diagnostic applications. The quest for migraine genes has only just started but hopefully the genetic approach and this thesis itself will be useful in revealing the primary pathways of this debilitating disease.

ACKNOWLEDGEMENTS

This thesis work was carried out during the years 1999 to 2005 at the Department of Clinical Chemistry, in the laboratory of Professor Aarno Palotie and as part of the Research Program in Molecular Medicine, Biomedicum Helsinki, University of Helsinki. I want to thank Professor Ulf-Håkan Stenman, Head of the Department, and Professors Kimmo Kontula and Leena Peltonen-Palotie, Coordinators of the Research Program, for providing me with excellent working facilities.

I wish to express my deepest gratitude to my immediate supervisor, Docent Maija Wessman. I truly admire her wide knowledge in human genetics and enthusiasm in science as well as her way of noticing everybody around. It is amazing how after discussing with her for a few minutes you start to feel like a Very Important Person. Without her continuous support and positive guidance this thesis work would never have been accomplished.

I further wish to thank Professor Aarno Palotie, the Head of the migraine project and my second supervisor, for the opportunity to work in this project and his guidance and interest towards my work especially after returning back to Finland. His visions and connections in the complex disease field have been vital for this thesis.

I want to thank Professor Jim Schröder and Professor Tapio Palva, the Head of the Division of Genetics, University of Helsinki for their supportive attitude towards my thesis. Professor Jim Schröder is also thanked for accepting the role of custos in my defense.

I am truly grateful to Docent Tiina Paunio and Assistant Professor Joanna Jen for carefully pre-examining my thesis. Special thanks go to Tiina Paunio for many valuable comments and discussions and always finding time for me in her tight schedule. I am grateful to Jodie Painter for carefully revising the language of this thesis.

This thesis work would not have been possible without the help of many skilled neurologists. I wish to warmly thank Docent Markus Färkkilä for his encouraging and friendly attitude towards my work. I am especially indebted to Mikko Kallela whose role in my thesis has been invaluable. In addition to diagnosing thousands of migraine patients he has always been full of energy and great ideas and thus made this work much more interesting. I owe very special thanks to Hanna Harno as well. Collaboration with her in studies concerning the FHM and EA-2 patients has been very fruitful and pleasant and her friendship and support during these years has been very important to me. Furthermore, I want to acknowledge the help of many other Finnish headache specialists; Doctors Ville Artto, Hannele Havanka, Matti Ilmavirta, Jarmo Liukkonen, Markku Nissilä, Erkki Säkö and Heikki Teirmaa. Doctors Elina Liukkonen and Hannu Somer are acknowledged for their role in identifying the families with EA-2 and FHM symptoms.

I am indebted to the patients and families who have participated in this study.

I owe very special thanks to our laboratory superwoman, Eija Hämäläinen. She has taught me basically every wet-lab method I know and helped me with numerous practical problems during these years. Thank you for your patience! To gain her confidence has been the best feedback. I also want to warmly thank our former and present laboratory personnel, Maritta Putkiranta and Minna Veini, for willingly helping me with many practical matters and for expert technical assistance. Further, I am truly grateful to our former and present research nurses, Liisa Aaltoila, Tanja Moilanen and Raija-Leena Halme, for their expert assistance in contacting patients and for their ever positive and helpful attitude.

I am most indebted to Pia Marttila for supervision and guidance during the early parts of the work and her true friendship ever since. I also want to thank Iiris Hovatta for her pioneering work in this migraine genetics project and for encouraging me to start this thesis work.

I am truly grateful to Professor Jaakko Kaprio for his help in statistical analysis and for letting us use the valuable twin samples. I also want to acknowledge the help of Riika Kilpikari in statistical matters, especially using SPSS. Docent Elisabeth Widén, Päivi Lahermo and the personnel of the Finnish Genome Center as well as Assistant Professor Jeanette Papp and the personnel of the UCLA Genotyping Core are thanked for all their help with genotyping and practical matters. The guidance provided by Kaisa Silander concerning Sequenom has also been invaluable. Further I want to thank Outi Törnwall, Minttu Jussila, and the personnel of the DNA Isolation Unit of the National Public Health Institute for their help with sample handling issues. The BBU team, especially Tero Hiekkalinna and Juri Ahokas, have also been very helpful in practical matters. Docent Päivi Miettinen and Jouni Vesa are thanked for their kind help with the EA-2 study.

I truly appreciate the help of all our collaborators abroad. I warmly thank the Dutch migraine group, especially Professor Rune Frants, Assistant Professor Arn van den Maagdenberg and Kaate Vanmolkot, for their assistance and hospitality and for many interesting discussions. I further wish to acknowledge the help of Assistant Professor Roel Ophoff, Professor Jay Gargus, Professor Rhoda Blostein and Laura Segall. For fruitful collaboration in statistical matters I want to thank Professors Joseph Terwilliger, Suzanne Leal, Eric Sobel, Rita Cantor, and Jurg Ott. Special thanks go to Joe for numerous thought-provoking meetings as well as his habit of producing us new migraine patients among PhD students working with complex disease genetics during his courses.

I have been fortunate to work with many talented young scientists in Aarno's research group. My dearest thanks go to my former and present closest colleagues, Riitta Sallinen and Päivi Tikka-Kleemola. Thank you so much for your friendship and company! Riitta – sharing an office and the ups and downs of a PhD student with you has been a pleasure. Your energy and enthusiasm are something to admire! Päivi – as we both know, migraine genetics and a thesis project are not a perfect match. However, as a famous proverb says: "Shared joy is a double joy; shared sorrow is half a sorrow". Your amazingly systematic way of working as well as your rather ironic but great sense of humor has made our common projects enjoyable. I further wish to thank Greg Oswell for many useful discussions and for patiently helping me with English grammar. The two new, bright, Ph.D. students in our migraine project, Verneri Anttila and Annika Sarahonka, I want to thank for interest towards my studies and migraine in general. I have really enjoyed the numerous vivid conversations we have had and I am certain that you both have a bright future in science. I know that I have been rather preoccupied lately – thank you also for your understanding. I also want to thank Aarno's former Ph.D. students, Docent Nina Horelli-Kuitunen, Jaakko Kaukonen, Elina Virolainen, and Ulla Wartiovaara-Kautto, for their help when I was at the beginning of my studies and for many nice moments at the Meilahti Hospital cafeteria.

I am truly grateful to Riitta, Päivi, and Annika who found time to read this thesis manuscript and whose comments eliminated many mistakes.

At the very beginning of this project our lab was part of the Laboratory of the Molecular Genetics, Helsinki University Central Hospital. I truly appreciate the help of Docent Arto Orpana and his lab team, Agneta Blomqvist-Kaksonen, Kaisu Korhonen, Sinikka Laine, Katriina Melasniemi, and Tuula Salmivaara. Arto Orpana's knowledge in molecular genetic methods is admirable.

The working environment both in the Meilahti hospital and Biomedicum has been enjoyable and stimulating. In particular, I want to thank Professor Kimmo Kontula's "geneticist-girls" Heidi Fodstad, Päivi Forsblom (Laitinen), Maarit Lappalainen and Kaisa Valli-Jaakola. Your friendship, help and tips have probably meant a lot more to me than vice versa. I especially want to thank Heidi for sharing the thesis anxiety with me and for giving me so much good advice lately. I further wish to thank the "post-doc Kirsis" Piippo and Paukku, Tuula Hannila-Handelberg, Annukka Lehtonen, Tuula Soppela, Susanna Tverin and Saara Nyqvist as well as Susanna Mehtälä for being such nice persons to work with.

For financial support I express my sincere gratitude to the Research Funds of the Helsinki University Central Hospital, the Research Foundation of the University of Helsinki (Young Scientist's Award), the Sigrid Juselius Foundation, the Biomedicum Helsinki Foundation, the Finnish Cultural Foundation, the Maud Kuistila Memorial Foundation, the Finnish Neurology Foundation, the Biomedicum Helsinki Foundation and the Research and Science Foundation of Farmos.

During my student years I've had the pleasure of making friends with several gifted biologists. My "Viisikko friends", Anna, Anna Maria, Hanna & Leena and my "Viikki friends" Hanna, Heikki, Tarja, and Tuula are warmly thanked for their great company both during the lab courses and various Symbioosi occasions.

I want to express my warmest thanks to all my dear friends outside the lab. Eeva, Kirsi, Nina, and Saara – our long friendships mean a lot to me! Special hugs also to Saara's family, Tomi and Arttu. I am truly grateful to my "exercise buddies", Sanna & Riitta, for numerous hilarious though sweaty moments, therapeutic discussions, and useful tips concerning bringing up children (and husbands).

I wish to thank my dear parents, Seija and Kari, with all my heart for their love and support. Äiti, my big moments wouldn't feel the same without you there shedding tears… I owe big hugs also to my always helpful little sisters Päivi and Elina and their families. Special thanks go to Elina for such patiently drawing outlines for the cover picture of this thesis. I am also deeply grateful to my mother- and father-in-law Tuulikki and Touko for all their support and help and for accepting me as part of their fabulous family. Further, I wish to warmly thank Heikki's sisters Elina and Tiina and their families for numerous unforgettable moments. I also want to acknowledge Family Kaunisto for gradually preparing me for my dissertation since a get-together with them will almost invariably lead to a long and lively debate. Without this whole supporting team always ready for baby-sitting I would not have been able to finish this thesis.

Finally, words alone cannot express the thanks I owe to my husband, Heikki, for his love and company as well as taking care of everything during my most intense writing periods. His encouragement and support carried me through the roughest times. Our daughter, Kaisla, has brought an amazing amount of joy and action as well as the most unselfish kind of love to my life. I am lucky to have such a pleasant way of putting things into perspective since you are the most important thing in my life!

Helsinki, September 2005

REFERENCES

Abecasis GR, Cherny SS, Cookson WO and Cardon LR (2002). Merlin--rapid analysis of dense genetic maps using sparse gene flow trees. *Nat Genet* 30: 97-101.

Abreu PC, Greenberg DA and Hodge SE (1999). Direct power comparisons between simple LOD scores and NPL scores for linkage analysis in complex diseases. *Am J Hum Genet* 65: 847-857.

Abu-Arefeh I and Russell G (1994). Prevalence of headache and migraine in schoolchildren. *BMJ* 309: 765-769.

Ackerman MJ and Clapham DE (1997). Ion channels--basic science and clinical disease. *N Engl J Med* 336: 1575-1586.

Akerman S, Williamson DJ and Goadsby PJ (2003). Voltage-dependent calcium channels are involved in neurogenic dural vasodilatation via a presynaptic transmitter release mechanism. *Br J Pharmacol* 140: 558-566.

Almasy L and Blangero J (1998). Multipoint quantitative-trait linkage analysis in general pedigrees. *Am J Hum Genet* 62: 1198-1211.

Alonso I, Barros J, Tuna A, Coelho J, Sequeiros J, Silveira I *et al.* (2003). Phenotypes of spinocerebellar ataxia type 6 and familial hemiplegic migraine caused by a unique *CACNA1A* missense mutation in patients from a large family. *Arch Neurol* 60: 610-614.

Alonso I, Barros J, Tuna A, Seixas A, Coutinho P, Sequeiros J *et al.* (2004). A novel R1347Q mutation in the predicted voltage sensor segment of the P/Q-type calcium-channel alpha-subunit in a family with progressive cerebellar ataxia and hemiplegic migraine. *Clin Genet* 65: 70-72.

Altmuller J, Palmer LJ, Fischer G, Scherb H and Wjst M (2001). Genomewide scans of complex human diseases: true linkage is hard to find. *Am J Hum Genet* 69: 936-950.

Altshuler D, Hirschhorn JN, Klannemark M, Lindgren CM, Vohl MC, Nemesh J *et al.* (2000). The common *PPAR*gamma Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes. *Nat Genet* 26: 76-80.

Ambrosini A and Schoenen J (2003). The electrophysiology of migraine. *Curr Opin Neurol* 16: 327-331.

Anderson PA and Greenberg RM (2001). Phylogeny of ion channels: clues to structure and function. *Comp Biochem Physiol B Biochem Mol Biol* 129: 17-28.

Ardlie KG, Kruglyak L and Seielstad M (2002). Patterns of linkage disequilibrium in the human genome. *Nat Rev Genet* 3: 299-309.

Armstrong CM and Hille B (1998). Voltage-gated ion channels and electrical excitability. *Neuron* 20: 371-380.

Asumalahti K, Veal C, Laitinen T, Suomela S, Allen M, Elomaa O *et al.* (2002). Coding haplotype analysis supports *HCR* as the putative susceptibility gene for psoriasis at the MHC PSORS1 locus. *Hum Mol Genet* 11: 589-597.

Augustine GJ, Santamaria F and Tanaka K (2003). Local calcium signaling in neurons. *Neuron* 40: 331-346.

Ayata C, Shimizu-Sasamata M, Lo EH, Noebels JL and Moskowitz MA (2000). Impaired neurotransmitter release and elevated threshold for cortical spreading depression in mice with mutations in the alpha 1A subunit of P/Q type calcium channels. *Neuroscience* 95: 639-645.

Bahra A, Matharu MS, Buchel C, Frackowiak RS and Goadsby PJ (2001). Brainstem activation specific to migraine headache. *Lancet* 357: 1016-1017.

Baker KE and Parker R (2004). Nonsense-mediated mRNA decay: terminating erroneous gene expression. *Curr Opin Cell Biol* 16: 293-299.

Balestrino M, Young J and Aitken P (1999). Block of Na^+,K^+ATPase with ouabain induces spreading depression-like depolarization in hippocampal slices. *Brain Res* 838: 37-44.

Baloh RW, Yue Q, Furman JM and Nelson SF (1997). Familial episodic ataxia: clinical heterogeneity in four families linked to chromosome 19p. *Ann Neurol* 41: 8-16.

Barrett CF, Cao YQ and Tsien RW (2005a). Gating deficiency in a familial hemiplegic migraine type 1 mutant P/Q-type calcium channel. *J Biol Chem* 280: 24064-24071.

Barrett JC, Fry B, Maller J and Daly MJ (2005b). Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21: 263-265.

Bartsch T, Knight YE and Goadsby PJ (2004). Activation of 5-HT(1B/1D) receptor in the periaqueductal gray inhibits nociception. *Ann Neurol* 56: 371-381.

Bassi MT, Bresolin N, Tonelli A, Nazos K, Crippa F, Baschirotto C *et al.* (2004). A novel mutation in the *ATP1A2* gene causes alternating hemiplegia of childhood. *J Med Genet* 41: 621-628.

Battistini S, Stenirri S, Piatti M, Gelfi C, Righetti PG, Rocchi R *et al.* (1999). A new *CACNA1A* gene mutation in acetazolamide-responsive familial hemiplegic migraine and ataxia. *Neurology* 53: 38-43.

Bearden CE, Reus VI and Freimer NB (2004). Why genetic investigation of psychiatric disorders is so difficult. *Curr Opin Genet Dev* 14: 280-286.

Beauvais K, Cave-Riant F, De Barace C, Tardieu M, Tournier-Lasserve E and Furby A (2004). New *CACNA1A* gene mutation in a case of familial hemiplegic migraine with status epilepticus. *Eur Neurol* 52: 58-61.

Bech-Hansen NT, Naylor MJ, Maybaum TA, Pearce WG, Koop B, Fishman GA *et al.* (1998). Loss-of-function mutations in a calcium-channel alpha1-subunit gene in Xp11.23 cause incomplete X-linked congenital stationary night blindness. *Nat Genet* 19: 264-267.

Bell GI, Horita S and Karam JH (1984). A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus. *Diabetes* 33: 176-183.

Bennett EA, Coleman LE, Tsui C, Pittard WS and Devine SE (2004). Natural genetic variation caused by transposable elements in humans. *Genetics* 168: 933-951.

Berridge MJ (1998). Neuronal calcium signaling. *Neuron* 21: 13-26.

Berridge MJ, Lipp P and Bootman MD (2000). The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 1: 11-21.

Bhangale TR, Rieder MJ, Livingston RJ and Nickerson DA (2005). Comprehensive identification and characterization of diallelic insertion-deletion polymorphisms in 330 human candidate genes. *Hum Mol Genet* 14: 59-69.

Bichet D, Cornet V, Geib S, Carlier E, Volsen S, Hoshi T *et al.* (2000). The I-II loop of the Ca2+ channel alpha1 subunit contains an endoplasmic reticulum retention signal antagonized by the beta subunit. *Neuron* 25: 177-190.

Björnsson A, Gudmundsson G, Gudfinnsson E, Hrafnsdottir M, Benedikz J, Skuladottir S *et al.* (2003). Localization of a gene for migraine without aura to chromosome 4q21. *Am J Hum Genet* 73: 986-993.

Blau JN (1995). Migraine with aura and migraine without aura are not different entities. *Cephalalgia* 15: 186-190.

Blin N and Stafford DW (1976). A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res* 3: 2303-2308.

Blostein R, Dunbar L, Mense M, Scanzano R, Wilczynska A and Caplan MJ (1999). Cation selectivity of gastric H,K-ATPase and Na,K-ATPase chimeras. *J Biol Chem* 274: 18374-18381.

Bolay H, Reuter U, Dunn AK, Huang Z, Boas DA and Moskowitz MA (2002). Intrinsic brain activity triggers trigeminal meningeal afferents in a migraine model. *Nat Med* 8: 136-142.

Boomsma D, Busjahn A and Peltonen L (2002). Classical twin studies and beyond. *Nat Rev Genet* 3: 872-882.

Botstein D, White RL, Skolnick M and Davis RW (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am J Hum Genet* 32: 314-331.

Bottini N, Musumeci L, Alonso A, Rahmouni S, Nika K, Rostamkhani M *et al.* (2004). A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. *Nat Genet* 36: 337-338.

Bousser MG (2004). Estrogens, migraine, and stroke. *Stroke* 35: 2652-2656.

Bowyer SM, Aurora KS, Moran JE, Tepley N and Welch KM (2001). Magnetoencephalographic fields from patients with spontaneous and induced migraine aura. *Ann Neurol* 50: 582-587.

Breslau N and Rasmussen BK (2001). The impact of migraine: Epidemiology, risk factors, and co-morbidities. *Neurology* 56 (Suppl 1): S4-S12.

Broman KW, Murray JC, Sheffield VC, White RL and Weber JL (1998). Comprehensive human genetic maps: Individual and sex-specific variation in recombination. *Am J Hum Genet* 63: 681-689.

Browne DL, Gancher ST, Nutt JG, Brunt ER, Smith EA, Kramer P *et al.* (1994). Episodic ataxia/myokymia syndrome is associated with point mutations in the human potassium channel gene, *KCNA1*. *Nat Genet* 8: 136-140.

Brugnoni R, Leone M, Rigamonti A, Moranduzzo E, Cornelio F, Mantegazza R *et al.* (2002). Is the *CACNA1A* gene involved in familial migraine with aura? *Neurol Sci* 23: 1-5.

Buchner DA, Trudeau M and Meisler MH (2003). SCNM1, a putative RNA splicing factor that modifies disease severity in mice. *Science* 301: 967-969.

Bulman DE (1997). Phenotype variation and newcomers in ion channel disorders. *Hum Mol Genet* 6: 1679-1685.

Burset M, Seledtsov IA and Solovyev VV (2000). Analysis of canonical and non-canonical splice sites in mammalian genomes. *Nucleic Acids Res* 28: 4364-4375.

Burstein R, Yarnitsky D, Goor-Aryeh I, Ransil BJ and Bajwa ZH (2000). An association between migraine and cutaneous allodynia. *Ann Neurol* 47: 614-624.

Burstein R, Collins B and Jakubowski M (2004). Defeating migraine pain with triptans: a race against the development of cutaneous allodynia. *Ann Neurol* 55: 19-26.

The *C. elegans* Sequencing Consortium (1998). Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* 282: 2012-2018.

Caddick SJ, Wang C, Fletcher CF, Jenkins NA, Copeland NG and Hosford DA (1999). Excitatory but not inhibitory synaptic transmission is reduced in lethargic (*Cacnb4*^{lh}) and tottering (*Cacna1a*^{tg}) mouse thalami. *J Neurophysiol* 81: 2066-2074.

Cader ZM, Noble-Topham S, Dyment DA, Cherny SS, Brown JD, Rice GP *et al.* (2003). Significant linkage to migraine with aura on chromosome 11q24. *Hum Mol Genet* 12: 2511-2517.

Cao Y, Welch KM, Aurora S and Vikingstad EM (1999). Functional MRI-BOLD of visually triggered headache in patients with migraine. *Arch Neurol* 56: 548-554.

Cao Y, Aurora SK, Nagesh V, Patel SC and Welch KM (2002). Functional MRI-BOLD of brainstem structures during visually triggered migraine. *Neurology* 59: 72-78.

Cao YQ, Piedras-Renteria ES, Smith GB, Chen G, Harata NC and Tsien RW (2004). Presynaptic Ca^{2+} channels compete for channel type-preferring slots in altered neurotransmission arising from Ca^{2+} channelopathy. *Neuron* 43: 387-400.

Cao YQ and Tsien RW (2005). Effects of familial hemiplegic migraine type 1 mutations on neuronal P/Q-type Ca^{2+} channel activity and inhibitory synaptic transmission. *Proc Natl Acad Sci USA* 102: 2590-2595.

Cardon LR and Bell JI (2001). Association study designs for complex diseases. *Nat Rev Genet* 2: 91-99.

Cargill M, Altshuler D, Ireland J, Sklar P, Ardlie K, Patil N *et al.* (1999). Characterization of single-nucleotide polymorphisms in coding regions of human genes. *Nat Genet* 22: 231-238.

Carlson CS, Eberle MA, Kruglyak L and Nickerson DA (2004). Mapping complex disease loci in whole-genome association studies. *Nature* 429: 446-452.

Carlsson A, Forsgren L, Nylander PO, Hellman U, Forsman-Semb K, Holmgren G *et al.* (2002). Identification of a susceptibility locus for migraine with and without aura on 6p12.2-p21.1. *Neurology* 59: 1804-1807.

Carrera P, Piatti M, Stenirri S, Grimaldi LM, Marchioni E, Curcio M *et al.* (1999). Genetic heterogeneity in Italian families with familial hemiplegic migraine. *Neurology* 53: 26-33.

Castro M, Lemos C, Barros J, Fraga C, Guimaraes L, Maciel P *et al.* (2004). A mutation in the *ATP1A2* gene in a large family with migraine without aura. *American Journal of Human Genetics* 75 (Suppl): 382, abstract 2089.

Catterall WA (1995). Structure and function of voltage-gated ion channels. *Annu Rev Biochem* 64: 493-531.

Catterall WA (1998). Structure and function of neuronal Ca^{2+} channels and their role in neurotransmitter release. *Cell Calcium* 24: 307-323.

Catterall WA (2000). Structure and regulation of voltage-gated Ca^{2+} channels. *Annu Rev Cell Dev Biol* 16: 521-555.

Cevoli S, Pierangeli G, Monari L, Valentino ML, Bernardoni P, Mochi M *et al.* (2002). Familial hemiplegic migraine: clinical features and probable linkage to chromosome 1 in an Italian family. *Neurol Sci* 23: 7-10.

Chandy KG, Fantino E, Wittekindt O, Kalman K, Tong LL, Ho TH *et al.* (1998). Isolation of a novel potassium channel gene *hSKCa3* containing a polymorphic CAG repeat: a candidate for schizophrenia and bipolar disorder? *Mol Psychiatr* 3: 32-37.

Chang CL, Donaghy M and Poulter N (1999). Migraine and stroke in young women: case-control study. The World Health Organisation Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception. *BMJ* 318: 13-18.

Charlier C, Singh NA, Ryan SG, Lewis TB, Reus BE, Leach RJ *et al.* (1998). A pore mutation in a novel KQT-like potassium channel gene in an idiopathic epilepsy family. *Nat Genet* 18: 53-55.

Chaudhuri D, Chang SY, DeMaria CD, Alvania RS, Soong TW and Yue DT (2004). Alternative splicing as a molecular switch for Ca^{2+}/calmodulin-dependent facilitation of P/Q-type Ca^{2+} channels. *J Neurosci* 24: 6334-6342.

Chen Y, Lu J, Pan H, Zhang Y, Wu H, Xu K *et al.* (2003). Association between genetic variation of *CACNA1H* and childhood absence epilepsy. *Ann Neurol* 54: 239-243.

Chiano MN and Yates JR (1995). Linkage detection under heterogeneity and the mixture problem. *Ann Hum Genet* 59: 83-95.

Chioza B, Wilkie H, Nashef L, Blower J, McCormick D, Sham P *et al.* (2001). Association between the alpha(1a) calcium channel gene *CACNA1A* and idiopathic generalized epilepsy. *Neurology* 56: 1245-1246.

Chioza B, Osei-Lah A, Nashef L, Suarez-Merino B, Wilkie H, Sham P *et al.* (2002). Haplotype and linkage disequilibrium analysis to characterise a region in the calcium channel gene *CACNA1A* associated with idiopathic generalised epilepsy. *Eur J Hum Genet* 10: 857-864.

Choudhuri R, Cui L, Yong C, Bowyer S, Klein RM, Welch KM *et al.* (2002). Cortical spreading depression and gene regulation: relevance to migraine. *Ann Neurol* 51: 499-506.

Clark AG (2004). The role of haplotypes in candidate gene studies. *Genet Epidemiol* 27: 321-333.

Clarke JM (1910). On recurrent motor paralysis in migraine. *BMJ* 1: 1534-1538.

Clayton D (1999). A generalization of the transmission/disequilibrium test for uncertain-haplotype transmission. *Am J Hum Genet* 65: 1170-1177.

Cohen JC, Kiss RS, Pertsemlidis A, Marcel YL, McPherson R and Hobbs HH (2004). Multiple rare alleles contribute to low plasma levels of HDL cholesterol. *Science* 305: 869-872.

Colhoun HM, McKeigue PM and Davey Smith G (2003). Problems of reporting genetic associations with complex outcomes. *Lancet* 361: 865-872.

Collins FS, Guyer MS and Charkravarti A (1997). Variations on a theme: cataloging human DNA sequence variation. *Science* 278: 1580-1581.

Colson NJ, Lea RA, Quinlan S, MacMillan J and Griffiths LR (2004). The estrogen receptor 1 G594A polymorphism is associated with migraine susceptibility in two independent case/control groups. *Neurogenetics* 5: 129-133.

Colson NJ, Lea RA, Quinlan S, Macmillan J and Griffiths LR (2005). Investigation of hormone receptor genes in migraine. *Neurogenetics* 6: 17-23.

Cordell HJ (2002). Epistasis: what it means, what it doesn't mean, and statistical methods to detect it in humans. *Hum Mol Genet* 11: 2463-2468.

Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW *et al.* (1993). Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* 261: 921-923.

Cossette P, Liu L, Brisebois K, Dong H, Lortie A, Vanasse M *et al.* (2002). Mutation of *GABRA1* in an autosomal dominant form of juvenile myoclonic epilepsy. *Nat Genet* 31: 184-189.

Cox RD and Brown SD (2003). Rodent models of genetic disease. *Curr Opin Genet Dev* 13: 278-283.

Curtis D (1997). Use of siblings as controls in case-control association studies. *Ann Hum Genet* 61: 319-333.

Cutrer FM, Sorensen AG, Weisskoff RM, Ostergaard L, Sanchez del Rio M, Lee EJ *et al.* (1998). Perfusion-weighted imaging defects during spontaneous migrainous aura. *Ann Neurol* 43: 25-31.

D'Onofrio M, Ambrosini A, Santorelli F, Buzzi M, Grieco G, Di Mambro A *et al.* (2004). A new mutation on the *ATP1A2* gene in familial basilar migraine. *Cephalalgia* 24: 147, abstract LMOP147.

Daly MJ, Rioux JD, Schaffner SF, Hudson TJ and Lander ES (2001). High-resolution haplotype structure in the human genome. *Nat Genet* 29: 229-232.

de Carvalho Aguiar P, Sweadner KJ, Penniston JT, Zaremba J, Liu L, Caton M *et al.* (2004). Mutations in the Na^+/K^+ -ATPase alpha3 gene *ATP1A3* are associated with rapid-onset dystonia parkinsonism. *Neuron* 43: 169-175.

De Fusco M, Becchetti A, Patrignani A, Annesi G, Gambardella A, Quattrone A *et al.* (2000). The nicotinic receptor beta 2 subunit is mutant in nocturnal frontal lobe epilepsy. *Nat Genet* 26: 275-276.

De Fusco M, Marconi R, Silvestri L, Atorino L, Rampoldi L, Morgante L *et al.* (2003). Haploinsufficiency of *ATP1A2* encoding the Na+/K+ pump alpha2 subunit associated with familial hemiplegic migraine type 2. *Nat Genet* 33: 192-196.

de la Chapelle A (1993). Disease gene mapping in isolated human populations: the example of Finland. *J Med Genet* 30: 857-865.

de la Chapelle A and Peltomäki P (1998). The genetics of hereditary common cancers. *Curr Opin Genet Dev* 8: 298-303.

De Waard M, Liu H, Walker D, Scott VE, Gurnett CA and Campbell KP (1997). Direct binding of G-protein betagamma complex to voltage-dependent calcium channels. *Nature* 385: 446-450.

Denier C, Ducros A, Vahedi K, Joutel A, Thierry P, Ritz A *et al.* (1999). High prevalence of *CACNA1A* truncations and broader clinical spectrum in episodic ataxia type 2. *Neurology* 52: 1816-1821.

Denier C, Ducros A, Durr A, Eymard B, Chassande B and Tournier-Lasserve E (2001). Missense *CACNA1A* mutation causing episodic ataxia type 2. *Arch Neurol* 58: 292-295.

Denoeud F, Vergnaud G and Benson G (2003). Predicting human minisatellite polymorphism. *Genome Res* 13: 856-867.

Dent W, Spiss H, Helbok R, Matuja W, Scheunemann S and Schmutzhard E (2004). Prevalence of migraine in a rural area in South Tanzania: a door-to-door survey. *Cephalalgia* 24: 960-966.

Dermitzakis ET, Reymond A and Antonarakis SE (2005). Conserved non-genic sequences - an unexpected feature of mammalian genomes. *Nat Rev Genet* 6: 151-157.

Dib C, Faure S, Fizames C, Samson D, Drouot N, Vignal A *et al.* (1996). A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature* 380: 152-154.

Dichgans M, Freilinger T, Eckstein G, Babini E, Lorenz-Depiereux B, Biskup S *et al.* (2005). Mutation in the neuronal voltage-gated sodium channel *SCN1A* in familial hemiplegic migraine. *Lancet* 366: 371-377.

Diriong S, Lory P, Williams ME, Ellis SB, Harpold MM and Taviaux S (1995). Chromosomal localization of the human genes for alpha 1A, alpha 1B, and alpha 1E voltage-dependent Ca^{2+} channel subunits. *Genomics* 30: 605-609.

Du W, Bautista JF, Yang H, Diez-Sampedro A, You SA, Wang L *et al.* (2005). Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat Genet* 37: 733-738.

Ducros A, Joutel A, Labauge P, Pages M, Bousser MG and Tournier-Lasserve E (1995). Monozygotic twins discordant for familial hemiplegic migraine. *Neurology* 45: 1222.

Ducros A, Joutel A, Vahedi K, Cecillon M, Ferreira A, Bernard E *et al.* (1997). Mapping of a second locus for familial hemiplegic migraine to 1q21-q23 and evidence of further heterogeneity. *Ann Neurol* 42: 885-890.

Ducros A, Denier C, Joutel A, Vahedi K, Michel A, Darcel F *et al.* (1999). Recurrence of the T666M calcium channel *CACNA1A* gene mutation in familial hemiplegic migraine with progressive cerebellar ataxia. *Am J Hum Genet* 64: 89-98.

Ducros A, Denier C, Joutel A, Cecillon M, Lescoat C, Vahedi K *et al.* (2001). The clinical spectrum of familial hemiplegic migraine associated with mutations in a neuronal calcium channel. *N Engl J Med* 345: 17-24.

Eaves IA, Merriman TR, Barber RA, Nutland S, Tuomilehto-Wolf E, Tuomilehto J *et al.* (2000). The genetically isolated populations of Finland and Sardinia may not be a panacea for linkage disequilibrium mapping of common disease genes. *Nat Genet* 25: 320-323.

Edwards AO, Ritter R, 3rd, Abel KJ, Manning A, Panhuysen C and Farrer LA (2005). Complement factor H polymorphism and age-related macular degeneration. *Science* 308: 421-424.

Ekelund J, Hovatta I, Parker A, Paunio T, Varilo T, Martin R *et al.* (2001). Chromosome 1 loci in Finnish schizophrenia families. *Hum Mol Genet* 10: 1611-1617.

Enattah NS, Sahi T, Savilahti E, Terwilliger JD, Peltonen L and Jarvela I (2002). Identification of a variant associated with adult-type hypolactasia. *Nat Genet* 30: 233-237.

Ertel EA, Campbell KP, Harpold MM, Hofmann F, Mori Y, Perez-Reyes E *et al.* (2000). Nomenclature of voltage-gated calcium channels. *Neuron* 25: 533-535.

Escayg A, MacDonald BT, Meisler MH, Baulac S, Huberfeld G, An-Gourfinkel I *et al.* (2000a). Mutations of *SCN1A*, encoding a neuronal sodium channel, in two families with GEFS+2. *Nat Genet* 24: 343-345.

Escayg A, De Waard M, Lee DD, Bichet D, Wolf P, Mayer T *et al.* (2000b). Coding and noncoding variation of the human calcium-channel beta4-subunit gene *CACNB4* in patients with idiopathic generalized epilepsy and episodic ataxia. *Am J Hum Genet* 66: 1531-1539.

Etminan M, Takkouche B, Isorna FC and Samii A (2005). Risk of ischaemic stroke in people with migraine: systematic review and meta-analysis of observational studies. *BMJ* 330: 63.

Eunson LH, Graves TD and Hanna MG (2005). New calcium channel mutations predict aberrant RNA splicing in episodic ataxia. *Neurology* 65: 308-310.

Evans A, Salomaa V, Kulathinal S, Asplund K, Cambien F, Ferrario M *et al.* (2005). MORGAM (an international pooling of cardiovascular cohorts). *Int J Epidemiol* 34: 21-27.

Ewens WJ and Spielman RS (2001). Locating genes by linkage and association. *Theor Popul Biol* 60: 135-139.

Fajans SS, Bell GI and Polonsky KS (2001). Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young. *N Engl J Med* 345: 971-980.

Falk CT and Rubinstein P (1987). Haplotype relative risks: an easy reliable way to construct a proper control sample for risk calculations. *Ann Hum Genet* 51: 227-233.

Fearnhead NS, Wilding JL, Winney B, Tonks S, Bartlett S, Bicknell DC *et al.* (2004). Multiple rare variants in different genes account for multifactorial inherited susceptibility to colorectal adenomas. *Proc Natl Acad Sci USA* 101: 15992-15997.

Featherstone HJ (1985). Migraine and muscle contraction headaches: a continuum. *Headache* 25: 194-198.

Felix R (2000). Channelopathies: ion channel defects linked to heritable clinical disorders. *J Med Genet* 37: 729-740.

Ferrari MD (1998). Migraine. *Lancet* 351: 1043-1051.

Fletcher CF, Lutz CM, O'Sullivan TN, Shaughnessy JD, Jr., Hawkes R, Frankel WN *et al.* (1996). Absence epilepsy in tottering mutant mice is associated with calcium channel defects. *Cell* 87: 607-617.

Fletcher CF, Tottene A, Lennon VA, Wilson SM, Dubel SJ, Paylor R *et al.* (2001). Dystonia and cerebellar atrophy in *Cacna1a* null mice lacking P/Q calcium channel activity. *Faseb J* 15: 1288-1290.

Fredman D, White SJ, Potter S, Eichler EE, Den Dunnen JT and Brookes AJ (2004). Complex SNP-related sequence variation in segmental genome duplications. *Nat Genet* 36: 861-866.

Freimer N and Sabatti C (2004). The use of pedigree, sib-pair and association studies of common diseases for genetic mapping and epidemiology. *Nat Genet* 36: 1045-1051.

Friend KL, Crimmins D, Phan TG, Sue CM, Colley A, Fung VS *et al.* (1999). Detection of a novel missense mutation and second recurrent mutation in the *CACNA1A* gene in individuals with EA-2 and FHM. *Hum Genet* 105: 261-265.

Frontali M (2001). Spinocerebellar ataxia type 6: channelopathy or glutamine repeat disorder? *Brain Res Bull* 56: 227-231.

Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG *et al.* (1995). A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 10: 111-113.

Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B *et al.* (2002). The structure of haplotype blocks in the human genome. *Science* 296: 2225-2229.

Gaist D, Pedersen L, Madsen C, Tsiropoulos I, Bak S, Sindrup S *et al.* (2005). Long-term effects of migraine on cognitive function: a population-based study of Danish twins. *Neurology* 64: 600-607.

Gardner K, Barmada MM, Ptacek LJ and Hoffman EP (1997). A new locus for hemiplegic migraine maps to chromosome 1q31. *Neurology* 49: 1231-1238.

Gardner K, Bernal O, Keegan M, Badger J, Gerber O, Lowry N *et al.* (1999). A new mutation in the Chr19p calcium channel gene *CACNL1A4* causing hemiplegic migraine with ataxia. *Neurology* 52 (Suppl 2): A115.

Gardner K, Estewez M, Keryanov S, Estevrz A, Barmada M, Badger J *et al.* (2004). A two-locus FHM2 family with unique *ATP1A2* mutation and comparative study in C. elegans showing regulation of tryptophan hydroxylase by EAT-6 and UNC-2, the orthologous FHM2 and FHM1 genes. *Cephalalgia* 24: 149-150, abstract LMOP148.

Gargus JJ (2003). Unraveling monogenic channelopathies and their implications for complex polygenic disease. *Am J Hum Genet* 72: 785-803.

Gervil M, Ulrich V, Kaprio J, Olesen J and Russell MB (1999a). The relative role of genetic and environmental factors in migraine without aura. *Neurology* 53: 995-999.

Gervil M, Ulrich V, Kyvik KO, Olesen J and Russell MB (1999b). Migraine without aura: A population-based twin study. *Ann Neurol* 46: 606-611.

Ghosh S, Watanabe RM, Hauser ER, Valle T, Magnuson VL, Erdos MR *et al.* (1999). Type 2 diabetes: evidence for linkage on chromosome 20 in 716 Finnish affected sib pairs. *Proc Natl Acad Sci USA* 96: 2198-2203.

Glazier AM, Nadeau JH and Aitman TJ (2002). Finding genes that underlie complex traits. *Science* 298: 2345-2349.

Goadsby PJ (2001). Migraine, aura, and cortical spreading depression: why are we still talking about it? *Ann Neurol* 49: 4-6.

Goldstein DB, Ahmadi KR, Weale ME and Wood NW (2003). Genome scans and candidate gene approaches in the study of common diseases and variable drug responses. *Trends Genet* 19: 615-622.

Golovina VA, Song H, James PF, Lingrel JB and Blaustein MP (2003). Na^+ pump alpha 2-subunit expression modulates Ca^{2+} signaling. *Am J Physiol Cell Physiol* 284: C475-486.

Gretarsdottir S, Thorleifsson G, Reynisdottir ST, Manolescu A, Jonsdottir S, Jonsdottir T *et al.* (2003). The gene encoding phosphodiesterase 4D confers risk of ischemic stroke. *Nat Genet* 35: 131-138.

Griggs RC, Moxley RT, 3rd, Lafrance RA and McQuillen J (1978). Hereditary paroxysmal ataxia: response to acetazolamide. *Neurology* 28: 1259-1264.

Gudbjartsson DF, Jonasson K, Frigge ML and Kong A (2000). Allegro, a new computer program for multipoint linkage analysis. *Nat Genet* 25: 12-13.

Guida S, Trettel F, Pagnutti S, Mantuano E, Tottene A, Veneziano L *et al.* (2001). Complete loss of P/Q calcium channel activity caused by a *CACNA1A* missense mutation carried by patients with episodic ataxia type 2. *Am J Hum Genet* 68: 759-764.

Gulcher JR and Stefansson K (2000). The Icelandic Healthcare Database and informed consent. *N Engl J Med* 342: 1827-1830.

Gyapay G, Morissette J, Vignal A, Dib C, Fizames C, Millasseau P *et al.* (1994). The 1993-94 Genethon human genetic linkage map. *Nat Genet* 7: 246-339.

Göring HH and Terwilliger JD (2000). Linkage analysis in the presence of errors IV: joint pseudomarker analysis of linkage and/or linkage disequilibrium on a mixture of pedigrees and singletons when the mode of inheritance cannot be accurately specified. *Am J Hum Genet* 66: 1310-1327.

Göring HH, Terwilliger JD and Blangero J (2001). Large upward bias in estimation of locus-specific effects from genomewide scans. *Am J Hum Genet* 69: 1357-1369.

Hadjikhani N, Sanchez Del Rio M, Wu O, Schwartz D, Bakker D, Fischl B *et al.* (2001). Mechanisms of migraine aura revealed by functional MRI in human visual cortex. *Proc Natl Acad Sci USA* 98: 4687-4692.

Hagen K, Zwart JA, Vatten L, Stovner LJ and Bovim G (2000). Prevalence of migraine and non-migrainous headache--head-HUNT, a large population-based study. *Cephalalgia* 20: 900-906.

Haines JL, Hauser MA, Schmidt S, Scott WK, Olson LM, Gallins P *et al.* (2005). Complement factor H variant increases the risk of age-related macular degeneration. *Science* 308: 419-421.

Hans M, Luvisetto S, Williams ME, Spagnolo M, Urrutia A, Tottene A *et al.* (1999). Functional consequences of mutations in the human alpha1A calcium channel subunit linked to familial hemiplegic migraine. *J Neurosci* 19: 1610-1619.

Haug K, Warnstedt M, Alekov AK, Sander T, Ramirez A, Poser B *et al.* (2003). Mutations in *CLCN2* encoding a voltage-gated chloride channel are associated with idiopathic generalized epilepsies. *Nat Genet* 33: 527-532.

He S, Shelly DA, Moseley AE, James PF, James JH, Paul RJ *et al.* (2001). The $alpha_1$- and $alpha_2$-isoforms of Na-K-ATPase play different roles in skeletal muscle contractility. *Am J Physiol Regul Integr Comp Physiol* 281: 917-925.

Headache Classification Committee of the International Headache Society (1988). Classification and diagnostic criteria for headache disorders, cranial neuralgias and facial pain. *Cephalalgia* 8: 1-96.

Headache Classification Committee of the International Headache Society (2004). The International Classification of Headache Disorders: 2nd edition. *Cephalalgia* 24 (Suppl 1): 9-160.

Helgadottir A, Manolescu A, Thorleifsson G, Gretarsdottir S, Jonsdottir H, Thorsteinsdottir U *et al.* (2004). The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat Genet* 36: 233-239.

Hennah W, Varilo T, Kestila M, Paunio T, Arajarvi R, Haukka J *et al.* (2003). Haplotype transmission analysis provides evidence of association for *DISC1* to schizophrenia and suggests sex-dependent effects. *Hum Mol Genet* 12: 3151-3159.

Hennah W, Varilo T, Paunio T and Peltonen L (2004). Haplotype analysis and identification of genes for a complex trait: examples from schizophrenia. *Ann Med* 36: 322-331.

Hiekkalinna T and Peltonen L (1999). New program: AUTOSCAN 1.0 automated use of linkage analysis programs. *Am J Hum Genet* 65 (Suppl): A254, abstract 1416.

Hirschhorn JN and Daly MJ (2005). Genome-wide association studies for common diseases and complex traits. *Nat Rev Genet* 6: 95-108.

Hodge SE (2001). Model-free vs. model-based linkage analysis: a false dichotomy? *Am J Med Genet* 105: 62-64.

Hodgkin AL and Huxley AF (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J Physiol* 117: 500-544.

Honkasalo ML, Kaprio J, Heikkila K, Sillanpaa M and Koskenvuo M (1993). A population-based survey of headache and migraine in 22,809 adults. *Headache* 33: 403-412.

Honkasalo ML, Kaprio J, Winter T, Heikkila K, Sillanpaa M and Koskenvuo M (1995). Migraine and concomitant symptoms among 8167 adult twin pairs. *Headache* 35: 70-78.

Horikawa Y, Oda N, Cox NJ, Li X, Orho-Melander M, Hara M *et al.* (2000). Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. *Nat Genet* 26: 163-175.

Horvath S, Xu X and Laird NM (2001). The family based association test method: strategies for studying general genotype--phenotype associations. *Eur J Hum Genet* 9: 301-306.

Hovatta I, Kallela M, Farkkila M and Peltonen L (1994). Familial migraine: exclusion of the susceptibility gene from the reported locus of familial hemiplegic migraine on 19p. *Genomics* 23: 707-709.

Hovatta I, Terwilliger JD, Lichtermann D, Makikyro T, Suvisaari J, Peltonen L *et al.* (1997). Schizophrenia in the genetic isolate of Finland. *Am J Med Genet* 74: 353-360.

Hugot JP, Laurent-Puig P, Gower-Rousseau C, Olson JM, Lee JC, Beaugerie L *et al.* (1996). Mapping of a susceptibility locus for Crohn's disease on chromosome 16. *Nature* 379: 821-823.

Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J *et al.* (2001). Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411: 599-603.

Hübner CA and Jentsch TJ (2002). Ion channel diseases. *Hum Mol Genet* 11: 2435-2445.

Hästbacka J, de la Chapelle A, Mahtani MM, Clines G, Reeve-Daly MP, Daly M *et al.* (1994). The diastrophic dysplasia gene encodes a novel sulfate transporter: positional cloning by fine-structure linkage disequilibrium mapping. *Cell* 78: 1073-1087.

Iafrate AJ, Feuk L, Rivera MN, Listewnik ML, Donahoe PK, Qi Y *et al.* (2004). Detection of large-scale variation in the human genome. *Nat Genet* 36: 949-951.

Ikeda K, Onaka T, Yamakado M, Nakai J, Ishikawa TO, Taketo MM *et al.* (2003). Degeneration of the amygdala/piriform cortex and enhanced fear/anxiety behaviors in sodium pump alpha$_2$ subunit (*Atp1a2*)-deficient mice. *J Neurosci* 23: 4667-4676.

Imbrici P, Jaffe SL, Eunson LH, Davies NP, Herd C, Robertson R *et al.* (2004). Dysfunction of the brain calcium channel $Ca_V2.1$ in absence epilepsy and episodic ataxia. *Brain* 127: 2682-2692.

The International HapMap Consortium (2003). The International HapMap Project. *Nature* 426: 789-796.

International Human Genome Sequencing Consortium (2004). Finishing the euchromatic sequence of the human genome. *Nature* 431: 931-945.

Ioannidis JP, Ntzani EE, Trikalinos TA and Contopoulos-Ioannidis DG (2001). Replication validity of genetic association studies. *Nat Genet* 29: 306-309.

Ioannidis JP, Ntzani EE and Trikalinos TA (2004). 'Racial' differences in genetic effects for complex diseases. *Nat Genet* 36: 1312-1318.

Jen J, Yue Q, Nelson SF, Yu H, Litt M, Nutt J *et al.* (1999). A novel nonsense mutation in *CACNA1A* causes episodic ataxia and hemiplegia. *Neurology* 53: 34-37.

Jen J, Wan J, Graves M, Yu H, Mock AF, Coulin CJ *et al.* (2001). Loss-of-function EA2 mutations are associated with impaired neuromuscular transmission. *Neurology* 57: 1843-1848.

Jen J, Kim GW and Baloh RW (2004a). Clinical spectrum of episodic ataxia type 2. *Neurology* 62: 17-22.

Jen JC, Kim GW, Dudding KA and Baloh RW (2004b). No mutations in *CACNA1A* and *ATP1A2* in probands with common types of migraine. *Arch Neurol* 61: 926-928.

Jen JC, Wan J, Palos TP, Howard BD and Baloh RW (2005). Mutation in the glutamate transporter *EAAT1* causes episodic ataxia, hemiplegia, and seizures. *Neurology* 65: 529-534.

Jodice C, Mantuano E, Veneziano L, Trettel F, Sabbadini G, Calandriello L *et al.* (1997). Episodic ataxia type 2 (EA2) and spinocerebellar ataxia type 6 (SCA6) due to CAG repeat expansion in the *CACNA1A* gene on chromosome 19p. *Hum Mol Genet* 6: 1973-1978.

Johnson GC, Esposito L, Barratt BJ, Smith AN, Heward J, Di Genova G *et al.* (2001). Haplotype tagging for the identification of common disease genes. *Nat Genet* 29: 233-237.

Johnson MP, Lea RA, Curtain RP, MacMillan JC and Griffiths LR (2003). An investigation of the 5-HT2C receptor gene as a migraine candidate gene. *Am J Med Genet B Neuropsychiatr Genet* 117: 86-89.

Joiner WJ, Wang LY, Tang MD and Kaczmarek LK (1997). hSK4, a member of a novel subfamily of calcium-activated potassium channels. *Proc Natl Acad Sci USA* 94: 11013-11018.

Jones KW, Ehm MG, Pericak-Vance MA, Haines JL, Boyd PR and Peroutka SJ (2001). Migraine with aura susceptibility locus on chromosome 19p13 is distinct from the familial hemiplegic migraine locus. *Genomics* 78: 150-154.

Joutel A, Bousser MG, Biousse V, Labauge P, Chabriat H, Nibbio A *et al.* (1993). A gene for familial hemiplegic migraine maps to chromosome 19. *Nat Genet* 5: 40-45.

Joutel A, Ducros A, Vahedi K, Labauge P, Delrieu O, Pinsard N *et al.* (1994). Genetic heterogeneity of familial hemiplegic migraine. *Am J Hum Genet* 55: 1166-1172.

Jouvenceau A, Eunson LH, Spauschus A, Ramesh V, Zuberi SM, Kullmann DM *et al.* (2001). Human epilepsy associated with dysfunction of the brain P/Q-type calcium channel. *Lancet* 358: 801-807.

Juhaszova M and Blaustein MP (1997). Na^+ pump low and high ouabain affinity alpha subunit isoforms are differently distributed in cells. *Proc Natl Acad Sci USA* 94: 1800-1805.

Jun K, Piedras-Renteria ES, Smith SM, Wheeler DB, Lee SB, Lee TG *et al.* (1999). Ablation of P/Q-type Ca^{2+} channel currents, altered synaptic transmission, and progressive ataxia in mice lacking the alpha$_{1A}$-subunit. *Proc Natl Acad Sci USA* 96: 15245-15250.

Jurkat-Rott K, Freilinger T, Dreier JP, Herzog J, Gobel H, Petzold GC *et al.* (2004). Variability of familial hemiplegic migraine with novel A1A2 Na^+/K^+-ATPase variants. *Neurology* 62: 1857-1861.

Järveläinen HA, Orpana A, Perola M, Savolainen VT, Karhunen PJ and Lindros KO (2001). Promoter polymorphism of the CD14 endotoxin receptor gene as a risk factor for alcoholic liver disease. *Hepatology* 33: 1148-1153.

Kallela M, Wessman M and Färkkilä M (2001a). Validation of a migraine specific questionnaire for use in family studies. *Eur J Neurol* 8: 61-66.

Kallela M, Wessman M, Havanka H, Palotie A and Färkkilä M (2001b). Familial migraine with and without aura: clinical characteristics and co-occurrence. *Eur J Neurol* 8: 441-449.

Kaplan JH (2002). Biochemistry of Na,K-ATPase. *Annu Rev Biochem* 71: 511-535.

Kaprio J, Sarna S, Koskenvuo M and Rantasalo I (1978). The Finnish Twin Registry: formation and compilation, questionnaire study, zygocity, determination procedures, and research program. *Prog Clin Biol Res* 24: 179-184.

Kaprio J (2000). General principles of twin studies: Applications to research on migraine. In: Olesen IJ and Bousser M eds. *Genetics of Headache Disorders*. Philadelphia, Lippincott Williams &Wilkins.

Kara I, Sazci A, Ergul E, Kaya G and Kilic G (2003). Association of the C677T and A1298C polymorphisms in the 5,10 methylenetetrahydrofolate reductase gene in patients with migraine risk. *Brain Res Mol Brain Res* 111: 84-90.

Kaunisto MA (1999). Familiaaliselle hemiplegiselle migreenille altistavien geenilokusten kytkeytyminen suomalaisiin FHM-sukuihin. *Pro gradu -work*. Department of Biosciences, University of Helsinki, Helsinki, Finland.

Kaunisto MA, Harno H, Kallela M, Somer H, Sallinen R, Hamalainen E *et al.* (2004a). Novel splice site *CACNA1A* mutation causing episodic ataxia type 2. *Neurogenetics* 5: 69-73.

Kaunisto MA, Harno H, Vanmolkot KR, Gargus JJ, Sun G, Hamalainen E *et al.* (2004b). A novel missense *ATP1A2* mutation in a Finnish family with familial hemiplegic migraine type 2. *Neurogenetics* 5: 141-146.

Kaunisto MA, Tikka PJ, Kallela M, Leal SM, Papp JC, Korhonen A *et al.* (2005). Chromosome 19p13 loci in Finnish migraine with aura families. *Am J Med Genet B Neuropsychiatr Genet* 132: 85-89.

Ke X, Durrant C, Morris AP, Hunt S, Bentley DR, Deloukas P *et al.* (2004). Efficiency and consistency of haplotype tagging of dense SNP maps in multiple samples. *Hum Mol Genet* 13: 2557-2565.

Kelman L (2004). The premonitory symptoms (prodrome): a tertiary care study of 893 migraineurs. *Headache* 44: 865-872.

Kere J (2001). Human population genetics: lessons from Finland. *Annu Rev Genomics Hum Genet* 2: 103-128.

Kim JS, Yue Q, Jen JC, Nelson SF and Baloh RW (1998). Familial migraine with vertigo: no mutations found in *CACNA1A*. *Am J Med Genet* 79: 148-151.

Kittles RA, Perola M, Peltonen L, Bergen AW, Aragon RA, Virkkunen M *et al.* (1998). Dual origins of Finns revealed by Y chromosome haplotype variation. *Am J Hum Genet* 62: 1171-1179.

Klein RJ, Zeiss C, Chew EY, Tsai JY, Sackler RS, Haynes C *et al.* (2005). Complement factor H polymorphism in age-related macular degeneration. *Science* 308: 385-389.

Knapp M, Seuchter SA and Baur MP (1994). Linkage analysis in nuclear families. 2: Relationship between affected sib-pair tests and lod score analysis. *Hum Hered* 44: 44-51.

Knight YE and Goadsby PJ (2001). The periaqueductal grey matter modulates trigeminovascular input: a role in migraine? *Neuroscience* 106: 793-800.

Knight YE, Bartsch T, Kaube H and Goadsby PJ (2002). P/Q-type calcium-channel blockade in the periaqueductal gray facilitates trigeminal nociception: a functional genetic link for migraine? *J Neurosci* 22: RC213.

Kohler M, Hirschberg B, Bond CT, Kinzie JM, Marrion NV, Maylie J *et al.* (1996). Small-conductance, calcium-activated potassium channels from mammalian brain. *Science* 273: 1709-1714.

Koivisto M, Perola M, Varilo T, Hennah W, Ekelund J, Lukk M *et al.* (2003). An MDL method for finding haplotype blocks and for estimating the strength of haplotype block boundaries. *Pac Symp Biocomput*: 502-513.

Kong A and Cox NJ (1997). Allele-sharing models: LOD scores and accurate linkage tests. *Am J Hum Genet* 61: 1179-1188.

Kong A, Gudbjartsson DF, Sainz J, Jonsdottir GM, Gudjonsson SA, Richardsson B *et al.* (2002). A high-resolution recombination map of the human genome. *Nat Genet* 31: 241-247.

Kors EE, Terwindt GM, Vermeulen FL, Fitzsimons RB, Jardine PE, Heywood P *et al.* (2001). Delayed cerebral edema and fatal coma after minor head trauma: role of the *CACNA1A*

calcium channel subunit gene and relationship with familial hemiplegic migraine. *Ann Neurol* 49: 753-760.

Kors EE, Haan J, Giffin NJ, Pazdera L, Schnittger C, Lennox GG *et al.* (2003). Expanding the phenotypic spectrum of the *CACNA1A* gene T666M mutation: a description of 5 families with familial hemiplegic migraine. *Arch Neurol* 60: 684-688.

Kors EE (2004). Familial hemiplegic migraine Exploring the clinical and molecular spectrum of *CACNA1A* and *ATP1A2* mutations. *Thesis work.* Department of Neurology, Leiden University Medical Center, Leiden, The Netherlands.

Kors EE, Melberg A, Vanmolkot KR, Kumlien E, Haan J, Raininko R *et al.* (2004a). Childhood epilepsy, familial hemiplegic migraine, cerebellar ataxia, and a new *CACNA1A* mutation. *Neurology* 63: 1136-1137.

Kors EE, Vanmolkot KR, Haan J, Frants RR, van den Maagdenberg AM and Ferrari MD (2004b). Recent findings in headache genetics. *Curr Opin Neurol* 17: 283-288.

Kors EE, Vanmolkot KR, Haan J, Kheradmand Kia S, Stroink H, Laan LA *et al.* (2004c). Alternating hemiplegia of childhood: no mutations in the second familial hemiplegic migraine gene *ATP1A2*. *Neuropediatrics* 35: 293-296.

Kowa H, Yasui K, Takeshima T, Urakami K, Sakai F and Nakashima K (2000). The homozygous C677T mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for migraine. *Am J Med Genet* 96: 762-764.

Kramer PL, Yue Q, Gancher ST, Nutt JG, Baloh R, Smith E *et al.* (1995). A locus for the nystagmus-associated form of episodic ataxia maps to an 11-cM region on chromosome 19p. *Am J Hum Genet* 57: 182-185.

Kraus RL, Sinnegger MJ, Glossmann H, Hering S and Striessnig J (1998). Familial hemiplegic migraine mutations change alpha1A Ca2+ channel kinetics. *J Biol Chem* 273: 5586-5590.

Kraus RL, Sinnegger MJ, Koschak A, Glossmann H, Stenirri S, Carrera P *et al.* (2000). Three new familial hemiplegic migraine mutants affect P/Q-type Ca2+ channel kinetics. *J Biol Chem* 275: 9239-9243.

Krawczak M (1992). The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum Genet* 90: 41-54.

Kruglyak L, Daly MJ, Reeve-Daly MP and Lander ES (1996). Parametric and nonparametric linkage analysis: a unified multipoint approach. *Am J Hum Genet* 58: 1347-1363.

Kruglyak L and Nickerson DA (2001). Variation is the spice of life. *Nat Genet* 27: 234-236.

Kruit MC, van Buchem MA, Hofman PA, Bakkers JT, Terwindt GM, Ferrari MD *et al.* (2004). Migraine as a risk factor for subclinical brain lesions. *JAMA* 291: 427-434.

Kullmann DM and Hanna MG (2002). Neurological disorders caused by inherited ion-channel mutations. *Lancet Neurol* 1: 157-166.

Kuokkanen S, Sundvall M, Terwilliger JD, Tienari PJ, Wikstrom J, Holmdahl R *et al.* (1996). A putative vulnerability locus to multiple sclerosis maps to 5p14-p12 in a region syntenic to the murine locus *Eae2*. *Nat Genet* 13: 477-480.

Kuokkanen S, Gschwend M, Rioux JD, Daly MJ, Terwilliger JD, Tienari PJ *et al.* (1997). Genomewide scan of multiple sclerosis in Finnish multiplex families. *Am J Hum Genet* 61: 1379-1387.

Laan M and Pääbo S (1997). Demographic history and linkage disequilibrium in human populations. *Nat Genet* 17: 435-438.

Laitinen T, Daly MJ, Rioux JD, Kauppi P, Laprise C, Petays T *et al.* (2001). A susceptibility locus for asthma-related traits on chromosome 7 revealed by genome-wide scan in a founder population. *Nat Genet* 28: 87-91.

Laitinen T, Polvi A, Rydman P, Vendelin J, Pulkkinen V, Salmikangas P *et al.* (2004). Characterization of a common susceptibility locus for asthma-related traits. *Science* 304: 300-304.

Lalouel JM and Rohrwasser A (2002). Power and replication in case-control studies. *Am J Hypertens* 15: 201-205.

Lander E and Kruglyak L (1995). Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet* 11: 241-247.

Lander ES (1996). The new genomics: global views of biology. *Science* 274: 536-539.

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J *et al.* (2001). Initial sequencing and analysis of the human genome. *Nature* 409: 860-921.

Larsson B, Bille B and Pederson NL (1995). Genetic influence in headaches: a Swedish twin study. *Headache* 35: 513-519.

Lashley K (1941). Patterns of cerebral integration indicated by the scotomas of migraine. *Arch Neurol Psych* 46: 333-339.

Lathrop GM and Lalouel JM (1984). Easy calculations of lod scores and genetic risks on small computers. *Am J Hum Genet* 36: 460-465.

Launer LJ, Terwindt GM and Ferrari MD (1999). The prevalence and characteristics of migraine in a population-based cohort: The GEM Study. *Neurology* 53: 537-542.

Lauritzen M (1994). Pathophysiology of the migraine aura. The spreading depression theory. *Brain* 117: 199-210.

Lea RA, Curtain RP, Shepherd AG, Brimage PJ and Griffiths LR (2001a). No evidence for involvement of the human inducible nitric oxide synthase (iNOS) gene in susceptibility to typical migraine. *Am J Med Genet* 105: 110-113.

Lea RA, Curtain RP, Hutchins C, Brimage PJ and Griffiths LR (2001b). Investigation of the *CACNA1A* gene as a candidate for typical migraine susceptibility. *Am J Med Genet* 105: 707-712.

Lea RA, Shepherd AG, Curtain RP, Nyholt DR, Quinlan S, Brimage PJ *et al.* (2002). A typical migraine susceptibility region localizes to chromosome 1q31. *Neurogenetics* 4: 17-22.

Lea RA, Ovcaric M, Sundholm J, MacMillan J and Griffiths LR (2004). The methylenetetrahydrofolate reductase gene variant C677T influences susceptibility to migraine with aura. *BMC Med* 2: 3.

Lea RA, Nyholt DR, Curtain RP, Ovcaric M, Sciascia R, Bellis C *et al.* (2005). A genome-wide scan provides evidence for loci influencing a severe heritable form of common migraine. *Neurogenetics* 6: 67-72.

Leao AAP (1944). Spreading depression of activity in cerebral cortex. *J Neurophysiol* 7: 379-390.

Lee A, Wong ST, Gallagher D, Li B, Storm DR, Scheuer T *et al.* (1999). Ca^{2+}/calmodulin binds to and modulates P/Q-type calcium channels. *Nature* 399: 155-159.

Lee A, Zhou H, Scheuer T and Catterall WA (2003). Molecular determinants of Ca^{2+}/calmodulin-dependent regulation of $Ca_V2.1$ channels. *Proc Natl Acad Sci USA* 100: 16059-16064.

Lennon VA, Kryzer TJ, Griesmann GE, O'Suilleabhain PE, Windebank AJ, Woppmann A *et al.* (1995). Calcium-channel antibodies in the Lambert-Eaton syndrome and other paraneoplastic syndromes. *N Engl J Med* 332: 1467-1474.

Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I *et al.* (2003). Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: Schizophrenia. *Am J Hum Genet* 73: 34-48.

Levy D, Jakubowski M and Burstein R (2004). Disruption of communication between peripheral and central trigeminovascular neurons mediates the antimigraine action of 5HT 1B/1D receptor agonists. *Proc Natl Acad Sci USA* 101: 4274-4279.

Lingrel J, Moseley A, Dostanic I, Cougnon M, He S, James P *et al.* (2003). Functional roles of the alpha isoforms of the Na,K-ATPase. *Ann NY Acad Sci* 986: 354-359.

Lipton RB, Ottman R, Ehrenberg BL and Hauser WA (1994). Comorbidity of migraine: the connection between migraine and epilepsy. *Neurology* 44 (Suppl 7): S28-S32.

Lipton RB, Stewart WF and von Korff M (1997). Burden of migraine: societal costs and therapeutic opportunities. *Neurology* 48 (Suppl 3): S4-S9.

Lipton RB, Hamelsky SW, Kolodner KB, Steiner TJ and Stewart WF (2000). Migraine, quality of life, and depression: a population-based case- control study. *Neurology* 55: 629-635.

Lipton RB, Stewart WF, Diamond S, Diamond ML and Reed M (2001). Prevalence and burden of migraine in the United States: data from the American Migraine Study II. *Headache* 41: 646-657.

Lipton RB and Bigal ME (2005). Migraine: epidemiology, impact, and risk factors for progression. *Headache* 45 (Suppl 1): S3-S13.

Lohmueller KE, Pearce CL, Pike M, Lander ES and Hirschhorn JN (2003). Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet* 33: 177-182.

MacGregor EA (2004). Oestrogen and attacks of migraine with and without aura. *Lancet Neurol* 3: 354-361.

MacGregor EA and Hackshaw A (2004). Prevalence of migraine on each day of the natural menstrual cycle. *Neurology* 63: 351-353.

Mantuano E, Veneziano L, Spadaro M, Giunti P, Guida S, Leggio MG *et al.* (2004). Clusters of non-truncating mutations of P/Q type Ca^{2+} channel subunit $Ca_v2.1$ causing episodic ataxia 2. *J Med Genet* 41: e82.

Marconi R, De Fusco M, Aridon P, Plewnia K, Rossi M, Carapelli S *et al.* (2003). Familial hemiplegic migraine type 2 is linked to 0.9Mb region on chromosome 1q23. *Ann Neurol* 53: 376-381.

Markowitz S, Saito K and Moskowitz MA (1987). Neurogenically mediated leakage of plasma protein occurs from blood vessels in dura mater but not brain. *J Neurosci* 7: 4129-4136.

Marth G, Yeh R, Minton M, Donaldson R, Li Q, Duan S *et al.* (2001). Single-nucleotide polymorphisms in the public domain: how useful are they? *Nat Genet* 27: 371-372.

Marziniak M, Mossner R, Schmitt A, Lesch KP and Sommer C (2005). A functional serotonin transporter gene polymorphism is associated with migraine with aura. *Neurology* 64: 157-159.

Mathew NT, Kailasam J and Seifert T (2004). Clinical recognition of allodynia in migraine. *Neurology* 63: 848-852.

Matsuyama Z, Murase M, Shimizu H, Aoki Y, Hayashi M, Hozumi I *et al.* (2003). A novel insertion mutation of acetazolamide-responsive episodic ataxia in a Japanese family. *J Neurol Sci* 210: 91-93.

Matsuyama Z, Yanagisawa NK, Aoki Y, Black JL, 3rd, Lennon VA, Mori Y *et al.* (2004). Polyglutamine repeats of spinocerebellar ataxia 6 impair the cell-death-preventing effect of $Ca_V2.1$ Ca2+ channel--loss-of-function cellular model of SCA6. *Neurobiol Dis* 17: 198-204.

Mattsson P (2003). Hormonal factors in migraine: a population-based study of women aged 40 to 74 years. *Headache* 43: 27-35.

May A, Ophoff RA, Terwindt GM, Urban C, van Eijk R, Haan J *et al.* (1995). Familial hemiplegic migraine locus on 19p13 is involved in the common forms of migraine with and without aura. *Hum Genet* 96: 604-608.

McCarthy LC, Hosford DA, Riley JH, Bird MI, White NJ, Hewett DR *et al.* (2001). Single-nucleotide polymorphism alleles in the insulin receptor gene are associated with typical migraine. *Genomics* 78: 135-149.

McClatchey AI, Van den Bergh P, Pericak-Vance MA, Raskind W, Verellen C, McKenna-Yasek D *et al.* (1992). Temperature-sensitive mutations in the III-IV cytoplasmic loop region of the skeletal muscle sodium channel gene in paramyotonia congenita. *Cell* 68: 769-774.

Melliti K, Grabner M and Seabrook GR (2003). The familial hemiplegic migraine mutation R192Q reduces G-protein-mediated inhibition of P/Q-type ($Ca_V2.1$) calcium channels expressed in human embryonic kidney cells. *J Physiol* 546: 337-347.

Mercer RW, Biemesderfer D, Bliss DP, Jr., Collins JH and Forbush B, 3rd (1993). Molecular cloning and immunological characterization of the gamma polypeptide, a small protein associated with the Na,K-ATPase. *J Cell Biol* 121: 579-586.

Merikangas KR, Angst J and Isler H (1990). Migraine and psychopathology. Results of the Zurich cohort study of young adults. *Arch Gen Psychiatry* 47: 849-853.

Merikangas KR (1996). Sources of genetic complexity of migraine. In: Sandler M, Ferrari M and Harnett S eds. *Migraine: Pharmacology and Genetics*. London, Chapman & Hall.

Milner PM (1958). Note on a possible correspondence between scotomas of migraine and spreading depression of Leao. *Electroencephalogr Clin Neurophysiol* 10: 705.

Miyazaki T, Hashimoto K, Shin HS, Kano M and Watanabe M (2004). P/Q-type Ca^{2+} channel $alpha_{1A}$ regulates synaptic competition on developing cerebellar Purkinje cells. *J Neurosci* 24: 1734-1743.

Mohlke KL, Lange EM, Valle TT, Ghosh S, Magnuson VL, Silander K *et al.* (2001). Linkage disequilibrium between microsatellite markers extends beyond 1 cM on chromosome 20 in Finns. *Genome Res* 11: 1221-1226.

Mohlke KL, Erdos MR, Scott LJ, Fingerlin TE, Jackson AU, Silander K *et al.* (2002). High-throughput screening for evidence of association by using mass spectrometry genotyping on DNA pools. *Proc Natl Acad Sci USA* 99: 16928-16933.

Moller JV, Juul B and le Maire M (1996). Structural organization, ion transport, and energy transduction of P-type ATPases. *BBA-Rev Biomembranes* 1286: 1-51.

Monnier N, Procaccio V, Stieglitz P and Lunardi J (1997). Malignant-hyperthermia susceptibility is associated with a mutation of the alpha 1-subunit of the human dihydropyridine-sensitive L-type voltage-dependent calcium-channel receptor in skeletal muscle. *Am J Hum Genet* 60: 1316-1325.

Moreno H, Rudy B and Llinas R (1997). Beta subunits influence the biophysical and pharmacological differences between P- and Q-type calcium currents expressed in a mammalian cell line. *Proc Natl Acad Sci USA* 94: 14042-14047.

Mori Y, Friedrich T, Kim MS, Mikami A, Nakai J, Ruth P *et al.* (1991). Primary structure and functional expression from complementary DNA of a brain calcium channel. *Nature* 350: 398-402.

Mori Y, Wakamori M, Oda S, Fletcher CF, Sekiguchi N, Mori E *et al.* (2000). Reduced voltage sensitivity of activation of P/Q-type Ca^{2+} channels is associated with the ataxic mouse mutation *rolling Nagoya* (*tg*^{rol}). *J Neurosci* 20: 5654-5662.

Morris RW and Kaplan NL (2002). On the advantage of haplotype analysis in the presence of multiple disease susceptibility alleles. *Genet Epidemiol* 23: 221-233.

Morton NE (1955). Sequential tests for the detection of linkage. *Am J Hum Genet* 7: 277-318.

Moseley AE, Lieske SP, Wetzel RK, James PF, He S, Shelly DA *et al.* (2003). The Na,K-ATPase alpha 2 isoform is expressed in neurons, and its absence disrupts neuronal activity in newborn mice. *J Biol Chem* 278: 5317-5324.

Moskowitz MA, Reinhard JF, Jr., Romero J, Melamed E and Pettibone DJ (1979). Neurotransmitters and the fifth cranial nerve: is there a relation to the headache phase of migraine? *Lancet* 2: 883-885.

Moskowitz MA and Macfarlane R (1993). Neurovascular and molecular mechanisms in migraine headaches. *Cerebrovasc Brain Metab Rev* 5: 159-177.

Moskowitz MA, Bolay H and Dalkara T (2004). Deciphering migraine mechanisms: clues from familial hemiplegic migraine genotypes. *Ann Neurol* 55: 276-280.

Mulder EJ, Van Baal C, Gaist D, Kallela M, Kaprio J, Svensson DA *et al.* (2003). Genetic and environmental influences on migraine: a twin study across six countries. *Twin Res* 6: 422-431.

Müllner C, Broos LA, van den Maagdenberg AM and Striessnig J (2004). Familial hemiplegic migraine type 1 mutations K1336E, W1684R, and V1696I alter $Ca_v2.1$ Ca^{2+} channel gating: evidence for beta-subunit isoform-specific effects. *J Biol Chem* 279: 51844-51850.

Nakamura Y, Leppert M, O'Connell P, Wolff R, Holm T, Culver M *et al.* (1987). Variable number of tandem repeat (VNTR) markers for human gene mapping. *Science* 235: 1616-1622.

Nikali N, Suomalainen A, Terwilliger J, Koskinen T, Weissenbach J and Peltonen L (1995). Random search for shared chromosomal regions in four affected individuals: the assignment of a new hereditary ataxia locus. *Am J Hum Genet* 56: 1088-1095.

Noble-Topham SE, Dyment DA, Cader MZ, Ganapathy R, Brown JD, Rice GP *et al.* (2002). Migraine with aura is not linked to the FHM gene *CACNA1A* or the chromosomal region, 19p13. *Neurology* 59: 1099-1101.

Norio R, Nevanlinna HR and Perheentupa J (1973). Hereditary diseases in Finland; rare flora in rare soul. *Ann Clin Res* 5: 109-141.

Nussbaum RL and Ellis CE (2003). Alzheimer's disease and Parkinson's disease. *N Engl J Med* 348: 1356-1364.

Nyholt D, Lea R, Goadsby P, Brimage P and Griffiths L (1998a). Familial typical migraine. Linkage to chromosome 19p13 and evidence for genetic heterogeneity. *Neurology* 50: 1428-1432.

Nyholt DR, Dawkins JL, Brimage PJ, Goadsby PJ, Nicholson GA and Griffiths LR (1998b). Evidence for an X-linked genetic component in familial typical migraine. *Hum Mol Genet* 7: 459-463.

Nyholt DR, Curtain RP and Griffiths LR (2000). Familial typical migraine: significant linkage and localization of a gene to Xq24-28. *Hum Genet* 107: 18-23.

Nyholt DR (2000). All LODs are not created equal. *Am J Hum Genet* 67: 282-288.

Nyholt DR (2001). Genetic case-control association studies--correcting for multiple testing. *Hum Genet* 109: 564-567.

Nyholt DR, Gillespie NG, Heath AC, Merikangas KR, Duffy DL and Martin NG (2004). Latent class and genetic analysis does not support migraine with aura and migraine without aura as separate entities. *Genet Epidemiol* 26: 231-244.

Nyholt DR, Morley KI, Ferreira MA, Medland SE, Boomsma DI, Heath AC *et al.* (2005). Genomewide significant linkage to migrainous headache on chromosome 5q21. *Am J Hum Genet* 77: 500-512.

O'Connell JR and Weeks DE (1998). PedCheck: a program for identification of genotype incompatibilities in linkage analysis. *Am J Hum Genet* 63: 259-266.

O'Donovan MC, Williams NM and Owen MJ (2003). Recent advances in the genetics of schizophrenia. *Hum Mol Genet* 12: R125-R133.

Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R *et al.* (2001). A frameshift mutation in *NOD2* associated with susceptibility to Crohn's disease. *Nature* 411: 603-606.

Olesen J, Friberg L, Skyhoj-Olsen T, Iversen HK, Lassen NA, Andersen AR *et al.* (1990). Timing and topography of cerebral blood flow, aura and headache during migraine attacks. *Ann Neurol* 28: 791-798.

Ophoff RA, van Eijk R, Sandkuijl LA, Terwindt GM, Grubben CP, Haan J *et al.* (1994). Genetic heterogeneity of familial hemiplegic migraine. *Genomics* 22: 21-26.

Ophoff RA, Terwindt GM, Vergouwe MN, van Eijk R, Oefner PJ, Hoffman SM *et al.* (1996a). Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca2+ channel gene *CACNL1A4*. *Cell* 87: 543-552.

Ophoff RA, Terwindt GM, Vergouwe MN, van Eijk R, Mohrenweiser H, Litt M *et al.* (1996b). A 3-Mb region for the familial hemiplegic migraine locus on 19p13.1- p13.2: exclusion of *PRKCSH* as a candidate gene. *Eur J Hum Genet* 4: 321-328.

Ophoff RA, DeYoung J, Service SK, Joosse M, Caffo NA, Sandkuijl LA *et al.* (2001). Hereditary vascular retinopathy, cerebroretinal vasculopathy, and hereditary endotheliopathy with retinopathy, nephropathy, and stroke map to a single locus on chromosome 3p21.1-p21.3. *Am J Hum Genet* 69: 447-453.

Ophoff RA, Escamilla MA, Service SK, Spesny M, Meshi DB, Poon W *et al.* (2002). Genomewide linkage disequilibrium mapping of severe bipolar disorder in a population isolate. *Am J Hum Genet* 71: 565-574.

Orlowski J and Lingrel JB (1988). Tissue-specific and developmental regulation of rat Na,K-ATPase catalytic alpha isoform and beta subunit mRNAs. *J Biol Chem* 263: 10436-10442.

Oterino A, Valle N, Bravo Y, Munoz P, Sanchez-Velasco P, Ruiz-Alegria C *et al.* (2004). *MTHFR* T677 homozygosis influences the presence of aura in migraineurs. *Cephalalgia* 24: 491-494.

Ott J (1989). Computer-simulation methods in human linkage analysis. *Proc Natl Acad Sci USA* 86: 4175-4178.

Ott J (1991). *Analysis of Human Genetic Linkage*. Baltimore, The Johns Hopkins University Press.

Ottman R and Lipton R (1994). Comorbidity of migraine and epilepsy. *Neurology* 44: 2105-2110.

Page KM, Heblich F, Davies A, Butcher AJ, Leroy J, Bertaso F *et al.* (2004). Dominant-negative calcium channel suppression by truncated constructs involves a kinase implicated in the unfolded protein response. *J Neurosci* 24: 5400-5409.

Pajukanta P, Nuotio I, Terwilliger JD, Porkka KV, Ylitalo K, Pihlajamaki J *et al.* (1998). Linkage of familial combined hyperlipidaemia to chromosome 1q21-q23. *Nat Genet* 18: 369-373.

Pajukanta P, Lilja HE, Sinsheimer JS, Cantor RM, Lusis AJ, Gentile M *et al.* (2004). Familial combined hyperlipidemia is associated with upstream transcription factor 1 (*USF1*). *Nat Genet* 36: 371-376.

Palotie A, Baloh RW and Wessman M (2002). Migraine. In: King RA, Rotter JI and Motulsky AG eds. *The Genetic Basis of Common Diseases*. New York, Oxford University Press.

Parker HL (1946). Periodic ataxia. *Mayo Clin Proc*: 642-645.

Patil N, Berno AJ, Hinds DA, Barrett WA, Doshi JM, Hacker CR *et al.* (2001). Blocks of limited haplotype diversity revealed by high-resolution scanning of human chromosome 21. *Science* 294: 1719-1723.

Paunio T, Tuulio-Henriksson A, Hiekkalinna T, Perola M, Varilo T, Partonen T *et al.* (2004). Search for cognitive trait components of schizophrenia reveals a locus for verbal learning and memory on 4q and for visual working memory on 2q. *Hum Mol Genet* 13: 1693-1702.

Peltonen L, Jalanko A and Varilo T (1999). Molecular genetics of the Finnish disease heritage. *Hum Mol Genet* 8: 1913-1923.

Peltonen L, Palotie A and Lange K (2000). Use of population isolates for mapping complex traits. *Nat Rev Genet* 1: 182-190.

Peltonen L (2003). GenomEUtwin: a strategy to identify genetic influences on health and disease. *Twin Res* 6: 354-360.

Penrose LS (1953). The general purpose sibpair linkage test. *Ann Eugen* 18: 120-124.

Pietrobon D (2002). Calcium channels and channelopathies of the central nervous system. *Mol Neurobiol* 25: 31-50.

Pineda JC, Waters RS and Foehring RC (1998). Specificity in the interaction of HVA Ca^{2+} channel types with Ca^{2+}-dependent AHPs and firing behavior in neocortical pyramidal neurons. *J Neurophysiol* 79: 2522-2534.

Plomp JJ, Vergouwe MN, van den Maagdenberg AM, Ferrari MD, Frants RR and Molenaar PC (2000). Abnormal transmitter release at neuromuscular junctions of mice carrying the tottering $alpha_{1A}$ Ca^{2+} channel mutation. *Brain* 123: 463-471.

Pritchard JK and Rosenberg NA (1999). Use of unlinked genetic markers to detect population stratification in association studies. *Am J Hum Genet* 65: 220-228.

Pritchard JK and Cox NJ (2002). The allelic architecture of human disease genes: common disease-common variant… or not? *Hum Mol Genet* 11: 2417-2423.

Protti DA, Reisin R, Mackinley TA and Uchitel OD (1996). Calcium channel blockers and transmitter release at the normal human neuromuscular junction. *Neurology* 46: 1391-1396.

Ptacek LJ, George AL, Jr., Griggs RC, Tawil R, Kallen RG, Barchi RL *et al.* (1991). Identification of a mutation in the gene causing hyperkalemic periodic paralysis. *Cell* 67: 1021-1027.

Ptacek LJ, Tawil R, Griggs RC, Engel AG, Layzer RB, Kwiecinski H *et al.* (1994). Dihydropyridine receptor mutations cause hypokalemic periodic paralysis. *Cell* 77: 863-868.

Ptacek LJ (1998). The place of migraine as a channelopathy. *Curr Opin Neurol* 11: 217-226.

Ptacek LJ and Fu YH (2004). Channels and disease: past, present, and future. *Arch Neurol* 61: 1665-1668.

Purcell S, Cherny SS and Sham PC (2003). Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics* 19: 149-150.

Purves D, Augustine GJ, Fitzpatrick D, Katz LC, LaMantia A-S and McNamara JO (1997). *Neuroscience*. Sunderland, Sinauer Associates.

Radat F and Swendsen J (2005). Psychiatric comorbidity in migraine: a review. *Cephalalgia* 25: 165-178.

Rainero I, Gallone S, Valfre W, Ferrero M, Angilella G, Rivoiro C *et al.* (2004a). A polymorphism of the hypocretin receptor 2 gene is associated with cluster headache. *Neurology* 63: 1286-1288.

Rainero I, Grimaldi LM, Salani G, Valfre W, Rivoiro C, Savi L *et al.* (2004b). Association between the tumor necrosis factor-alpha -308 G/A gene polymorphism and migraine. *Neurology* 62: 141-143.

Rasmussen BK, Jensen R, Schroll M and Olesen J (1991). Epidemiology of headache in a general population--a prevalence study. *J Clin Epidemiol* 44: 1147-1157.

Rasmussen BK, Jensen R and Olesen J (1992). Impact of headache on sickness absence and utilisation of medical services: a Danish population study. *J Epidemiol Community Health* 46: 443-446.

Rasmussen BK (1992). Migraine and tension-type headache in a general population: psychosocial factors. *Int J Epidemiol* 21: 1138-1143.

Rasmussen BK (1993). Migraine and tension-type headache in a general population: precipitating factors, female hormones, sleep pattern and relation to lifestyle. *Pain* 53: 65-72.

Rasmussen BK (2001). Epidemiology of headache. *Cephalalgia* 21: 774-777.

Reich DE, Cargill M, Bolk S, Ireland J, Sabeti PC, Richter DJ *et al.* (2001). Linkage disequilibrium in the human genome. *Nature* 411: 199-204.

Reich DE and Lander ES (2001). On the allelic spectrum of human disease. *Trends Genet* 17: 502-510.

Riant F, De Fusco M, Aridon P, Ducros A, Ploton C, Marchelli F *et al.* (2005). *ATP1A2* mutations in 11 families with familial hemiplegic migraine. *Hum Mutat* 26: 281.

Risch N and Merikangas K (1996). The future of genetic studies of complex human diseases. *Science* 273: 1516-1517.

Risch NJ (2000). Searching for genetic determinants in the new millennium. *Nature* 405: 847-856.
Riva A and Kohane IS (2004). A SNP-centric database for the investigation of the human genome. *BMC Bioinformatics* 5: 33.
Roberts SB, MacLean CJ, Neale MC, Eaves LJ and Kendler KS (1999). Replication of linkage studies of complex traits: an examination of variation in location estimates. *Am J Hum Genet* 65: 876-884.
Rucker JC, Jen J, Stahl JS, Natesan N, Baloh RW and Leigh RJ (2005). Internuclear ophthalmoparesis in episodic ataxia type 2. *Ann NY Acad Sci* 1039: 571-574.
Russell MB and Olesen J (1995). Increased familial risk and evidence of genetic factor in migraine. *BMJ* 311: 541-544.
Russell MB, Rasmussen BK, Thorvaldsen P and Olesen J (1995a). Prevalence and sex-ratio of the subtypes of migraine. *Int J Epidemiol* 24: 612-618.
Russell MB, Iselius L and Olesen J (1995b). Inheritance of migraine investigated by complex segregation analysis. *Hum Genet* 96: 726-730.
Russell MB and Olesen J (1996). A nosographic analysis of the migraine aura in a general population. *Brain* 119: 355-361.
Russell MB, Rasmussen BK, Fenger K and Olesen J (1996). Migraine without aura and migraine with aura are distinct clinical entities: a study of four hundred and eighty-four male and female migraineurs from the general population. *Cephalalgia* 16: 239-245.
Russell MB, Ostergaard S, Bendtsen L and Olesen J (1999). Familial occurrence of chronic tension-type headache. *Cephalalgia* 19: 207-210.
Russell MB, Ulrich V, Gervil M and Olesen J (2002). Migraine without aura and migraine with aura are distinct disorders. A population-based twin survey. *Headache* 42: 332-336.
Russell MB (2004). Epidemiology and genetics of cluster headache. *Lancet Neurol* 3: 279-283.
Russo L, Mariotti P, Sangiorgi E, Giordano T, Ricci I, Lupi F *et al.* (2005). A new susceptibility locus for migraine with aura in the 15q11-q13 genomic region containing three GABA-A receptor genes. *Am J Hum Genet* 76: 327-333.
Saarela J, Schoenberg Fejzo M, Chen D, Finnila S, Parkkonen M, Kuokkanen S *et al.* (2002). Fine mapping of a multiple sclerosis locus to 2.5 Mb on chromosome 17q22-q24. *Hum Mol Genet* 11: 2257-2267.
Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G *et al.* (2001). A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature* 409: 928-933.
Sander T, Toliat MR, Heils A, Becker C and Nurnberg P (2002). Failure to replicate an allelic association between an exon 8 polymorphism of the human alpha$_{1A}$ calcium channel gene and common syndromes of idiopathic generalized epilepsy. *Epilepsy Res* 49: 173-177.
Sandor PS, Afra J, Proietti-Cecchini A, Albert A and Schoenen J (1999). Familial influences on cortical evoked potentials in migraine. *Neuroreport* 10: 1235-1238.
Sanger F, Nicklen S and Coulson AR (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74: 5463-5467.
Sawcer SJ, Maranian M, Singlehurst S, Yeo T, Compston A, Daly MJ *et al.* (2004). Enhancing linkage analysis of complex disorders: an evaluation of high-density genotyping. *Hum Mol Genet* 13: 1943-1949.
Schoenen J (1996). Deficient habituation of evoked cortical potentials in migraine: a link between brain biology, behavior and trigeminovascular activation? *Biomed Pharmacother* 50: 71-78.
Schwartz BS, Stewart WF and Lipton RB (1997). Lost workdays and decreased work effectiveness associated with headache in the workplace. *J Occup Environ Med* 39: 320-327.
Schöls L, Bauer P, Schmidt T, Schulte T and Riess O (2004). Autosomal dominant cerebellar ataxias: clinical features, genetics, and pathogenesis. *Lancet Neurol* 3: 291-304.
Scoggan KA, Chandra T, Nelson R, Hahn AF and Bulman DE (2001). Identification of two novel mutations in the *CACNA1A* gene responsible for episodic ataxia type 2. *J Med Genet* 38: 249-253.
Sebat J, Lakshmi B, Troge J, Alexander J, Young J, Lundin P *et al.* (2004). Large-scale copy number polymorphism in the human genome. *Science* 305: 525-528.

Segall L, Scanzano R, Kaunisto MA, Wessman M, Palotie A, Gargus JJ *et al.* (2004). Kinetic alterations due to a missense mutation in the Na,K-ATPase alpha2 subunit cause familial hemiplegic migraine type 2. *J Biol Chem* 279: 43692-43696.

Segall L, Mezzetti A, Scanzano R, Gargus JJ, Purisima E and Blostein R (2005). Alterations in the alpha2 isoform of Na,K-ATPase associated with familial hemiplegic migraine type 2. *Proc Natl Acad Sci USA* 102: 11106-11111.

Service SK, Ophoff RA and Freimer NB (2001). The genome-wide distribution of background linkage disequilibrium in a population isolate. *Hum Mol Genet* 10: 545-551.

Sham P, Bader JS, Craig I, O'Donovan M and Owen M (2002). DNA pooling: a tool for large-scale association studies. *Nat Rev Genet* 3: 862-871.

Sheffield VC, Stone EM and Carmi R (1998). Use of isolated inbred human populations for identification of disease genes. *Trends Genet* 14: 391-396.

Sherry ST, Ward M and Sirotkin K (1999). dbSNP-database for single nucleotide polymorphisms and other classes of minor genetic variation. *Genome Res* 9: 677-679.

Shiang R, Ryan SG, Zhu YZ, Hahn AF, O'Connell P and Wasmuth JJ (1993). Mutations in the alpha 1 subunit of the inhibitory glycine receptor cause the dominant neurologic disorder, hyperekplexia. *Nat Genet* 5: 351-358.

Shull MM, Pugh DG and Lingrel JB (1989). Characterization of the human Na,K-ATPase alpha 2 gene and identification of intragenic restriction fragment length polymorphisms. *J Biol Chem* 264: 17532-17543.

Silander K, Scott LJ, Valle TT, Mohlke KL, Stringham HM, Wiles KR *et al.* (2004). A large set of Finnish affected sibling pair families with type 2 diabetes suggests susceptibility loci on chromosomes 6, 11, and 14. *Diabetes* 53: 821-829.

Silberstein SD (1995). Migraine symptoms: results of a survey of self-reported migraineurs. *Headache* 35: 387-396.

Silberstein S, Lipton R and Goadsby PJ (1998). *Headache in Clinical Practice*. Oxford, Isis Medical Media.

Sillanpää M (1983). Changes in the prevalence of migraine and other headaches during the first seven school years. *Headache* 23: 15-19.

Siminovitch KA (2004). *PTPN22* and autoimmune disease. *Nat Genet* 36: 1248-1249.

Singh NA, Charlier C, Stauffer D, DuPont BR, Leach RJ, Melis R *et al.* (1998). A novel potassium channel gene, *KCNQ2*, is mutated in an inherited epilepsy of newborns. *Nat Genet* 18: 25-29.

Slager SL, Huang J and Vieland VJ (2000). Effect of allelic heterogeneity on the power of the transmission disequilibrium test. *Genet Epidemiol* 18: 143-156.

Smith DJ and Lusis AJ (2002). The allelic structure of common disease. *Hum Mol Genet* 11: 2455-2461.

Smith R (1998). Impact of migraine on the family. *Headache* 38: 423-426.

Sobel E and Lange K (1996). Descent graphs in pedigree analysis: application to haplotyping, location scores, and marker sharing statistics. *Am J Hum Genet* 58: 1323-1337.

Soong TW, DeMaria CD, Alvania RS, Zweifel LS, Liang MC, Mittman S *et al.* (2002). Systematic identification of splice variants in human P/Q-type channel alpha$_1$(2.1) subunits: implications for current density and Ca^{2+}-dependent inactivation. *J Neurosci* 22: 10142-10152.

Soragna D, Vettori A, Carraro G, Marchioni E, Vazza G, Bellini S *et al.* (2003). A locus for migraine without aura maps on chromosome 14q21.2-q22.3. *Am J Hum Genet* 72: 161-167.

Spacey SD, Hildebrand ME, Materek LA, Bird TD and Snutch TP (2004). Functional implications of a novel EA2 mutation in the P/Q-type calcium channel. *Ann Neurol* 56: 213-220.

Spacey SD, Materek LA, Szczygielski BI and Bird TD (2005). Two novel *CACNA1A* gene mutations associated with episodic ataxia type 2 and interictal dystonia. *Arch Neurol* 62: 314-316.

Spadaro M, Ursu S, Lehmann-Horn F, Veneziano L, Antonini G, Giunti P *et al.* (2004). A G301R Na^+/K^+ -ATPase mutation causes familial hemiplegic migraine type 2 with cerebellar signs. *Neurogenetics* 5: 177-185.

Spielman RS, McGinnis RE and Ewens WJ (1993). Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 52: 506-516.

Spierings EL (1988). Recent advances in the understanding of migraine. *Headache* 28: 655-658.

Splawski I, Timothy KW, Sharpe LM, Decher N, Kumar P, Bloise R *et al.* (2004). $Ca_V1.2$ calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. *Cell* 119: 19-31.

Starr TV, Prystay W and Snutch TP (1991). Primary structure of a calcium channel that is highly expressed in the rat cerebellum. *Proc Natl Acad Sci USA* 88: 5621-5625.

Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S *et al.* (2002). Neuregulin 1 and susceptibility to schizophrenia. *Am J Hum Genet* 71: 877-892.

Stefansson H, Helgason A, Thorleifsson G, Steinthorsdottir V, Masson G, Barnard J *et al.* (2005). A common inversion under selection in Europeans. *Nat Genet* 37: 129-137.

Steinlein OK, Mulley JC, Propping P, Wallace RH, Phillips HA, Sutherland GR *et al.* (1995). A missense mutation in the neuronal nicotinic acetylcholine receptor alpha 4 subunit is associated with autosomal dominant nocturnal frontal lobe epilepsy. *Nat Genet* 11: 201-203.

Steinlein OK (2004). Genetic mechanisms that underlie epilepsy. *Nat Rev Neurosci* 5: 400-408.

Stephens M and Donnelly P (2003). A comparison of bayesian methods for haplotype reconstruction from population genotype data. *Am J Hum Genet* 73: 1162-1169.

Stewart WF, Lipton RB, Celentano DD and Reed ML (1992). Prevalence of migraine headache in the United States. Relation to age, income, race, and other sociodemographic factors. *JAMA* 267: 64-69.

Stewart WF, Lipton RB and Liberman J (1996). Variation in migraine prevalence by race. *Neurology* 47: 52-59.

Stewart WF, Staffa J, Lipton RB and Ottman R (1997). Familial risk of migraine: a population-based study. *Ann Neurol* 41: 166-172.

Strachan T and Read AP (1996). *Human Molecular Genetics*. Oxford, BIOS Scientific Publishers Limited.

Strassman AM, Raymond SA and Burstein R (1996). Sensitization of meningeal sensory neurons and the origin of headaches. *Nature* 384: 560-564.

Styrkarsdottir U, Cazier JB, Kong A, Rolfsson O, Larsen H, Bjarnadottir E *et al.* (2003). Linkage of osteoporosis to chromosome 20p12 and association to *BMP2*. *PLoS Biol* 1: E69.

Subramony SH, Schott K, Raike RS, Callahan J, Langford LR, Christova PS *et al.* (2003). Novel *CACNA1A* mutation causes febrile episodic ataxia with interictal cerebellar deficits. *Ann Neurol* 54: 725-731.

Sugawara T, Tsurubuchi Y, Agarwala KL, Ito M, Fukuma G, Mazaki-Miyazaki E *et al.* (2001). A missense mutation of the Na^+ channel alpha II subunit gene $Na_v1.2$ in a patient with febrile and afebrile seizures causes channel dysfunction. *Proc Natl Acad Sci USA* 98: 6384-6389.

Sun G, Tomita H, Shakkottai VG and Gargus JJ (2001). Genomic organization and promoter analysis of human *KCNN3* gene. *J Hum Genet* 46: 463-470.

Sutton KG, McRory JE, Guthrie H, Murphy TH and Snutch TP (1999). P/Q-type calcium channels mediate the activity-dependent feedback of syntaxin-1A. *Nature* 401: 800-804.

Suviolahti E, Oksanen LJ, Ohman M, Cantor RM, Ridderstrale M, Tuomi T *et al.* (2003). The *SLC6A14* gene shows evidence of association with obesity. *J Clin Invest* 112: 1762-1772.

Swartz RH and Kern RZ (2004). Migraine is associated with magnetic resonance imaging white matter abnormalities: a meta-analysis. *Arch Neurol* 61: 1366-1368.

Svensson DA, Larsson B, Waldenlind E and Pedersen NL (2003). Shared rearing environment in migraine: results from twins reared apart and twins reared together. *Headache* 43: 235-244.

Syvänen AC, Aalto-Setälä K, Harju L, Kontula K and Söderlund H (1990). A primer-guided nucleotide incorporation assay in the genotyping of apolipoprotein E. *Genomics* 8: 684-692.

Syvänen AC (2001). Accessing genetic variation: genotyping single nucleotide polymorphisms. *Nat Rev Genet* 2: 930-942.

Syvänen AC (2005). Toward genome-wide SNP genotyping. *Nat Genet* 37 (Suppl): S5-10.

Tabor HK, Risch NJ and Myers RM (2002). Candidate-gene approaches for studying complex genetic traits: practical considerations. *Nat Rev Genet* 3: 391-397.

Taipale M, Kaminen N, Nopola-Hemmi J, Haltia T, Myllyluoma B, Lyytinen H *et al.* (2003). A candidate gene for developmental dyslexia encodes a nuclear tetratricopeptide repeat domain protein dynamically regulated in brain. *Proc Natl Acad Sci USA* 100: 11553-11558.

Takahashi T, Igarashi S, Kimura T, Hozumi I, Kawachi I, Onodera O *et al.* (2002). Japanese cases of familial hemiplegic migraine with cerebellar ataxia carrying a T666M mutation in the *CACNA1A* gene. *J Neurol Neurosurg Psychiatry* 72: 676-677.

Takeshima T, Ishizaki K, Fukuhara Y, Ijiri T, Kusumi M, Wakutani Y *et al.* (2004). Population-based door-to-door survey of migraine in Japan: the Daisen study. *Headache* 44: 8-19.

Tanabe T, Takeshima H, Mikami A, Flockerzi V, Takahashi H, Kangawa K *et al.* (1987). Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature* 328: 313-318.

Teh BT, Silburn P, Lindblad K, Betz R, Boyle R, Schalling M *et al.* (1995). Familial periodic cerebellar ataxia without myokymia maps to a 19-cM region on 19p13. *Am J Hum Genet* 56: 1443-1449.

Terwilliger JD (1995). A powerful likelihood method for the analysis of linkage disequilibrium between trait loci and one or more polymorphic marker loci. *Am J Hum Genet* 56: 777-787.

Terwilliger JD, Shannon WD, Lathrop GM, Nolan JP, Goldin LR, Chase GA *et al.* (1997). True and false positive peaks in genomewide scans: applications of length-biased sampling to linkage mapping. *Am J Hum Genet* 61: 430-438.

Terwilliger JD and Weiss KM (1998). Linkage disequilibrium mapping of complex disease: fantasy or reality? *Curr Opin Biotechnol* 9: 578-594.

Terwindt G, Kors E, Haan J, Vermeulen F, van den Maagdenberg A, Frants R *et al.* (2002). Mutation analysis of the *CACNA1A* calcium channel subunit gene in 27 patients with sporadic hemiplegic migraine. *Arch Neurol* 59: 1016-1018.

Terwindt GM, Ferrari MD, Tijhuis M, Groenen SM, Picavet HS and Launer LJ (2000). The impact of migraine on quality of life in the general population: the GEM study. *Neurology* 55: 624-629.

Terwindt GM, Ophoff RA, van Eijk R, Vergouwe MN, Haan J, Frants RR *et al.* (2001). Involvement of the *CACNA1A* gene containing region on 19p13 in migraine with and without aura. *Neurology* 56: 1028-1032.

Thomas PD and Kejariwal A (2004). Coding single-nucleotide polymorphisms associated with complex vs. Mendelian disease: evolutionary evidence for differences in molecular effects. *Proc Natl Acad Sci USA* 101: 15398-15403.

Thomsen LL, Eriksen MK, Roemer SF, Andersen I, Olesen J and Russell MB (2002a). A population-based study of familial hemiplegic migraine suggests revised diagnostic criteria. *Brain* 125: 1379-1391.

Thomsen LL, Eriksen MK, Romer SF, Andersen I, Ostergaard E, Keiding N *et al.* (2002b). An epidemiological survey of hemiplegic migraine. *Cephalalgia* 22: 361-375.

Thomsen LL, Olesen J and Russell MB (2003). Increased risk of migraine with typical aura in probands with familial hemiplegic migraine and their relatives. *Eur J Neurol* 10: 421-427.

Thornton-Wells TA, Moore JH and Haines JL (2004). Genetics, statistics and human disease: analytical retooling for complexity. *Trends Genet* 20: 640-647.

Tienari PJ, Sumelahti ML, Rantamäki T and Wikström J (2004). Multiple sclerosis in western Finland: evidence for a founder effect. *Clin Neurol Neurosurg* 106: 175-179.

Todd JA (2001). Tackling common disease. *Nature* 411: 537, 539.

Todt U, Dichgans M, Jurkat-Rott K, Heinze A, Zifarelli G, Koenderink JB *et al.* (2005). Rare missense variants in *ATP1A2* in families with clustering of common forms of migraine. *Hum Mutat* 26: 315-321.

Toru S, Murakoshi T, Ishikawa K, Saegusa H, Fujigasaki H, Uchihara T *et al.* (2000). Spinocerebellar ataxia type 6 mutation alters P-type calcium channel function. *J Biol Chem* 275: 10893-10898.

Tottene A, Fellin T, Pagnutti S, Luvisetto S, Striessnig J, Fletcher C *et al.* (2002). Familial hemiplegic migraine mutations increase Ca^{2+} influx through single human $Ca_V2.1$ channels and decrease maximal $Ca_V2.1$ current density in neurons. *Proc Natl Acad Sci USA* 99: 13284-13289.

Tottene A, Pivotto F, Fellin T, Cesetti T, van den Maagdenberg AM and Pietrobon D (2005). Specific kinetic alterations of human $Ca_V2.1$ calcium channels produced by mutation S218L causing familial hemiplegic migrane and delayed cerebral edema and coma after minor head trauma. *J Biol Chem* 280: 17678-17686.

Trettel F, Mantuano E, Calabresi V, Veneziano L, Olsen AS, Georgescu A *et al.* (2000). A fine physical map of the *CACNA1A* gene region on 19p13.1-p13.2 chromosome. *Gene* 241: 45-50.

Turner TJ, Adams ME and Dunlap K (1992). Calcium channels coupled to glutamate release identified by omega-Aga-IVA. *Science* 258: 310-313.

Tzourio C, Iglesias S, Hubert JB, Visy JM, Alperovitch A, Tehindrazanarivelo A *et al.* (1993). Migraine and risk of ischaemic stroke: a case-control study. *BMJ* 307: 289-292.

Tzourio C, Tehindrazanarivelo A, Iglesias S, Alperovitch A, Chedru F, d'Anglejan-Chatillon J *et al.* (1995). Case-control study of migraine and risk of ischaemic stroke in young women. *BMJ* 310: 830-833.

Tzourio C, El Amrani M, Poirier O, Nicaud V, Bousser MG and Alperovitch A (2001). Association between migraine and endothelin type A receptor (ETA -231 A/G) gene polymorphism. *Neurology* 56: 1273-1277.

Ulrich V, Russell MB, Ostergaard S and Olesen J (1997). Analysis of 31 families with an apparently autosomal-dominant transmission of migraine with aura in the nuclear family. *Am J Med Genet* 74: 395-397.

Ulrich V, Gervil M, Kyvik KO, Olesen J and Russell MB (1999a). The inheritance of migraine with aura estimated by means of structural equation modelling. *J Med Genet* 36: 225-227.

Ulrich V, Gervil M, Kyvik KO, Olesen J and Russell MB (1999b). Evidence of a genetic factor in migraine with aura: a population-based Danish twin study. *Ann Neurol* 45: 242-246.

Ulrich V, Olesen J, Gervil M and Russell MB (2000). Possible risk factors and precipitants for migraine with aura in discordant twin-pairs: a population-based study. *Cephalalgia* 20: 821-825.

Ulrich V, Gervil M and Olesen J (2004). The relative influence of environment and genes in episodic tension-type headache. *Neurology* 62: 2065-2069.

Vahedi K, Joutel A, Van Bogaert P, Ducros A, Maciazeck J, Bach JF *et al.* (1995). A gene for hereditary paroxysmal cerebellar ataxia maps to chromosome 19p. *Ann Neurol* 37: 289-293.

Vahedi K, Denier C, Ducros A, Bousson PV, Levy C, Chabriat H *et al.* (2000). *CACNA1A* gene de novo mutation causing hemiplegic migraine, coma, and cerebellar atrophy. *Neurology* 55: 1040-1042.

van den Maagdenberg AM, Kors EE, Brunt ER, Van Paesschen W, Pascual J, Ravine D *et al.* (2002). Episodic ataxia type 2: Three novel truncating mutations and one novel missense mutation in the *CACNA1A* gene. *J Neurol* 249: 1515-1519.

van den Maagdenberg AM, Pietrobon D, Pizzorusso T, Kaja S, Broos LA, Cesetti T *et al.* (2004). A *Cacna1a* knockin migraine mouse model with increased susceptibility to cortical spreading depression. *Neuron* 41: 701-710.

Vanmolkot KR, Kors EE, Hottenga JJ, Terwindt GM, Haan J, Hoefnagels WA *et al.* (2003). Novel mutations in the Na^+, K^+-ATPase pump gene *ATP1A2* associated with familial hemiplegic migraine and benign familial infantile convulsions. *Ann Neurol* 54: 360-366.

Vanmolkot KRJ, Barone V, Turk U, Kors EE, Turkdogan D, Kia SK *et al.* (2004). Two novel de novo mutations in the Na^+, K^+-ATPase pump gene *ATP1A2* associated with Familial Hemiplegic Migraine type 2. *American Journal of Human Genetics* 75 (Suppl): 482, abstract 2689.

Varilo T and Peltonen L (2004). Isolates and their potential use in complex gene mapping efforts. *Curr Opin Genet Dev* 14: 316-323.

Varilo T, Paunio T, Parker A, Perola M, Meyer J, Terwilliger JD *et al.* (2003). The interval of linkage disequilibrium (LD) detected with microsatellite and SNP markers in chromosomes of Finnish populations with different histories. *Hum Mol Genet* 12: 51-59.

Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG *et al.* (2001). The sequence of the human genome. *Science* 291: 1304-1351.

von Brederlow B, Hahn AF, Koopman WJ, Ebers GC and Bulman DE (1995). Mapping the gene for acetazolamide responsive hereditary paryoxysmal cerebellar ataxia to chromosome 19p. *Hum Mol Genet* 4: 279-284.

Wada T, Kobayashi N, Takahashi Y, Aoki T, Watanabe T and Saitoh S (2002). Wide clinical variability in a family with a *CACNA1A* T666M mutation: hemiplegic migraine, coma, and progressive ataxia. *Pediatr Neurol* 26: 47-50.

Wakamori M, Yamazaki K, Matsunodaira H, Teramoto T, Tanaka I, Niidome T *et al.* (1998). Single tottering mutations responsible for the neuropathic phenotype of the P-type calcium channel. *J Biol Chem* 273: 34857-34867.

Wall JD and Pritchard JK (2003). Haplotype blocks and linkage disequilibrium in the human genome. *Nat Rev Genet* 4: 587-597.

Wallace RH, Wang DW, Singh R, Scheffer IE, George AL, Jr., Phillips HA *et al.* (1998). Febrile seizures and generalized epilepsy associated with a mutation in the Na^+-channel beta1 subunit gene *SCN1B*. *Nat Genet* 19: 366-370.

Wallace RH, Marini C, Petrou S, Harkin LA, Bowser DN, Panchal RG *et al.* (2001). Mutant $GABA_A$ receptor gamma2-subunit in childhood absence epilepsy and febrile seizures. *Nat Genet* 28: 49-52.

Wan J, Carr JR, Baloh RW and Jen JC (2005a). Nonconsensus intronic mutations cause episodic ataxia. *Ann Neurol* 57: 131-135.

Wan J, Khanna R, Sandusky M, Papazian DM, Jen JC and Baloh RW (2005b). *CACNA1A* mutations causing episodic and progressive ataxia alter channel trafficking and kinetics. *Neurology* 64: 2090-2097.

Wang WY, Barratt BJ, Clayton DG and Todd JA (2005). Genome-wide association studies: theoretical and practical concerns. *Nat Rev Genet* 6: 109-118.

Wappl E, Koschak A, Poteser M, Sinnegger MJ, Walter D, Eberhart A *et al.* (2002). Functional consequences of P/Q-type Ca2+ channel Cav2.1 missense mutations associated with episodic ataxia type 2 and progressive ataxia. *J Biol Chem* 277: 6960-6966.

Watts AG, Sanchez-Watts G, Emanuel JR and Levenson R (1991). Cell-specific expression of mRNAs encoding Na^+,K^+-ATPase alpha- and beta-subunit isoforms within the rat central nervous system. *Proc Natl Acad Sci USA* 88: 7425-7429.

Waxman SG (2001). Transcriptional channelopathies: an emerging class of disorders. *Nat Rev Neurosci* 2: 652-659.

Weber JL and May PE (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am J Hum Genet* 44: 388-396.

Weeks DE, Ott J and Lathrop GM (1994). Slink: A general simulation program for linkage analysis. *Am J Hum Genet* 47 (Suppl): A204.

Weiller C, May A, Limmroth V, Juptner M, Kaube H, Schayck RV *et al.* (1995). Brain stem activation in spontaneous human migraine attacks. *Nat Med* 1: 658-660.

Weiss KM and Terwilliger JD (2000). How many diseases does it take to map a gene with SNPs? *Nat Genet* 26: 151-157.

Weissenbach J, Gyapay G, Dib C, Vignal A, Morissette J, Millasseau P *et al.* (1992). A second-generation linkage map of the human genome. *Nature* 359: 794-801.

Welch KM, D'Andrea G, Tepley N, Barkley G and Ramadan NM (1990). The concept of migraine as a state of central neuronal hyperexcitability. *Neurol Clin* 8: 817-828.

Wessman M, Kallela M, Kaunisto MA, Marttila P, Sobel E, Hartiala J *et al.* (2002). A susceptibility locus for migraine with aura, on chromosome 4q24. *Am J Hum Genet* 70: 652-662.

Westenbroek RE, Sakurai T, Elliott EM, Hell JW, Starr TV, Snutch TP *et al.* (1995). Immunochemical identification and subcellular distribution of the alpha 1A subunits of brain calcium channels. *J Neurosci* 15: 6403-6418.

Wieser T, Mueller C, Evers S, Zierz S and Deufel T (2003). Absence of known familial hemiplegic migraine (FHM) mutations in the *CACNA1A* gene in patients with common migraine: implications for genetic testing. *Clin Chem Lab Med* 41: 272-275.

Wolff HG (1963). *Headache and Other Head Pain*. New York, Oxford University Press.

Womack MD, Chevez C and Khodakhah K (2004). Calcium-activated potassium channels are selectively coupled to P/Q-type calcium channels in cerebellar Purkinje neurons. *J Neurosci* 24: 8818-8822.

Woods RP, Iacoboni M and Mazziotta JC (1994). Bilateral spreading cerebral hypoperfusion during spontaneous migraine headache. *N Engl J Med* 331: 1689-1692.

Wright AF, Carothers AD and Pirastu M (1999). Population choice in mapping genes for complex diseases. *Nat Genet* 23: 397-404.

Yue Q, Jen JC, Nelson SF and Baloh RW (1997). Progressive ataxia due to a missense mutation in a calcium-channel gene. *Am J Hum Genet* 61: 1078-1087.

Yue Q, Jen JC, Thwe MM, Nelson SF and Baloh RW (1998). De novo mutation in *CACNA1A* caused acetazolamide-responsive episodic ataxia. *Am J Med Genet* 77: 298-301.

Zareparsi S, Branham KE, Li M, Shah S, Klein RJ, Ott J *et al.* (2005). Strong association of the Y402H variant in complement factor H at 1q32 with susceptibility to age-related macular degeneration. *Am J Hum Genet* 77: 149-153.

Zhang K, Calabrese P, Nordborg M and Sun F (2002). Haplotype block structure and its applications to association studies: power and study designs. *Am J Hum Genet* 71: 1386-1394.

Zhong H, Yokoyama CT, Scheuer T and Catterall WA (1999). Reciprocal regulation of P/Q-type Ca^{2+} channels by SNAP-25, syntaxin and synaptotagmin. *Nat Neurosci* 2: 939-941.

Zhuchenko O, Bailey J, Bonnen P, Ashizawa T, Stockton DW, Amos C *et al.* (1997). Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the alpha 1A-voltage-dependent calcium channel. *Nat Genet* 15: 62-69.

Ziegler DK, Hur YM, Bouchard TJ, Jr., Hassanein RS and Barter R (1998). Migraine in twins raised together and apart. *Headache* 38: 417-422.

Zondervan KT and Cardon LR (2004). The complex interplay among factors that influence allelic association. *Nat Rev Genet* 5: 89-100.

Zwingman TA, Neumann PE, Noebels JL and Herrup K (2001). Rocker is a new variant of the voltage-dependent calcium channel gene *Cacna1a*. *J Neurosci* 21: 1169-1178.