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Search for Susceptibility Genes in Schizophrenia

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A C A D E M I C D I S S E R T A T I O N

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ABSTRACT

Schizophrenia is a severe mental disorder affecting 0.4-1% of the population worldwide. It is characterized by impairments in the perception of reality and by significant social or occupational dysfunction. The disorder is one of the major contributors to the global burden of diseases. Studies of twins, families, and adopted children point to strong genetic components for schizophrenia, but environmental factors also play a role in the pathogenesis of disease.

Molecular genetic studies have identified several potential positional candidate genes. The strongest evidence for putative schizophrenia susceptibility loci relates to the genes encoding dysbindin (*DTNBP1*) and neuregulin (*NRG1*), but studies lack impressive consistency in the precise genetic regions and alleles implicated. We have studied the role of three potential candidate genes by genotyping 28 single nucleotide polymorphisms in the *DNTBP1*, *NRG1*, and *AKT1* genes in a large schizophrenia family sample consisting of 441 families with 865 affected individuals from Finland. Our results do not support a major role for these genes in the pathogenesis of schizophrenia in Finland.

We have previously identified a region on chromosome 5q21-34 as a susceptibility locus for schizophrenia in a Finnish family sample. Recently, two studies reported association between the γ-aminobutyric acid type A receptor cluster of genes in this region and one study showed suggestive evidence for association with another regional gene encoding clathrin interactor 1 (CLINT1, also called Epsin 4 and ENTH). To further address the significance of these genes under the linkage peak in the Finnish families, we genotyped SNPs of these genes, and observed statistically significant association of variants between GABRG2 and schizophrenia. Furthermore, these variants also seem to affect the functioning of the working memory.

Fetal events and obstetric complications are associated with schizophrenia. Rh incompatibility has been implicated as a risk factor for schizophrenia in several epidemiological studies. We conducted a family-based candidate-gene study that assessed the role of maternal-fetal genotype incompatibility at the RhD locus in schizophrenia. There was significant evidence for an RhD maternal-fetal genotype incompatibility, and the risk ratio was estimated at 2.3. This is the first candidate-

gene study to explicitly test for and provide evidence of a maternal-fetal genotype incompatibility mechanism in schizophrenia.

In conclusion, in this thesis we found evidence that one GABA receptor subunit, GABRG2, is significantly associated with schizophrenia. Furthermore, it also seems to affect to the functioning of the working memory. In addition, an RhD maternal-fetal genotype incompatibility increases the risk of schizophrenia by two-fold.

Keywords: Schizophrenia, genetic association analysis, GABA receptor, GABRG2, RhD, maternal-fetal incompatibility, DNTBP1, NRG1, AKT1, CLINT1

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TIIVISTELMÄ

Skitsofrenia on vakava mielenterveyden häiriö ja sitä esiintyy noin 0,4-1 % maailman väestöstä. Skitsofreniaa sairastavilla on ongelmia todellisuuden havainnoinnissa ja heidän sosiaalinen toimintansa on yleensä häiriintynyt. Perhe-, kaksois- ja adoptiotutkimusten perusteella skitsofrenialla on merkittävä perinnöllinen tausta, mutta ympäristötekijätkin vaikuttavat taudin syntyyn.

Olemme aikaisemmissa tutkimuksissa löytäneet kromosomista 5 alueen, joka kytkeytyi skitsofreniaan perhemateriaalissamme. Aineistomme koostui 441 perheestä käsittäen 865 skitsofreniaa sairastavaa ihmistä. Viime aikoina on tältä samalta alueelta löydetty useita geenejä, joiden variaatiot näyttäisivät altistavan skitsofrenialle. γ-aminovoihappo A-tyypin reseptoreiden alayksiköistä muodostuva geenirykelmä näyttäisi liittyvän suurentuneeseen riskiin sairastua tautiin. Halusimme tutkia näiden geenien roolia skitsofreniassa myös suomalaisessa väestössä analysoimalla niissä sijaisevia variaatioita. Havaitsimme, että *GABRG2* geenin variaatiot näyttäisivät liittyvän skitsofrenian sairastumisriskiin. Lisäksi nämä variaatiot näyttivät liittyvän myös työmuistin toimintaan. Skitsofreniaa sairastavilla on keskimäärin huonontuneet kongnitiiviset toiminnot, joten löydös voi tulevaisuudessa avata uusia mahdollisuuksia työmuistin toiminnan tutkimuksessa.

Viime aikaiset molkyyligeneettiset tutkimukset maailmalla ovat löytäneet useita lupaavia skitsofrenialle altistavia ehdokasgeenejä. Kaksi vahvinta ehdokasta ovat dysbindin (*DTNBP1*) ja neureguliini (*NRG1*), mutta tutkimukset eivät ole olleet aivan johdonmukaisia geenialueen ja alleelien suhteen. Tutkimme kolmen aikaisemmin skitsofreniaan yhdistetyn geenin (*DNTBP1*, *NRG1*, and *AKT1*) roolia suomalaisessa perhemateriaalissamme analysoimalla 28 yhden nukelotidin muutosta (SNP, single nucelotide polymorphism). Tulostemme valossa näiden kolmen geenin variaatiot eivät ole merkittäviä riskitekijöitä skitsofrenialle meidän väestössämme.

Raskauden aikaiset komplikaatiot lisäävät sikiön riskiä sairastua skitsofreniaan noin kaksinkertaiseksi myöhemmin elämässä. Rhesus -veriryhmän yhteenopimattomuus on epidemiologisissa tutkimuksissa yhdistetty skitsofreniaan. Tämän veriryhmän yhteensopimattomuus voi vakavammillaan johtaa sikiön pahaan hemolyyttiseen tautiin. Tutkimme tätä äidin ja sikiön RhD geenin yhteensopimattomuutta skitsofrenia perheaineistossamme. RhD geenin yhteensopimattomuus äidin ja sikiön

välillä nosti sikiön sairastumisriskiä 2,26-kertaiseksi meidän tutkimuksessamme. Tämä on maailmassa ensimmäin tutkimus, joka suoraan pystyi yhdistämään äidin ja sikiön yhteensopimattomuuden geenitasolla skitsofreniaan. On kuitenkin humioitava, että tutkimukseen osallistuneet henkilöt olivat syntyneet suurimmaksi osaksi ennen 70-lukua, joten anti-D-vasta-aineiden anto äidille ei ollut vakiintunut käytäntö Suomessa. RhD geenin yhteensopimattomuuden roolia skitsofrenialle altistavana tekijänä myöhemmin syntyneiden joukossa ei tiedetä.

Tässä väitöskirjassa löysimme viitteitä, että yhden GABA välittäjäaanereseptorin (*GABRG2*) variaatiot perimässä altistavat skitsofrenialle. Osoitimme myös, että RhD veriryhmän yhteensopimattomuus äidin ja sikiön välillä näyttäisi lisäävän sikiön riskiä sairastua myöhemmin skitsofrenialle.

Asiasanat: Skitsofrenia, geneettinen assosiaatio analyysi, GABA reseptori, GABRG2, RhD, DNTBP1, NRG1, AKT1, CLINT1

CONTENTS

ABBREVIATIONS

3AF	all Finland
CNV	copy-number variant
DNA	deoxyribonucleic acid
DNTBP1	dystrobrevin-binding protein dysbindin
DSM	Diagnostic and Statistical Manual of Mental Disorders
DZ	dizygotic
GABA	gamma-aminobutyric acid
HLA	human leukocyte antigen
HRR	haplotype-relative risk
ICD	International Classification of Diseases
IS	internal isolate
LC	liability class
LD	linkage disequilibrium
LOD	logarithm of odds
MFG	maternal-fetal genotype
MR	morbid risk
MZ	monozygotic
NA	not applicable
PCR	polymerase chain reaction
QTL	quantitative trait locus
Rh	rhesus
SNP	single nucleotide polymorphism
STR	short tandem repeats
TDT	transmission disequilibrium test
TRAX	translin-associated factor X
VCFS	Velo-cardia-facial syndrome
WAIS-R	Weschsler Adult Intelligence Scale – revised
WMS-R	Wechsler Memory Scale – revised

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Turunen JA*, Peltonen JO*, Pietilainen OP, Hennah W, Loukola A, Paunio T, Silander K, Ekelund J, Varilo T, Partonen T, Lönnqvist J, Peltonen L. The role of DTNBP1, NRG1, and AKT1 in the genetics of schizophrenia in Finland. Schizophr Res. 2007 Mar;91(1-3):27-36

II Turunen JA, Paunio T, Tuulio-Henriksson AM, Hennah W, Ekelund J, Varilo T, Ylisaukko-oja T, Partonen T, Loukola A, Silander K, Lönnqvist J, Peltonen L. Variants in the GABRG2 gene are associated with schizophrenia. *Submitted*

III Palmer CG, Turunen JA, Sinsheimer JS, Minassian S, Paunio T, Lonnqvist J, Peltonen L, Woodward JA. RHD maternal-fetal genotype incompatibility increases schizophrenia susceptibility. Am J Hum Genet. 2002 Dec;71(6):1312-9.

IV Minassian SL, Palmer CG, Turunen JA, Paunio T, Lönnqvist J, Peltonen L, Woodward JA, Sinsheimer JS. Incorporating serotypes into family based association studies using the MFG test. Ann Hum Genet. 2006 Jul;70(Pt 4):541-53.

*These authors contributed equally to this work

1 INTRODUCTION

Schizophrenia is a severe mental disorder characterized by impairments in the perception of reality and by significant social or occupational dysfunction. It is characterized by a constellation of features, including delusions, hallucinations, disorganized speech and behavior, flattened affect, and inability to initiate and persist in goal-directed activities. Schizophrenia has a lifetime prevalence of 0.4–1% worldwide (1-4). Studies of twins, families, and adopted children point to strong genetic components: for example, risk of the disease in a co-twin of an affected proband is 5-8 fold higher in monozygotic (46%) than in dizygotic (9%) twins (5-10). Several environmental risk factors have also been suggested for schizophrenia, among them obstetric complications (11-13).

The genetic mapping approach offers tools for identification of genetic components of diseases. Thus, it can eventually lead to better understanding of the biological basis of diseases, and ultimately to development of new treatments. The completion of the Human Genome Project and continuing cataloguing of variations within the human genome have offered effective tools for identification of the genetic risk factors for the disease.

The following review will focus on the existing knowledge of the genetic background of schizophrenia. In the thesis work, current knowledge of the human genome variations, the efficient genotyping techniques, and novel statistical methods have been utilized to enlighten the genetic background of schizophrenia.

2 REVIEW OF THE LITERATURE

2.1 Overview of schizophrenia

2.1.1 Diagnosis

Schizophrenia is a devastating psychiatric disorder affecting 0.4-1% of the population worldwide. The onset of the illness occurs typically in young adults, and leads to significant impairment in occupational and social functioning. The clinical picture of schizophrenia is heterogeneous, and no single symptom defines the disease. The disorder is equally prevalent in both sexes, but men tend to have slightly earlier onset, and a more severe course of the disorder. Even if the incidence of schizophrenia is relatively low, the disorder is one of the major contributors to the global burden of diseases (14).

Currently, two parallel diagnosis methods for schizophrenia exist: the Diagnostic and Statistical Manual for Mental Disorders, fourth edition (DSM-IV) (table 1), and the International Classification of Diseases, tenth edition (ICD-10). The methods are essentially similar, and lead to the same kind of diagnostic definition of the disease. In the scientific literature, the DSM-IV is more widely used, and in the current thesis all diagnoses are based on the DSM-IV criteria. Diagnosis is based on the self-reported experiences of the patient, in combination with secondary signs observed by psychiatrists, and documented information in patient records. No specific laboratory test for the disorder is available.

By definition, schizophrenia is characterized by psychotic symptoms, which include delusions, hallucinations and disorganized thought and behaviour. These symptoms are historically called “positive” symptoms. “Negative” symptoms, on the other hand, involve the absence of normal behaviours, and include affective flattening, lack of pleasure in everyday life, diminished ability to initiate and sustain planned activities, and social withdrawal. Patients should have continuous signs of the disturbance for at least 6 months. Symptoms have to be so severe that normal functioning in work, interpersonal relations, or self-care is greatly disturbed.

Although not directly included in the diagnostic criteria, schizophrenia is also characterized by cognitive deficits. Schizophrenia patients have severe problems with attention, memory, and executive functions. These deficits affect the functional capacity of the patients, and currently no treatment is available for definitely improving them. The studies have shown that the cognitive dysfunction observed in schizophrenia patients and their relatives is heritable. Thus, genetics can be used to study factors behind these traits.

Table 1. Diagnostic criteria for schizophrenia according to the DSM-IV (American Psychiatric Association 1994)

A. Characteristic symptoms: Two or more of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated):

1. Delusions
2. Hallucinations
3. Disorganized speech
4. Grossly disorganized or catatonic behaviour
5. Negative symptoms

Only one Criterion A symptom is required if delusions are bizarre or hallucinations consist of a voice keeping up a running commentary on the person's behaviour or thoughts, or two or more voices are conversing with each other.

B. Social/occupational dysfunction: for a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care are markedly below the level achieved prior to the onset (or, when the onset is in childhood or adolescence, failure to achieve the expected level).

C. Duration: Continuous signs of the disturbance persist for at least 6 months, of which at least one month should be of symptoms that meet Criterion A. The 6 months may include periods of prodromal and residual symptoms.

D. Schizoaffective and mood disorder exclusion: Schizoaffective disorder and mood disorder with psychotic features have been ruled out because either no major depressive, manic, or mixed episodes have occurred concurrently with the active-phase symptoms, or if mood episodes have occurred during active-phase symptoms, their total duration has been brief relative to the active and residual periods.

E. Substance/general medical condition exclusion: The disturbance is not due to the direct physiological effects of a substance or a general medical condition.

F. Relationship to a pervasive developmental disorder: if there is a history of autistic disorder or another pervasive developmental disorder, the additional diagnosis of schizophrenia is made only if prominent delusions or hallucinations are also present for at least a month (or less if successfully treated).

2.1.2 Epidemiology

Understanding the ‘‘epidemiological landscape’’ of schizophrenia requires many different types of descriptive studies in order to guide research to ultimately understand the etiology and pathology of the disease.

In a recently published systematic review the incidence of schizophrenia was found to be 15.2 persons per 100,000 per year with variation between geographical locations. Males were slightly more likely to develop schizophrenia than females, as were migrants compared to native-born individuals. Individuals in urban sites are at higher risk for the disease than those with mixed urban/rural sites (15). Also, individuals born in the winter and spring have a slightly increased risk of developing schizophrenia (16).

Prevalence measures the proportion of individuals who have a disorder at a specified time, or during a specified period. Lifetime prevalence is the proportion of individuals in the population who are alive on a given day and who have ever manifested a disorder, Saha and colleagues conducted a systematic review of the schizophrenia prevalence from 188 studies from 46 countries. They estimated that the median lifetime prevalence for schizophrenia is 4.0 per 1000 (3). The prevalence of schizophrenia did not vary between the sexes. The prevalence of the disease was higher in migrants than native-born individuals, and about 5% to 15% higher in people born during the winter than in those born at other times of the year (17).

The age of onset vary between men and women. Men tend to show the first signs of disease during their early 20s, women during their mid and late 20s. In the vast majority of schizophrenic people, onset occurs between the ages of 15 and 45.

2.1.3 Etiology and pathophysiology

2.1.3.1 Genetics of schizophrenia

Studies of twins, families, and adopted children point to strong genetic components for schizophrenia: for example, risk of the disease in a co-twin of an affected individual is higher in monozygotic (46%) than in dizygotic (9%) twins (5-10, 18, 19). Several candidate genes have been proposed in the recent years, but conclusive

evidence for any single gene is lacking. The genetics of schizophrenia is discussed in details in the section 2.3.

2.1.3.2 Environmental factors

There is now growing evidence that adult chronic diseases can be programmed prenatally or in early infancy (20, 21). Stressful situations during pregnancy have been linked to increased risk for schizophrenia. Several maternal infections during pregnancy have been associated with increased later risk for schizophrenia: influenza (22), other respiratory tract infections (23), polio (24), rubella (25), and herpes simplex virus (26). Furthermore, many other obstetric complications appear to increase the risk for schizophrenia. In a recent meta-analysis Cannon and colleagues (11) grouped the obstetric complications into three main categories: (1) complications of pregnancy (bleeding, diabetes, rhesus incompatibility, pre-eclampsia); (2) abnormal fetal growth and development (low birth weight, high birth weight, congenital malformations, reduced head circumference), and (3) complications of delivery (uterine atony, asphyxia, emergency caesarean section). The overall odds ratio of the obstetric complications is estimated to be 2.0 (95% confidence interval 1.5 - 2.4) (11, 27, 28). The individual risk factors for some of the most important obstetric risk factors are presented in table 2. It should be noted that obstetric complications are not specific, and these problems in the early life can also predispose to other chronic diseases. Nevertheless, the complications during pregnancy are a well-established risk factor for schizophrenia and should be taken into account in the future research.

Rhesus (Rh) incompatibility can cause of hemolytic disease of the fetus and newborn. Hemolytic disease results from the transplacentally transmitted maternal antibodies against Rh factor D and can cause permanent neurological damage in the affected newborn. Epidemiological study found that the rate of schizophrenia was significantly higher in the Rh-incompatible group compared with the Rh-compatible group (29), which indicates that Rh imcombatibility may be risk factor for schizophrenia.

People who later develop schizophrenia often appear to have more neuromotor, language and cognitive developmental deficits in the early childhood than control individuals. These individuals seem to have also social and emotional problems during childhood, but these may be a more general marker of risk for different kinds of psychiatric illnesses in adulthood (30). These developmental impairments have been thought to be genetically programmed, consistent with the hypothesis of schizophrenia as a neurodevelopmental disorder (31).

Use of cannabis is associated with a two-fold risk for later schizophrenia (32, 33), and the use of cannabis in the early adulthood is linked with even higher risk for later development of the disease. The connection between schizophrenia and cannabis is not direct, and these findings need to be studied in more detailed.

There have also been studies concerning the early rearing environment, which include whether or not the pregnancy was wanted (34), antenatal depression of the mother (35), atypical mother–infant interaction (11), poor mothering (35), and early parental loss (36), but evidence for these is rather weak and these results should be considered with caution.

Table 2. Meta-Analysis of Eight Prospective Population-Based Studies of the Association Between Obstetric Complications and Schizophrenia. Adapted from Cannon and colleagues (11).

Complication[a]	Odds ratio	95% CI	p-value
Diabetes in pregnancy	7.76	1.37-43.9	<0.03
Placental abruption	4.02	0.89-18.12	0.07
Birth weight <2000 g	3.89	1.40-10.84	0.009
Emergency Cesarean section	3.24	1.40-7.50	0.006
Congenital malformations	2.35	1.21-4.57	<0.02
Uterine atony	2.29	1.51-3.50	<0.001
Rhesus variables[b]	2.00	1.01-3.96	<0.05
Threatened premature delivery	1.98	0.79-4.90	0.14
Asphyxia	1.74	1.15-2.62	0.008
Bleeding in pregnancy	1.69	1.14-2.52	0.009
Birth weight <2500 g	1.67	1.22-2.29	0.002
Head circumference <32 cm	1.38	0.97-1.91	0.08
Smoking in pregnancy	1.38	0.88-2.14	0.16
Preeclampsia	1.36	0.99-1.85	0.05
Anemia in pregnancy	1.26	0.69-2.28	0.45
Gestational age <37 weeks	1.22	0.90-1.65	0.2
Small for gestational age	1.21	0.91-1.61	0.19
Induction of labor	1.18	0.89-1.56	0.25
Apgar score <7 at 1 minute after birth	1.09	0.62-1.92	0.76
Gestational age >42 weeks	1.08	0.69-1.68	0.72
Child stayed in hospital after mother discharged	1.07	0.79-1.44	0.65
Forceps delivery or vacuum extraction	1.07	0.85-1.35	0.48
Birth length <49 cm	1.06	0.86-1.31	0.59
Cephalopelvic disproportion	1.04	0.28-3.82	0.95
Cord around neck	1.03	0.81-1.31	0.83

[a]Complications presented in order of effect size.

[b]Includes rhesus incompatibility, rhesus-negative mother, and rhesus antibodies.

2.1.3.3 Structural and functional brain abnormalities

There are many kinds of macroscopic changes in the brains of schizophrenic individuals compared to the healthy individuals. The cerebral volume of the subjects with schizophrenia seems to be smaller, and the total ventricular volume of the subjects with schizophrenia is greater (37, 38). The following brain regions are most often implicated: the hippocampus (39, 40), the prefrontal and superior temporal neocortex (41, 42), and the thalamus (43). Several other brain abnormalities have been associated with schizophrenia; cortical thickness (44), cortical gyrification (45, 46), hippocampal shape (47, 48), and cerebral asymmetry (49, 50). However, none of these changes are specific for schizophrnenia.

The most repeated finding at the histological level is an abnormal distribution of neurons, especially interstitial white matter neurons, in the brains of the schizophrenics. Defects in the interstitial white matter neurons have been found in the dorsolateral prefrontal (51-53), middle and medial temporal (54) and inferior parietal (55) cortices. These kinds of abnormalities point to an early neurodevelopmental deficits in neuronal migration, survival, and connectivity (56). In the several studies, the cell bodies of pyramidal neurons in the hippocampus and neocortex were found to be smaller in brains of schizophrenic patients (57-61). Several other neuronal abnormalities have also been reported, but detailed description of them is beyond the scope of this review. An important finding is also that schizophrenia is not associated with an increased frequency of neurodegenerative disorders (62, 63).

Most of these differences in brain structure and histology are only detected when comparing groups of people, and are not always observed in all individuals with schizophrenia. In conclusion, schizophrenia is beyond doubt a brain disorder, but the disease does not have a clear neuropathological signature in the macroscopical or histological level.

2.2 Genetic mapping of complex diseases

2.2.1 Human genome

2.2.1.1 Structure of the human genome

The human genome, when referred to DNA content, consists of two genomes: a nuclear genome and a mitochondrial genome. The nuclear human genome contains a total of approximately 3 billion DNA base pairs in 24 distinct human chromosomes: 22 autosomal chromosomes, and the sex-determining X and Y chromosomes. The "finished" human genome sequence was announced in 2004, and it covered 99% of the euchromatic genome (64).

There are an estimated 20,000 protein-coding genes in the human genome. Genes make up of only about 2% of the human genome; the rest consists of noncoding regions, whose function is under an intense investigation. Comparative analyses of vertebrate sequences can identify coding and conserved non-coding regions, including regulatory elements (65, 66). Mammalian genomes contain highly conserved sequences that are not functionally transcribed, and these sequences comprise approximately 1–2% of the human genome (67). During evolution the human genome has accumulated a vast amount of repeated sequences that do not code for proteins (64, 68, 69). Repetitive sequences make up at least 50% of the human genome. Their role is not totally understood, but they are involved with chromosome structure and dynamics.

2.2.1.2 Variations in the human genome

Any two humans are approximately 99.9% identical in their DNA sequence (70, 71). Thus, the small fraction of DNA sequence that constitutes the genetic variation between individuals leads us to the roots of the disease susceptibility and the phenotypic variations. Historically, the first differences observed in the genome was aneuploidy, i.e. the presence of an abnormal number of chromosomes within a cell (72, 73). Later, larger rearrangements were observed using a microscope (74). These are usually larger than 3 Mb in size.

The sequencing of the human genome has made it possible to better catalogue the variations among humans. The most abundant variation type in the human genome

is a single nucleotide polymorphism (SNP). It has been estimated that there are at least 10 million SNPs within the human populations (75). The human genome also has various repetitive elements that involve relatively short DNA sequences (for example, micro- and minisatellites), and small (usually <1 kb) insertions, deletions, inversions and duplications. During the last years it has become evident that larger structural variations in the human genome are also relatively common (76, 77). These are usually referred to as a copy-number variants (CNV), which are segments of DNA that is 1 kb or larger and present at a variable number of copies in comparison with a reference genome. The structural genomic variants are likely as important as SNPs, short tandem repeats (STRs), and other small changes in their contribution to genetic variation in humans.

2.2.1.3 Epigenetics

Epigenetic modifications of the human genome are widely considered the important link between environment and the genome. The term 'epigenetics' accurately states 'above the genetics'. Nowadays, it is generally used to refer to changes in gene expression without changing of the DNA sequence. The most intensely studied epigenetic changes are DNA methylation and histone modifications (78, 79). Epigenetic changes can be inherited mitotically in somatic cells, and thus environmental effects on the genome can have long-term effects on gene expression in different tissues.

2.2.2 Linkage analysis

The general paradigm for disease gene mapping, so called positional cloning, has emerged for as an efficient application for single gene disorders. It consists of identifying families, collecting DNA samples, genome-wide genotyping markers, linkage analysis, fine genetic and physical mapping, and mutation identification. The same strategy has been applied to complex disorders with very limited success. Only a handful of truly believable “gene identification” has been published. The main reasons for unsuccessful studies are a lack of statistical power, difficult phenotypes, and the fact that the underlying genetic variants generally only have a low risk ratio.

Before starting genetic mapping, it should be evident that the disease has a genetic basis. It is not sufficient that the trait “runs in families”, rather there should also be data also from twin and adoption studies.

In this section I shortly review the linkage analysis, because it has been for years the main starting point for genetic studies of complex diseases. It should be mentioned that nowadays the genome-wide association studies are technically feasible, and even more cost-effective than linkage studies with multisatellite markers.

Traditionally, the most common markers used for genome-wide studies are multisatellites (short tandem repeats, STR), which are di-, tri-, or tetranucleotide repeats (80, 81). Today, linkage studies, if performed, are usually done with SNPs. The advantage is that several highly automated high-throughput methods have been developed for SNP genotyping (82).

Genetic linkage analysis refers to the ordering of genetic loci on a chromosome and then estimation of the genetic distances among them. The distances are determined on the basis of statistical findings. In linkage analysis one tests the hypothesis that a certain chromosomal region is transmitted to affected individuals in a non-random fashion. Linkage tests the recombination fraction (θ) between two loci, usually a marker and a disease locus. It measures if there is statistically significant deviation from free recombination between the disease and the marker loci. The linkage analysis is performed in families having multiple affected individuals, and the statistical analyses are used to detect if a particular marker seem to segregate with the disease at a greater frequency than predicted by chance. The statistical significance is traditionally measured using a logarithm of odds (LOD) score (83). In practice, linkage is assumed, if the LOD score is equal to or greater than 3, which means that the likelihood of observing the result if the two loci are not linked is less than 1 in 1000).

2.2.3 Association analysis

The basic principle of association studies in genetics is simple: we merely need to identify alleles that systematically and repeatedly differ between affected and control individuals. Allelic association is a nonparametric approach to mapping disease genes. The power of association analyses in detectecting genetic contributions to complex diseases can be much greater than that of linkage studies (84). Allelic association of the variant can be explained by direct biological action of the polymorphisms (i.e. causative variant), or by linkage disequilibrium (LD) with the nearby susceptibility variant. Linkage disequilibrium occurs when a particular marker allele lies so close to the disease susceptibility allele that these alleles will be inherited together over many generations. Therefore, the same allele will be detected in affected individuals in multiple apparently unrelated individuals or families.

2.2.3.1 Association analysis with dichotomised traits

Association analysis can be conducted with the individual markers (usually with SNPs or multiallelic markers) or a combination of them, i.e. haplotypes. The simplest way to perform association analysis is to utilize case-control samples, but today it is also possible to perform analyses in family material. Association studies are generally considered more powerful than linkage studies when applied to complex traits having many susceptibility variants with low risk rations. Still, there are a number of potential pitfalls and limitations of association studies (85), many of which depend on the particular design, study aims and analytical framework used (86). Common errors in performing association studies include small sample size, subgroup analysis and multiple testing, random error, a poorly matched control group, overinterpretation of results, and positive publication bias.

Two types of basic of settings for association analyses are case-control and family study. In basic case-control studies, a sample of affected individuals and a sample of well-matched unrelated controls are studied. The case-control study designs have been criticized for their potential spurious association owing to population stratification. Population stratification can occur when individuals are selected from two genetically different populations in different proportions in cases and controls, and thus the cases and controls are not matched for their genetic background (87).

To avoid the problem of selecting controls, family-based association studies were developed. In the classical haplotype-relative risk (HRR) method, parental alleles are classified into those transmitted to affected children and those not transmitted. It compares allele frequency between the proband group and a control group, which is constructed from non-transmitted alleles (83, 88). The transmission disequilibrium test (TDT) also utilizes the same kind of control group, but by extracting data only from heterozygous parents (89, 90). A variety of extensions of TDT are currently available for overcoming the problems caused by missing or homozygous parental data, eliminating the effect of linkage when multiple siblings are present in a family (91-94).

When testing for association, it is common to genotype multiple SNPs within the gene or gene regions. Testing each SNP separately leads to issues with multiple testing, and is not efficient when the SNPs are in high LD. One alternative is to use haplotypes rather than individual SNPs. Constructing haplotypes from individual SNPs can provide stronger evidence of a region being associated with the disease phenotype than can the use of individual markers. The Human Genome Project has produced an enormous amount of data on the haplotype structure of the human genome, which can now be utilized to better construct the haplotypes.

2.2.3.2 Association analysis with quantitative traits

Both linkage and association analysis programs traditionally use dichotomized traits which classify individuals in the analysis as either affected or unaffected. Recently, it has been made possible to also analyze traits that are continuous by nature. The quantitative trait locus (QTL) is any locus that contributes to a phenotype that is measured quantitatively. Currently, it is possible to perform linkage and association analyses with quantitative traits. It has been speculated that using quantitative traits would increase the power to detect linkage and association, because these traits may better capture the genetic nature of the examined trait. The classic example of such a trait is height. Variables can also be collected from tests of neurocognitive functions, which are deficient in individuals with schizophrenia and their relatives when compared to the general population (95). However, lessons from animal studies have shown that the number of loci, their relative effects, and how they interact with each other and the environment might vary considerably between phenotypes, indicating that some phenotypes might be better suited to QTL cloning than others (96).

2.3 Genetics of schizophrenia

2.3.1 Establishing the genetic component in schizophrenia

Over many years a large body of data has been collected demonstrating that schizophrenia and the schizophrenia spectrum disorders have a major genetic component. It is also evident that the genetic basis of psychoses is not simple, i.e. there is no single gene causing schizophrenia, and that the genetic susceptibility to the disease is likely to involve several predisposing variants in the genome.

Most studies of schizophrenia show that it runs in families. Twin studies of schizophrenia have shown consistently higher concordance in monozygotic (MZ, ~46%) than dizygotic (DZ, ~9%) twins (10). Meta-analyses of twin studies have estimated the heritability of schizophrenia to be approximately 80% (97). In the adoption studies, an increased risk of schizophrenia was present in the biological relatives of individuals with schizophrenia (98). The lifetime morbidity risks (MR) for relatives of schizophrenic patients are shown in table 3. Because twins also share prenatal and postnatal environments, the effect of genetics tends to be overestimated. However, across all adoption studies performed, increased risk of schizophrenia was present in the biological relatives of individuals with

schizophrenia (98). These findings indicate that schizophrenia have genetic basis, but it can not be explained by a single gene, rather of combination many risk genes.

Schizophrenia is genetically mediated disease, but the contribution of the genetic component of it seems to be broader. In general, it seems that risk for all psychotic spectrum disorders (i.e. schizoaffective, schziophreniform, delusional, paranoid personality and schizotypal personality disorders) is increased in the relatives of schizophrenic individuals (99). Relatives of schizophrenic patients are probably not at increased risk for anxiety or alcohol and drug dependence disorders (100), but evidence concerning bipolar disorder is not totally clear: bipolar disorder and schizophrenia might have shared predisposing genetic factors (101).

In conclusion, there is plenty of evidence that schizophrenia is at least genetically mediated although not necessarily genetically determined, making it reasonable to perform genetic studies on the disease.

Table 3. Morbid risk of schizophrenia for relatives of schizophrenic patients
(adapted from Tsuang 2000 (10))

Relationship	Shared genes (%)	Risk (%)
General population	N.A.	1
Spouses of patients	N.A.	2
Third-degree relatives	12.5	
First cousins		2
Second degree relatives	25	
Uncles/aunts		2
Nieces/nephews		4
Grandchildren		5
Half-Siblings		6
First-degree relatives	50	
Parents		6
Siblings		9
Children		13
Siblings with 1 schizophrenic parent		17
Dizygotic twin		17
Monozygotic twin	100	48
Children with 2 schizophrenic parents	100	46

2.3.2 Previous genetic findings for schizophrenia

2.3.2.1 Chromosomal abnormalities

Individuals having Velo-cardia-facial syndrome (VCFS) carry a deletion in the chromosome 22q11 and have an increased risk for psychosis. Recent studies have suggested that about 25% of patients develop psychoses (102). Several studies have also demonstrated an increased prevalence of chromosome 22q11 deletions in people with schizophrenia (103-105). Pooled analyses from these studies suggested that 0.65% of people with schizophrenia had deletions at chromosome 22q11 (105). When samples were restricted to childhood-onset schizophrenia, 5.3% of patients carried the 22q11 deletion (106). The causative genes in this region remains unidentified, but three candidate genes are intensively studied: catechol-*O*-methyl transferase (*COMT*), proline dehydrogenase (*PRODH2*), and *ZDHHC8*. More discussion on this is found in the candidate genes section.

A large multi-generational Scottish pedigree carries the chromosome 1 breakpoint of a balanced t(1;11) translocation that co-segregates with schizophrenia and related mood disorders (107, 108). The translocation disrupts three genes, the disrupted in schizophrenia 1 and 2 (*DISC1*, *DISC2*), as well as translin-associated factor X (*TRAX*). The *DISC1* gene is the most intensely studied, and there is now growing evidence that this gene is involved in schizophrenia and other psychiatric diseases (108, 109). See the candidate genes section for more discussion. Furthermore, the gene encoding phosphodiesterase 4B (PDE4B) interacting with DISC1 was reported to be disrupted by a balanced translocation in a subject diagnosed with schizophrenia and a relative with chronic psychiatric illness (110).

2.3.2.2 Linkage studies

Genetic linkage studies of schizophrenia have been carried out for more than two decades. The findings are inconsistent, and loci for predisposing genes for schizophrenia have been reported in almost all chromosomes (111). I omit a more detailed review of the linkage studies in this thesis, because evidence from linkage studies has not been very fruitful. Some linkage studies are mentioned in the following section, if the proposed candidate genes are supposed to locate under previously identified linkage peaks.

2.3.2.3 Candidate genes

Schizophrenia—like most other complex traits in biomedicine—has been the subject of a number of genetic association studies (112, 113). A schizophrenia gene database (SchizophreniaGene) of the Schizophrenia Research Forum has already included more than 1,500 association studies which include almost 500 different genes (in April of year 2007). Hard evidence for any gene is still missing, but reports supporting the role of many proposed genes have appeared in respected journals known for rigorous peer review. Table 4 lists some promising candidate genes predisposing to schizophrenia. Below is a review of those candidate genes with most potential in playing a role in the molecular pathogenesis of schizophrenia.

Studies of a Scottish pedigree with individuals with the balanced translocation between chromosomes 1 and 11 identified that the *DISC1* and *DISC2* genes are disrupted in chromosome 1 (114). There is as yet nothing more concrete to say about any possible impact of *DISC2* on the psychiatric phenotype, but DISC1 is intensively studied. Several family studies, including two Finnish studies (115-120), have reported suggestive linkage between psychiatric illness and this 1q42 region using broad phenotype models. In our sample Hennah et al. reported association between haplotypes and schizophrenia in *DISC1* (121). Several studies have also demonstrated association between *DISC1* and schizophrenia, but inconsistency exists between markers, haplotypes, and the gene region (122-124). DISC1 is widely expressed, and seems to have a role in cytoskeletal regulation, and thus may affect neuronal migration, neurite outgrowth and intracellular transport (125, 126). A recent study demonstrated that mice with Disc1 missense mutations exhibited physiological, pharmacological, neuroanatomical, and behavioural features similar to schizophrenia phenotype. Futher these features could be reversed by antipsychotic treatment (127).

The *COMT* gene works in the synthesis and degradation of catecholamines, and functional polymorphism (Val158Met) in the gene is under marked scrutiny for its role in schizophrenia. It is a functionally relevant candidate gene, and is located at the 22q11 deletion region of VCFS syndrome. Three meta-analyses of the association studies have been published with mixed results. Generally, the results have been negative (128, 129), but in one study COMT was associated with schizophrenia in European individuals (130).

The deCODE Genetics group identified several risk haplotypes for schizophrenia around the neuregulin 1 (*NRG1*) gene on a linkage region they had identified on 8p (131) in Icelandic families. The findings have been replicated (132-137), although contradictory reports also exist (138-141), and there are inconsistencies concerning the associated markers, risk alleles, and haplotypes in both genes. The specific risk alleles and pathogenic mechanisms are unknown. NRG1 is expressed in synapses in

the brain and appears to be involved with expression and activation of neurotransmitter receptors (142).

Several studies have found evidence for linkage of schizophrenia on 6p24-22 (143). Follow-up of the linkage peak in the Irish family set demonstrated a positive association in the dystrobrevin binding protein 1 or dysbindin (*DTNBP1*) gene (144). The association in this gene region has been replicated in several independent populations, but also? negative studies exist (145-150). The causative variant remains unidentified, and associated alleles and haplotypes have not always been macthing in all studies. The function of *DTNBP1* in the brain is largely unknown. Expression of *DTNBP1* is reduced in certain brain regions of schizophrenic patients at both RNA and protein levels (151, 152). Reduced protein expression is associated with additional changes consistent with a role in glutamatergic neurotransmission. The glutamatergic system is of great interest for schizophrenia liability. Overexpression of DTNBP1 is associated with increasedphosphorylation and activity of AKT1 in neuronal culture, suggesting that DTNBP1 also interacts with the AKT signaling pathway, which mediates cell survival (153). AKT1 is recently also associated with schizophrenia.

The G-protein signaling 4 (*RGS4*) gene maps to the 1q21–q22 linkage region. Microarray studies of post-mortem brain samples of schizophrenic patients found altered expression of *RGS4* in schizophrenia (154). A study of US and Indian pedigrees showed association with this gene (155). One replication study supported the *RGS4* association with schizophrenia liability (156), whereas others have not (157, 158).

Emamian and colleagues found evidence for the involvement of the *AKT* signaling pathway in the pathogenesis of schizophrenia (159). In addition to impairment of protein levels and phosphorylation patterns in the pathway, they detected association of v-akt murine thymoma viral oncogene homolog 1 (*AKT1*) SNPs and their corresponding haplotypes with the disease. Findings from this gene region have been replicated (160, 161), but a negative report also exists (162).

The chromosome 13q14-32 linkage region harbors the D-amino acid oxidase activator (*DAOA* or *G72*) and *G30* genes, which have been reported to be associated with schizophrenia (163). Evidence for epistasis was also observed for one pair of *G30* and *G72* genotypes, supporting a potential interaction between them in the risk for schizophrenia. There are now positive and negative reports on the role of these genes in schizophrenia (164-171).

Several lines of evidence indicate that disturbances in γ-aminobutyric acid (GABA) neurotransmission may contribute to the development of psychiatric disorders, in particular schizophrenia (172-174). Among different receptor subunit combinations, the α1/β2/γ2-containing heteropentamer is the most abundant GABA-A receptor subtype in mammalian brains (175), and the corresponding genes - the *GABRA1*,

GABRB2 and *GABRG2* genes - are located in the chromosomal region 5q34. This region have been linked in several genome-wide scans to schizophrenia, including ours (176-182). The study conducted with Portuguese and German families reported association between markers of three cluster genes, *GABRA1*, *GABRP*, and *GABRA6*, and schizophrenia (183). The study with Han Chinese detected association between *GABRB2* and schizophrenia (184), and the observation was replicated in a family-based study of Chinese origin (185), but at least one negative report also exists in a Japanese schizophrenia sample (186).

In conclusion, none of the genes listed above is proven to be "the schizophrenia gene". Nowadays, the *DISC1, DTNBP1, NRG1* genes are the most promising, but further studies are needed.

Table 4. Candidate susceptibility genes for schizophrenia.

Gene	Location
5HT-2A receptor	13q14
AKT1	14q32
CHRNA7	15q13
CLINT1 (Epsin4)	5q33
COMT	22q11
DAOA (G72)	13q33
DISC1	1q42
DTNBP1	6p22
G30	13q33
GABA-A receptors	5q34
GRM3	7q21
NDE1	16p13
NRG1	8p12
PPP2CC	8p21
PRODH	22q11
RELN	7q22
RGS4	1q23
TRAR4	6q23
ZDHHC8	22q11

3 AIMS OF THE STUDY

The aim of the present study was to investigate the genetic basis of schizophrenia and schizophrenia spectrum disorders in Finland by addressing the following specific aims:

- To examine the role of the common variants in the three previously associated schizophrenia candidate genes, *DTNBP1*, *NRG1*, and *AKT1* in the Finnish family sample (I)
- To investigate the regional candidate genes on chromosome 5q31-34, previously linked to schizophrenia in the Finnish family sample. The analysed genes were five GABA-A receptor genes (*GABRA1*, *GABRA6*, *GABRB2*, *GABRG2*, and *GABRP*) and the Epsin 4 gene (*ENTH*) (II)
- To explore the role of the maternal-fetal genotype incompatibility at the *RhD* locus in the susceptibilty of schizophrenia, and to develop statistical methods to enable these analyses (III, IV)

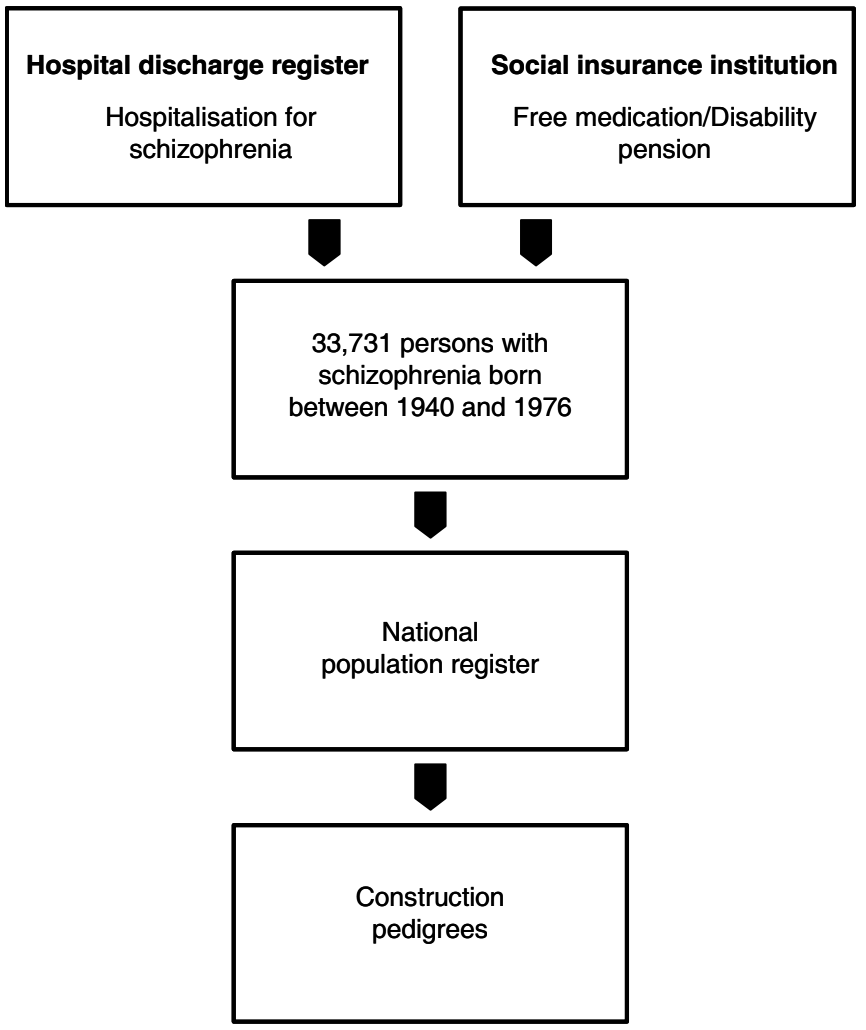
4 SUBJECTS AND METHODS

4.1 Study subjects

4.1.1 Schizophrenia family sample

The Finnish schizophrenia study sample used here has been collected as part of a larger project that has been carried out in Finland from the beginning of the late 1980s. Briefly, it includes individuals with schizophrenia born between 1940 and 1976 who had a diagnosis of schizophrenia spectrum disorders at any time point between 1969 and 1991. Probands were contacted by their treating physician, with additional family members contacted only if the proband provided written informed consent. The research was approved by the Ministry of Social Affairs and Health (Finland) and the appropriate institutional review boards, and informed consent was obtained from the study subjects. Two psychiatrists or psychiatric residents made independent DSM-IV best-estimate lifetime diagnoses from all available inpatient and outpatient records for probands and their relatives. The schematic of the methods used for identifying probands is presented in figure 1.

Figure 1. Flow-chart describing the identification of subjects for the schizophrenia study from the registers.



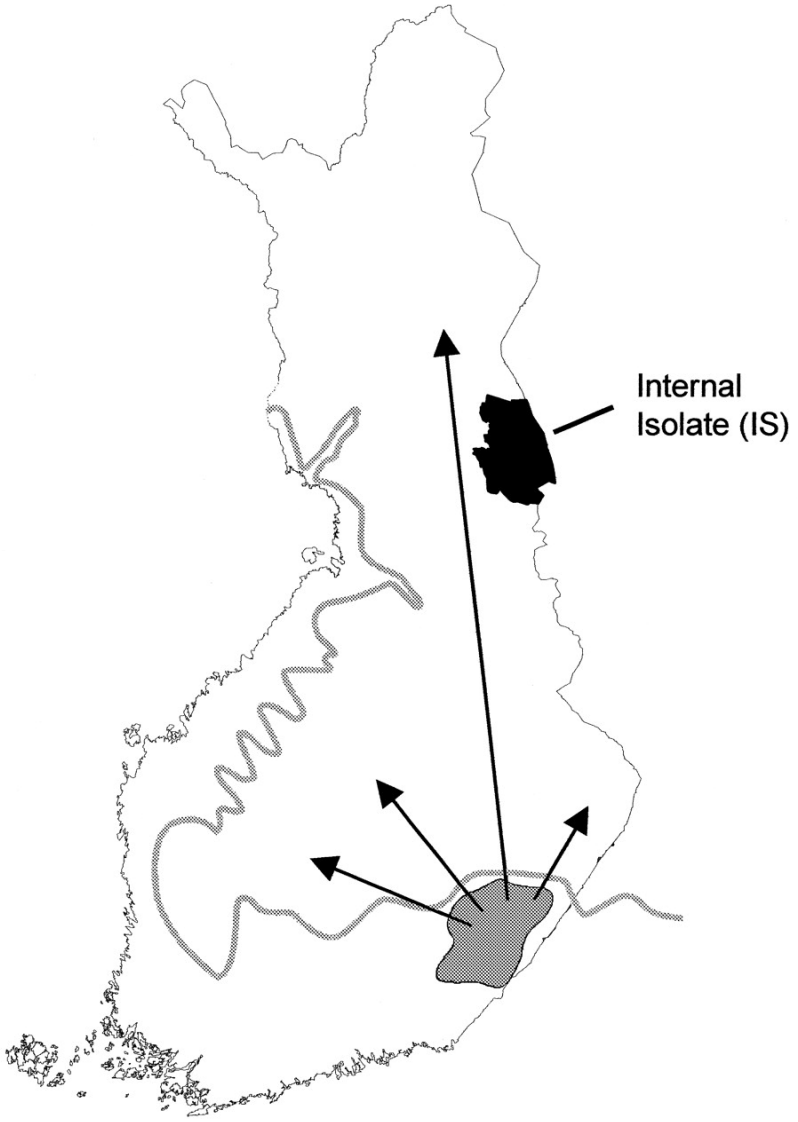
4.1.1.1 Sample used in candidate gene studies (I, II)

The complete study sample for the candidate gene analysis included 441 nuclear families consisting of 2,605 individuals, of which DNA was available for 1,864 individuals. For analysis in this study we used only two of the liability classes, LC1, which represents core schizophrenia, and LC3, which is representative of the schizophrenia spectrum disorders, enlisted in 4.1.2. Of the 865 affected individuals, 638 (74 %) and 865 (100 %) fulfilled the criteria for LC1 and LC3, respectively. 137 out of the 441 nuclear families had one affected individual according to LC3, 214 had two affected individuals, 67 had 3, 18 had 6, 4 had 5, and one family had seven, respectively. Of the affected individuals, 525 were males and 340 were females (LC3). There are slight differences in the numbers of the individuals in two papers (I, II) because of the difference in genotyping platforms used, but basically the sample is the same.

In the first study (I), we divided the study sample into two geographical sub-samples, one from a young internal isolate (IS) (181 families) and the other derived from the rest of Finland (AF) (268 families) (Figure 2). This division was based on the settlement history of Finland. The Finnish inhabitation of the internal isolate is well documented as beginning with the first immigrant in 1676, owing to legal records documenting inevitable conflicts with the native forager population, the Saami. About half of the immigrants of the region perished in the great famine of 1695–1697, which also resulted in the gradual disappearance of the Saami people from the district. When the parish registers were established in 1718, the population in the 165 houses consisted of 615 individuals belonging to 39 families. During the subsequent century, when diseases repeatedly swept through more densely populated parts of Europe, population growth was rapid in rural parts of Finland; this expansion eventually made possible the growth of the internal isolate to its present population of over 18 000 (Figure 3).

Figure 2. The sample used in the studies (I, II).

Figure 3. The settlement of the internal isolate utilized in the study. The inhabitation of the wilderness began in the 1500s in a small southeastern area of South Savo (shaded gray) to the central, western and finally northern parts of the country. Kuusamo was inhabited towards the end of this internal migration movement mainly by families from Ostrobothnia and from South Kainuu; both groups have their ancestral roots in South Savo.



4.1.1.2 Sample used in maternal-fetal incompatibility in the RhD locus studies (III, IV)

The study sample consists of the sub-sample of the previously described sample. The sample includes 181 trios with 450 individuals, and was composed of 88 patient-parent trios, 72 patient-mother pairs, and 21 patient-father pairs. Of the 181 patients, 112 were male (62%), 147 were second- or subsequently-born (81%), 145 had received a diagnosis of schizophrenia (80%), 25 had received a diagnosis of schizoaffective psychosis disorder (14%), and 11 had received a schizophrenia spectrum diagnosis (6%). Approximately 12% of mothers and 6% of fathers fell into one of those three diagnostic categories. Patients were born during the period 1937–1973, with a median birth year of 1957. Prophylaxis against maternal isoimmunization was started after 1969, and thus was not available for the vast majority of the patients in the study since only three patients were born after it (187).

4.1.2 Clinical diagnosis

All available inpatient and outpatient records were collected for probands and relatives with any psychiatric diagnosis in any of the three registers. Two independent psychiatrists made Diagnostic and Statistical Manual of Mental Disorder, Fourth Edition (DSM-IV), (American Psychiatric Association 1994) best-estimate lifetime diagnoses. If these two psychiatrists provided conflicting diagnoses, a consensus was obtained using a third reviewer. Owing to the difficulty in determining whether an individual is truly unaffected, all unaffecteds were given a disease status of unknown. The register diagnosis of schizophrenia has been shown, in several studies, to have a high reliability. Agreement between different psychiatrists on lifetime diagnosis has been shown to be good using the method described above (5, 188-190).

Affected individuals were divided into three increasingly inclusive liability classes (LC) according to the consensus diagnoses. The narrowest class was LC1, consisting only of individuals with core schizophrenia. LC2 added individuals with schizoaffective disorder, and LC3 added those with schizophrenia spectrum conditions (schizoid, schizotypal and paranoid personality disorders, schizophreniform, delusional and brief psychotic disorders, and psychotic disorder not otherwise specified). The complete study sample included 441 nuclear families consisting of 2,605 individuals, of which DNA was available for 1,864 individuals. For analysis we used only two of the liability classes, LC1 which represents core schizophrenia and LC3 which is representative of the schizophrenia spectrum that

are most likely to have similar underlying genetic variants (191). Of the 865 affected individuals, 638 (74 %) and 865 (100 %) fulfilled the criteria for LC1 and LC3, respectively. 137 out of the 441 nuclear families had one affected individual according to LC3, 214 had two affected individuals, 67 had 3, 18 had 6, 4 had 5, and one family had seven, respectively. Of the affected individuals, 525 were males and 340 were females (LC3).

4.1.3 Neurocognitive variables

A neuropsychological test battery was administered to 746 subjects in a fixed order. The test battery is a series of tests that uses well validated, internationally used neuropsychological instruments to evaluate an individual's cognitive ability. These tests have been shown to fullfil most of the criteria of endophenotypes for schizophrenia (192-194). Shortly, following criteria are proposed for endophenotypes: 1) the endophenotype is associated with illness in the population, 2) the endophenotype is significantly heritable, 3) the endophenotype is present in individuals with and without an active phase of the illness, 4) in families with the illness, also the unaffected relatives have the same endophenotypic trait, and 5) the endophenotype that is present in the affecteds, is more prevalent in the unaffecteds in the family than in general population (195). Test includes auditory verbal attention and verbal working memory which measured with the Digit Span forward and backward tests from the Wechsler Memory Scale – Revised (WMS-R), respectively (196). Visual attention and working memory were measured with the Visual Span forward and backward tests, respectively, from the WMS-R. The California Verbal Learning Test (CVLT) (197) was used as a test for verbal learning and memory. From the CVLT, we included the following variables: total recall from trials 1-5 (learning), semantic clustering as a learning strategy, perseverative and intrusive recall errors, and recognition memory. In addition, the interference score of the Stroop Task (197) was used as a measure of executive functioning. Basic cognitive ability was examined with the Vocabulary subtest from the Wechsler Adult Intelligence Scale-Revised (WAIS-R) (196).

4.2 Laboratory methods and statistical analysis

Methods used in this study have been described in detail in the original articles (I-IV) and are listed table 5. The methods used were generally common in the field of the human genetics. The maternal-fetal-genotype test was developed for this study.

Table 5. Methods used in the present study

Method	Original publication
Laboratory procedures	
DNA extraction	I, II, III, IV
Polymerase Chain Reaction (PCR)	I, II, III, IV
Agarose gel electrophoresis	I, II, III, IV
Restriction enzymes	III, IV
Sequenom MALDI-TOF mass spectrometry	I, II
TaqMan with an ABI Prism 7900	II
Statistical methods	
Pedcheck 1.1	I, II, III, IV
Downfreq 2.1	I, II
MLINK/LINKAGE	I, II
Homog 3.35	I, II
Analyze	I, II
FBAT	I, II
Haploview	I, II
QTDT	II
Simwalk 2.81	II
TDT	III, IV
Mendel 4.0	III, IV
MFG test	III, IV

5 RESULTS AND DISCUSSION

5.1 Association analysis of three candidate genes (I)

After years of candidate gene studies with inadequate sample sizes, and without consistent replications, two genes were reported to associate with schizophrenia with rather convincing data in the year 2002. The dystrobrevin binding protein 1 or dysbindin (*DTNBP1*) gene was located under the linkage peak on chromosome 6p24-22 (198). The deCODE Genetics group form Iceland found association to neuregulin 1 (*NRG1*) gene on a linkage region on 8p (131). After initial studies several replication attempts have been carried out with somewhat contradictory results.

The majority of the studies for both genes have found evidence of association with at least a couple of markers in the same gene regions, but no single high-risk haplotype is associated across all samples. The general rules for replications studies are still unspecified by the scientific community, and it is unclear whether we should expect to observe association with the same specific haplotype, or only with the gene, across samples. For common disorders such as schizophrenia, it is crucial to try to replicate the findings of others so that we can evaluate the validity of the proposed susceptibility genes. Further, the genetic studies are not sufficient, and also functional evidence is needed to support the role of certain variants in the gene.

We wanted to study the role of these two genes in the background of schizophrenia in Finland in our relatively large family material and possibly find support that *NRG1* and *DTNBP1* could be genetic risk factors in our population. Later in 2004 Emamian and colleagues reported in *Nature Genetics* that the AKT signaling pathway is possibly involved in the pathogenesis of the disease (159). The variants in the v-akt murine thymoma viral oncogene homolog 1 (*AKT1*) were associated with schizophrenia. Because our assay design was at a stage where new SNPs could still be added, we also included the SNPs from the AKT1 gene.

We studied 15 SNPs from *DTNBP1*, 10 form *NRG1*, and 3 from *AKT1*. The coverage of the most common haplotypes with these SNPs in *DTNBP1* was relatively good, and in addition, eight SNPs of our study were also included in the original article published by Straub and colleagues (144). We ended up with only 10 SNPs in *NRG1*, most likely not capturing all of the common allelic diversity. *NRG1* is a very large gene spanning approximately 1.4 Mb, and it has more than 20 exons giving rise to at least 15 isoforms. Nevertheless, we had two of the SNPs from the originally associated Icelandic haplotypes (131), and quite a few from the previously associated 3´end of the gene region (133, 136, 199, 200). *AKT1* is a very small gene, and with 3 SNPs we acquired enough information to cover common haplotypes from

the gene region. The LD diagrams and the location of studied SNPs in relation of previous findings are presented in the first article.

We did not detect any association with *DTNBP1* or with *AKT1* to DSM-IV-based diagnosis of schizophrenia or schizophrenia spectrum disorder. We found suggestive evidence of association in the 3′end of *NRG1*, but these results are not significant after correction for multiple testing. Haplotype analyses yielded no further evidence for association. The modest association seems to come from the All Finland sub-sample, since no association was detected in the Isolate sub-sample. The exact p-values are presented in the table 6, which is also published in the article. It is noteworthy, that there is a suggestive evidence of association between the core schizophrenia and *DTNBP1* when performing mixture model clustering of extensive clinical and neurobehavioural data collected from schizophrenia families to produce novel subclasses in a non-supervised manner (unpublished data).

Table 6. Association results of *DTNBP1* , AKT1, and NRG1 for the whole study sample and the two sub-samples: isolate (IS) and the rest of Finland (AF). (Original publication I).

		Combined		AF		IS	
Gene	Marker	LC1	LC3	LC1	LC3	LC1	LC3
DTNBP1	D6S1721	0.363	0.637	0.129	0.526	0.872	0.908
	rs1047631	0.853	0.487	0.558	0.434	0.483	0.935
	rs742106	0.19	0.306	0.263	0.386	0.62	0.647
	rs875462	0.374	0.37	0.353	0.289	0.815	0.972
	rs1040410	0.417	0.269	0.91	0.838	0.109	0.08
	rs742105	0.726	0.575	0.562	0.323	0.659	0.505
	rs760666	0.852	0.632	0.936	0.727	0.832	0.733
	rs2619539	0.833	0.429	0.668	0.378	0.851	0.975
	rs2743867	0.541	0.896	0.188	0.255	0.355	0.12
	rs1011313	0.436	0.546	0.076	0.053	0.295	0.092
	rs2619528	0.473	0.347	0.872	0.896	0.189	0.047
	rs2619522	0.22	0.086	0.558	0.419	0.135	0.024
	rs2743854	0.435	0.238	0.912	0.751	0.219	0.085
	rs1997679	0.647	0.678	0.332	0.282	0.531	0.395
	rs909706	0.67	0.573	0.546	0.208	0.698	0.258
	rs2769561	0.219	0.204	0.636	0.632	0.115	0.078
	D6S285	0.052	**0.01**	**0.047**	**0.016**	0.553	0.652
NRG1	D8S1820	0.398	0.4	0.358	0.469	0.895	0.832
	SNP8NRG221132	0.414	0.698	0.315	0.241	0.918	0.224
	rs6994992	0.268	0.165	0.323	0.141	0.684	0.776
	rs1503487	**0.046**	0.331	**0.045**	0.246	0.763	0.929
	rs1023911	0.16	0.187	**0.027**	**0.05**	0.354	0.502
	rs1481747	0.481	0.788	0.892	0.665	0.317	0.754
	D8S1477	0.065	0.216	0.403	0.613	0.209	0.431
	rs3924999	**0.026**	**0.048**	**0.035**	**0.04**	0.359	0.497
	rs3808368	0.838	0.979	0.995	0.581	1	0.448
	rs764059	**0.019**	**0.012**	**0.03**	**0.012**	0.36	0.485
	rs2919378	0.093	**0.029**	0.239	**0.019**	0.212	0.588
	rs3735782	**0.021**	0.093	0.101	0.138	0.101	0.42
	D8S1110	0.309	0.191	0.42	0.28	0.865	0.808
AKT1	D14S260	0.582	0.769	0.611	0.549	0.731	0.564
	rs2494732	0.612	0.293	0.615	0.389	0.879	0.547
	rs2498799	0.462	0.699	0.118	0.172	0.296	0.264
	rs1130214	0.52	0.414	0.258	0.106	0.44	0.178

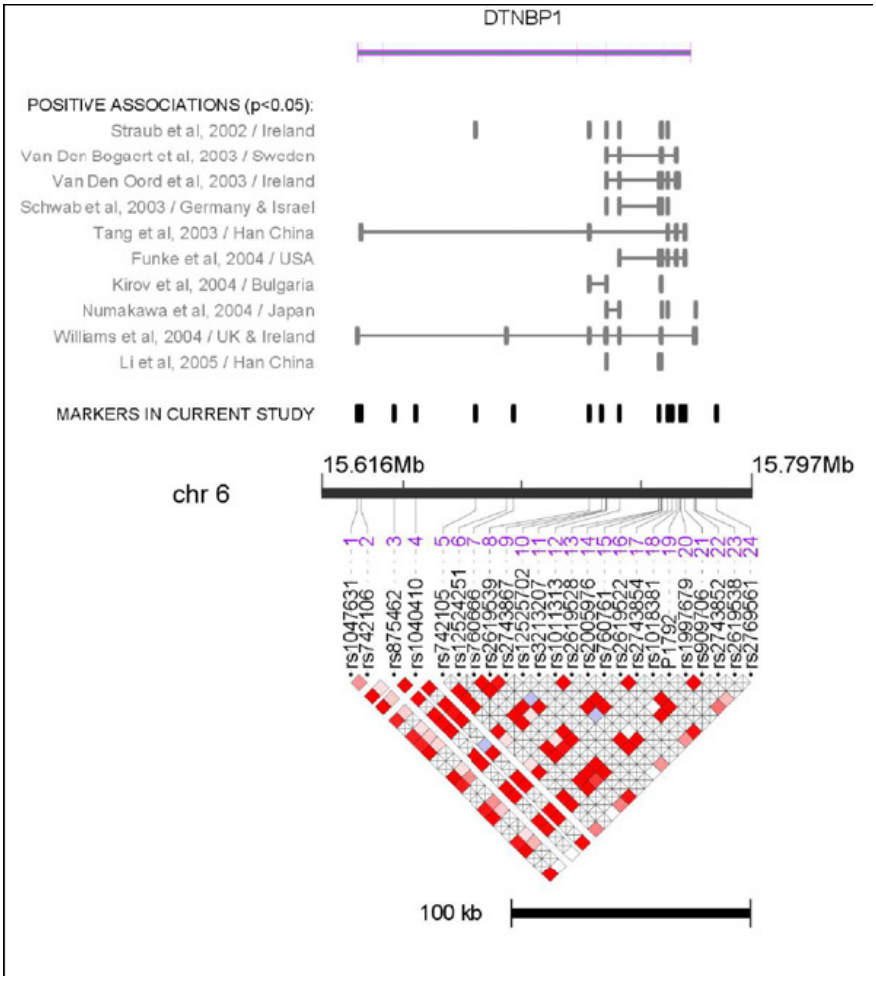
In conclusion, it seems that *DTNBP1*, *AKT1*, and *NRG1* do not play major roles in the pathogenesis of schizophrenia in Finland, at least in our family material. The allelic variants in *NRG1* should be studied in more detail to better address the potential role of these variants. Even though our sample size is fairly large, it is still possible that these three genes are susceptibility genes for psychosis in Finland and that our study merely lacked the detection power to show this. Regardless, our study is for now 'a case against' that these genes are predisposing for schizophrenia.

Generally speaking, there is no solid agreement in psychiatric genetics on what constitutes a true replication. There are several possible definitions: 1) same gene region, 2) same haplotypes, 3) same variants, and 4) same alleles. Strictly speaking, a true replication should consist of the same alleles of the same marker being associated with the phenotype, as observed in the original report. In figure 3 is a summary of published positive findings for *DTNBP1*. Even if positive findings outnumber the negative ones, there is no single marker that has been consistently associated with the disease. It can be argued, that because we do not have the 'causative' variant, it could be that the haplotype structure of the gene region is different in every populations, and thus resulting inconsistent alleles or haplotypes. Mutsuddi and colleagues showed that this is not the case with *DTNBP1* (201). They demonstrated that the studied European-derived populations have haplotype patterns and frequencies that are consistent with HapMap CEU samples (and each other). Thus, it is unlikely that population differences are creating the inconsistency of the association studies.

It should be noted that even if the register diagnosis of schizophrenia has been shown to have a high reliability, there has been studies that question this method. The Finnish studies showed clinicians do not make the diagnosis of schizophrenia as often as the application of operational criteria would suggest they should (202, 203). The discordance between clinical diagnosis and the research, operational diagnosis was in particular likely in patients having late onset and few contacts to psychiatric hospital. However, this should not bias our results extensively, because we usually only use affected individuals in our analysis and treat others as unknown of their status of disease.

Based on these pieces of evidence, it appears that the evidence of association between *DTNBP1*, *AKT1*, and *NRG1* with schizophrenia is at present, equivocal and unsatisfactory and that further studies are needed. Ideally, all the samples should be genotyped in the same place and the analyses carried out on the combined material. Only then can we truly evaluate the role of these genes.

Figure 4. Specific *DTNBP1* alleles and haplotypes associated with schizophrenia across studies. *DNTBP1* markers tested for association with schizophrenia in previous reports ($p<0.05$) and in the current study (original publication I).



5.2 Association analysis of the linkage region on chromosome 5q31-34 (II)

We have conducted a genome-wide scan in a study sample of 238 pedigrees collected nationwide in Finland and identified linkage to loci on 1q42, 2q37 and 5q21-33 (176, 204). Encouragingly, the chromosome 5q linkage finding is replicated in several studies and multiple independent populations (205), and was among one of the regions likely to contain loci that increase susceptibility to schizophrenia in a recent meta-analysis of 20 schizophrenia genome scans from diverse populations (206). Our original aim was to identify allelic variants of genes on 5q31-34 that associated with schizophrenia, as suggested by the previous linkage finding in 238 Finnish schizophrenia families. In the figure 4, linkage results from chromosome 5 from the late settlement region of the Finland are presented.

Figure 5. Profile for the multipoint non-parametric linkage (NPL) scores at 5q31-34 calculated using Simwalk2 software. Only one statistic (STAT A) is presented. The x-axis presents location on the chromosome in Mb.

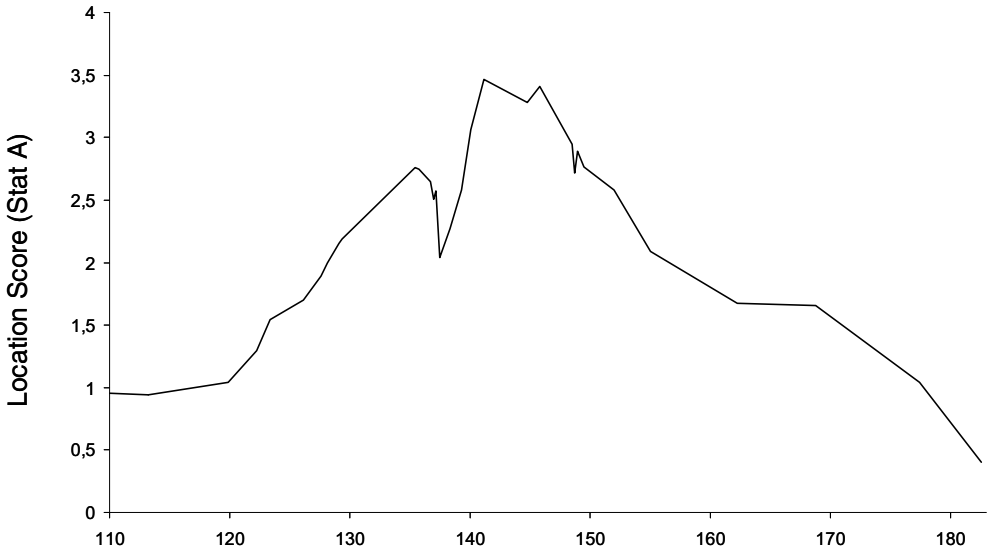
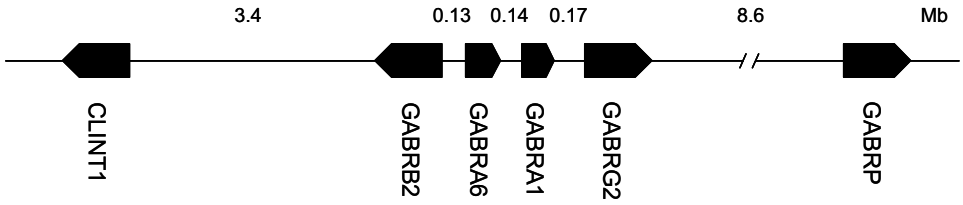


Figure 6. Genomic structure of the chromosome 5q $GABA_A$ receptor subunit genes and the CLINT1 gene. Gene Orientation is indicated by the direction of the arrows. Physical distances between genes are shown (Mb).



The GABA signalling system has been widely proposed to be involved with schizophrenia pathogenesis (for a detailed review see (207)). Although, we lack definitive evidence for involvement of the GABA signalling pathway, multiple line of evidence are building up. Postmortem schizophrenic brain samples exhibit a spectrum of alterations with respect to $GABA_A$ receptor binding (172), $GABA_A$ subunit mRNA levels (183, 208-211), and also some subunit protein expression (212, 213). Recently, suggestive genetic evidence for involvement of chromosome 5 $GABA_A$-receptor subunits has been published. Petryshen and colleagues reported association between schizophrenia and SNPs of the chromosome 5 $GABA_A$ cluster genes, *GABRA1*, *GABRP*, and *GABRA6* (183). Subsequently, Lo and colleagues detected an association between *GABRB2* and schizophrenia among Han Chinese (184), and the finding was confirmed in Chinese schizophrenic patients (185). After these findings were published we focused our attention on these functionally and genetically promising genes. Later, Pimm and colleagues reported association to Epsin 4 (*CLINT1*) in the same region, and we decided to add this gene to our study.

GABA is the major inhibitory neurotransmitter found in the brain. GABA is synthesized from its precursor, L-glutamate, by the enzyme glutamate decarboxylase (GAD). Three GABA receptors types have been identified. $GABA_A$ and $GABA_C$ receptors are ionotropic (i.e. composed of subunits which cluster together to form an ion channel), but the $GABA_B$ receptor is metabotropic and linked via a G-protein to a signalling pathway. $GABA_A$ is a heteropentameric receptor composed of five subunits: two copies of α and β each and one copy of either γ, δ, or ε subunits. The most common receptor configuration of the total $GABA_A$ receptor subtypes is $\alpha1\beta2\gamma2$. It is widespread throughout the brain compared with other configurations located in more selective regions (214). It is noteworthy that genes encoding these three subunits are all located in the chromosome 5 $GABA_A$-receptor cluster (figure 5).

Epsin 4 encodes a protein known as Enthoprotin, which links clathrin coated neurotransmitter vesicles to the neuronal membrane. It has a function in regulating the reuptake and storage of neurotransmitters in the brain.

We found association between individuals SNPs and haplotypes in the *GABRG2* gene ($p = 0.002$ for LC1 and $p = 0.005$ for LC3). Results are presented in the table 7, which is also published in the article 2. Locations of the SNPs in *GABRG2* are presented in figure 6. We also found evidence for association in GABRB2 with single SNPs, but the haplotype analysis did not support these findings. We found that the most common haplotype, having a frequency of 0.65 was most significantly associated with the disease, both for schizophrenia ($p=0.00014$ for LC1) and for schizophrenia spectrum disorders ($p = 0.00062$ for LC3), and the haplotype was overtransmitted to affected individuals. We have data of neuropsychological tests from 664 individuals, and used them as quantitative variables. We found suggestive

evidence that the same variants are associated with working memory (Table 3 in II). No association was observed with *CLINT1*.

In recent studies concerning the chromosome 5 GABA receptor subunits, different subunits were found to be associated with schizophrenia. This can be due to these being false positive findings, or may represent the complex genetic background of this region in relation to susceptibility to schizophrenia. It should be noted that causative variants remain to be identified, and furthermore, there is no solid proof that the found association has anything to do with GABA-receptors. They can merely be associated with this genomic region, or it can harbour a regulatory region which controls genes in other locations. If these associations truly point to a gene in this region, the $GABA_A$-receptor is as of now the most likely candidate.

Deficits in working memory are among the cognitive disturbances reported in schizophrenia (192, 194, 215-217). Working memory has been linked to dorsolateral prefrontal cortex activity (218, 219) as well as to the temporal cortex and a variety of subcortical structures. The dorsolateral prefrontal cortex has been shown to be damaged in schizophrenic patients (220), and imaging studies have linked prefrontal disturbances to working memory deficits in the individuals with schizophrenia (216, 217, 221). The GABA signaling pathway has been associated with working memory processes in the prefrontal cortex in monkeys (222, 223), and GABA has also been implicated in the temporal cortex and subcortical brain areas involved in working memory (224, 225). Our suggestive association between working memory and *GABRG2* is in line with earlier functional studies.

In conclusion, this study provides evidence of involvement of the GABAA receptor genes, particularly *GABRG2*, in pathogeneisis of schizophrenia in Finland. Furthermore, *GABRG2* seems to affect working memory functions of the brain. The evidence for the involvement of chromosome 5 $GABA_A$-receptor genes in the pathogenesis for schizophrenia is suggestive, and further studies are needed. All common variants should be identified and the genomic structure of the region clarified. After this, extensive collaborative studies should be carried out to address the role of this region and these genes in the etiopathogenesis of psychosis.

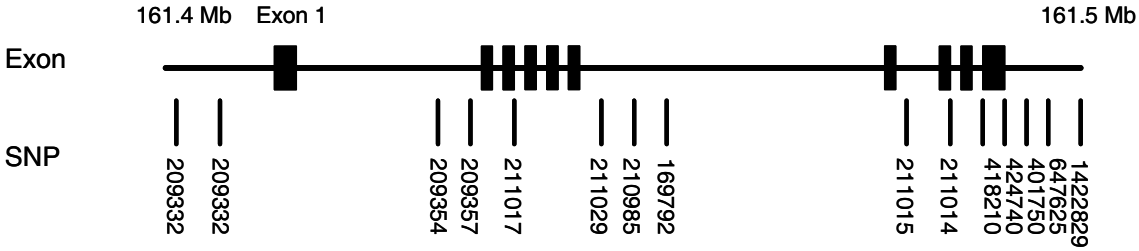
Table 7. Association results of the *CLINT1* and GABA-A receptor genes. Black and grey bars indicate haplotype blocks. For each haplotype, global haplotype association result is presented, and if it is significant (p<0.05) the best individual haplotype association result is also given. P-values below 0.05are indicated in bold.

		Single SNP		Haplotype			
Gene	rs#	LC1	LC3	LC1		LC3	
				Global	Individual	Global	Individual
CLINT1	rs2270811	0.589	0.987				
	rs406150	0.864	0.566	0.34		0.247	
	rs10515754	0.13	0.402				
	rs10515753	0.906	0.351				
	rs1186930	0.695	0.556				
	rs10046055	0.9	0.813				
GABRB2	rs168697	0.883	0.407				
	rs252965	0.242	0.368				
	rs252980	0.073	0.149	0.11		0.066	
	rs153296	0.064	**0.009**				
	rs173766	**0.004**	**0.002**	0.15		0.197	
	rs187269	0.072	0.092				
	rs194072	0.508	0.865				
	rs1816071	0.078	0.056				
	rs2617504	0.123	0.208				
	rs2910305	0.499	0.823	0.79		0.364	
	rs7714930	0.065	**0.038**				
	rs7724146	0.504	0.123				
	rs2964775	0.223	0.29				
	rs2962406	0.312	0.523				
	rs11748071	0.432	0.848	0.36		0.502	
	rs4426954	0.617	0.846				
GABRA6	rs2197414	0.957	0.795	0.83		0.769	
	rs3811992	0.984	0.906				
	rs3811991	0.721	0.521				

Table 7. Continues.

		Single SNP		Haplotype			
Gene	rs#	LC1	LC3	LC1		LC3	
				Global	Individual	Global	Individual
GABRA1	rs4340950	0.828	0.77	0.94		0.972	
	rs4263535	0.748	0.9				
	rs7734447	0.194	0.368	0.3		**0.013**	**0.01178**
	rs4260711	0.36	0.545				
	rs6878494	0.473	0.943				
	rs1350376	0.254	0.252				
	rs998754	0.136	0.132				
GABRG2	rs209332	0.454	0.95				
	rs209336	0.594	0.991				
	rs209354	0.659	0.282	0.18		0.101	
	rs209357	0.143	**0.035**				
	rs211017	0.583	0.888				
	rs211029	0.191	0.087	0.34		0.191	
	rs210985	0.472	0.886				
	rs169792	**0.027**	**0.028**				
	rs211015	0.114	0.113	**0.0022**	**0.00014**	**0.005**	**0.00062**
	rs211014	0.114	0.386				
	rs418210	0.093	0.083				
	rs424740	0.228	0.34				
	rs401750	**0.011**	**0.018**				
	rs647625	0.338	0.791				
	rs1422829	0.494	0.058				
GABRP	rs732157	0.664	0.898	0.98		0.76	
	rs1158443	0.807	0.739				
	rs9313525	0.377	0.613				
	rs1063310	0.475	0.681				

Figure 7. Genomic structure of the *GABRG2* gene and the locations of SNPs.



5.3 Maternal-fetal genotype incompatibility at the RhD locus (III, IV)

Epidemiological studies have demonstrated that problems in the environment of the developing fetus during pregnancy can increase the risk for schizophrenia (11) with a general estimate for risk ratio of about 2.0. Genes can be associated with disease through an individual's inherited genotype, the maternal genotype or via an interaction between these two. Rh (Rhesus) incompatibility has been implicated as a risk factor among obstetric complications for schizophrenia (11, 29). In a meta-analysis including three studies the overall odds ratio was estimated to be 2.0 (11). When these kinds of study designs are used, it is impossible to evaluate whether the effect of the RhD locus results from a maternal-fetal genotype incompatibility, or from linkage and association with a susceptibility allele at or near the RhD locus.

Because existing statistical models and tests for gene detection are not optimal or even appropriate for identifying maternal-fetal incompatibility loci, a maternal-fetal genotype incompatibility test (MFG) was developed (226), and method applied to genotype data from our schizophrenia family material. We genotyped the RhD locus in our family sample including 181 trios with 450 individuals. The MFG model is a modification of the case-parent-trio log-linear modelling approach (227, 228), and is sensitive to the effects of RhD maternal-fetal genotype incompatibility. It is able to identify the effects of the risk allele located at or near the RhD locus.

We tested the hypothesis of an RhD maternal-fetal genotype incompatibility effect in the possible presence of LD. We observed significant evidence for an RhD maternal-fetal genotype incompatibility ($p = .027$). A one-sided test was used in the analysis, because we were replicating previous findings, and our hypothesis was that an RhD maternal-fetal genotype incompatibility increases risk to the developing fetus for later schizophrenia. We also ruled out the possibility that the finding is due to linkage or association. There was no evidence of linkage/association with schizophrenia at or near the RhD locus using a modification of the MFG test. Furthermore, we tested for linkage or association between schizophrenia and the RhD locus and nearby microsatellite markers nearby (D1S368, D1S552, RHD, D1S1622, and D1S513). We detected no evidence for linkage or association with schizophrenia at any of these loci. These results demonstrated that observed association is due to maternal-fetal genotype incompatibility.

Our finding replicates earlier epidemiological findings, and demonstrates that the effect comes from to maternal-fetal genotype incompatibility rather than from an association with nearby markers. Furthermore, our estimate of the incompatibility parameter, in other words the modelling risk ratio, was 2.6. Cannon and colleagues estimated in the meta-analysis that the risk ratio is 2.0 (11), and Hollister and

colleagues found it to be 1.8 in their study (29). These findings are fairly similar, suggesting that the overall risk ratio of Rh incompatibility leads to a two-fold increased in the risk for schizophrenia.

This finding has encouraged us also to investigate other blood groups that can lead to maternal-fetal genotype imcompatibilities. Schizophrenia and human leukocyte antigen (HLA) matching between couples or between mothers and offspring have independently been associated with prenatal/obstetric complications. We analysed HLA-A, -B, and -DRB1 as risk factors for schizophrenia. We detected significant HLA-B maternal-fetal genotype-matching effects on schizophrenia in female offspring (229). Furhermore, we are currently analysing the ABO blood group.

Our study was conducted with individuals born before RhD prophylaxis in Finland and thus it is not possible to evaluate the role of prophylaxis in decreasing the relative risk for schizophrenia. These kinds of studies should be conducted to better understand the relation of RhD maternal-fetal genotype incompatibilities to schizophrenia risk.

Sometimes DNA from the all family members is not available, but serotypes of individuals are. Adding these serotypes to the analysis could increase the power to detect maternal-fetal genotype incompatibilities. Consideration of incomplete data resulting from using non-codominant data requires substantial modification to the MFG statistical model so that serotypes can be used in the analyses. In the fourth article, we have made extensions to the MFG model in order to analyze serotypes along with genotypes. We demonstrated the usefulness of this model with our data from Finnish schizophrenia families. In addition, simulations show that the power to detect the MFG incompatibility effect is similar for trio samples comprised of genotypes alone and for trio samples comprised of a combination of genotypes and serotypes.

In conclusion, the need to better elucidate the genetic basis of complex traits led us to develop a new statistical method, the MFG test, in order to test more realistic hypotheses regarding genetic effects. Because large samples are typically needed, the optimal use of different kinds of incomplete data is important. We have shown that the MFG method can be used to detect maternal-fetal genotype incompatibilities, and further that the statistical power can be increased by also including incomplete data such as serotypes.

6 CONCLUDING REMARKS AND FUTURE PROSPECTS

The basis of this study has been the collaboration of many experts from several fields to collect a schizophrenia family sample from Finland. Without the extensive work of many people these kinds of studies could not be conducted.

In recent years association studies have been leading the way toward characterization of the genetic background of schizophrenia. Unfortunately, lack of consistent replication for the same markers and haplotypes across studies has complicated the field of neuropsychiatric genetics. The collaboration of groups studying psychiatric illnesses throughout the world should be encouraged, and replication attempts should always preferably be conducted with the same markers as in the original studies. In this work, we have tried to replicate findings implicating some proposed schizophrenia candidate genes (*DTNBP1*, *NRG1*, and *AKT1*), but we conclude that these genes are not major risk factors for DSMIV-based diagnosis of schizophrenia or spectrum disorder in Finland. In the previously linked region on chromosome 5q, we studied the $GABA_A$ receptor cluster genes and *CLINT1*, and found evidence of association between *GABRG2* and schizophrenia. Further studies in different populations are warranted to evaluate the significance of this finding, and eventually meta-analysis should be conducted.

There is growing evidence that obstetric complications increase a child's risk for developing schizophrenia in later life. In the search for predisposing genes to complex diseases, we have to develop better analysis methods to capture the underlying genetic mechanism. In this work, we developed the maternal-fetal incompatibility (MFG) method in order to analyze genotype mismatch effects between mother and fetus. We demonstrate that incompatibility at the RhD locus increases the fetus' risk for schizophrenia two-fold.

In general, association studies of some complex human genetic diseases have produced unambiguous, consistent, and clear-cut replication. These diseases include type 1 and type 2 diabetes as well as other autoimmune diseases, inflammatory bowel diseases, and age-related macular degeneration. The schizophrenia literature supports the connection between genetic variations at least in the *NRG1*, *DNTBP1*, and *DISC1* genes and schizophrenia, but lacks impressive consistency in the precise genetic regions and alleles implicated. In the future, the study of the genetics of complex diseases will undoubtedly shed light on the pathogenesis of many ailments. However, whether these approaches will work for all diseases, especially in the case of psychiatric disorders, remains unknown.

There is growing appreciation that the human genome contains significant numbers of structural rearrangements, such as insertions, deletions, inversions, and large

tandem repeats. Recent studies have found that approximately 5% of the human genome is structurally variable in the normal population, involving more than 800 independent genes. As schizophrenia seems to be at least partly a neurodevelopmental disorder and some individuals with schizophrenia show cognitive decline, it could be that part of the disease is explained by rare structural rearrangements disturbing important genes for brain development.

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Joni Turunen

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