Common Gene Variants in Schizophrenia Susceptibility with Focus on Neurodevelopment

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2. List of Papers

This thesis is based on the following publications, which will be referred to by their roman numerals:

**Paper I.**
Association analysis of schizophrenia on 18 genes involved in neuronal migration: *MDGA1* as a new susceptibility gene.
*American Journal of Medical Genetics: Part B Neuropsychiatric Genetics 147B:1089-100.*

**Paper II.**
Association study of *PDE4B* Gene Variants in Scandinavian Schizophrenia and Bipolar Disorder multicenter case-control samples.
*American Journal of Medical Genetics: Part B Neuropsychiatric Genetics [Epub ahead of print]*

**Paper III.**
A Study of Ten Genes in the HNK-1 Pathway and Perineuronal Nets: *B3GAT2* is associated with schizophrenia in two large European Multi-Center Case Control Samples submitted

**Paper IV.**
A possible association between schizophrenia and *GRIK3* polymorphisms in a multicenter sample of Scandinavian origin (SCOPE).
3. Abstract

Schizophrenia is a severe multifactorial mental disorder with an important and complex genetic component, and the understanding of the underlying biological mechanisms is limited. Several lines of evidence support that abnormal neurodevelopment is involved, such as cognitive deficits in children who later develop schizophrenia, abnormalities in brain structure in the early phase of disease, and aberrant neuronal distributions. Also, glutamatergic dysfunctions are suggested in the schizophrenia etiology, and glutamate signalling is important during neurodevelopment. Perineuronal nets are extracellular matrix structures involved in brain maturation, which includes the characteristic neural epitope Human Natural Killer-1 (HNK-1).

To investigate if common gene variants important for neurodevelopment are involved in schizophrenia etiology, we used candidate gene-based association studies of tagSNPs spanning thirty genes, genotyped in a large Scandinavian case-control sample (SCOPE). Nineteen, out of the 289 tagSNPs in 18 neuronal migration genes, were nominally significant, and the strongest finding was a tagSNP located in MAM domain containing glycosylphosphatidylinositol anchor 1 (MDGA1), but no findings were significant after correction.

Phosphodiesterase 4B (PDE4B) is a Disrupted-in-Schizophrenia-1 (DISC1) interactor, with previously reported genetic associations only in women. Six and 16, out of 40 and 72 PDE4B tagSNPs, were nominally associated with schizophrenia and bipolar disorder, respectively, in the combined samples or in gender-specific subgroups. No findings were significant after correction. However, two of the tagSNPs nominally associated in schizophrenia females had proxies which were nominally associated in the total bipolar disorder sample, and the four SNPs were located in the same block, surrounding the splice site for the PDE4B3 isoform.

Five out of 104 tagSNPs in ten genes involved in perineuronal net formation and HNK-1 biosynthesis, located in beta-1,3-glucuronyltransferase 2 (B3GAT2), were nominally associated with schizophrenia. The association signal for tagSNPs in one of the LD blocks was replicated by proxy SNPs in a much larger European sample (SGENE-plus).

Six out of 30 tagSNPs in glutamate receptor ionotrophic kainate 3 (GRIK3) were nominally associated, and the best tagSNP were significant after correction, with increased significance in the Swedish subsample, as well as when the risk allele was combined with another tagSNP risk allele.

When investigating clinical characteristics, including positive and negative symptom scores, age at onset, and cognitive measures of learning, memory and IQ, for association with a subset of the tagSNPs and genes included in the thesis studies, there were no significant associations after correction.

The current results indicate that gene variants involved in neurodevelopment are associated with schizophrenia, which further supports the neurodevelopmental hypothesis.
### 4. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADHD</td>
<td>Attention Deficit Hyperactivity Disorder</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate</td>
</tr>
<tr>
<td>BD</td>
<td>Breslow Day</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine Mono Phosphate</td>
</tr>
<tr>
<td>CEPH</td>
<td>Centre d'Etude du Polymorphisme Humain</td>
</tr>
<tr>
<td>CEU</td>
<td>CEPH individuals from Utah, USA, with northern and western European ancestry</td>
</tr>
<tr>
<td>CDCV</td>
<td>Common Disease-Common Variant</td>
</tr>
<tr>
<td>CNV</td>
<td>Copy Number Variation</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-O-methyltransferase</td>
</tr>
<tr>
<td>CPT</td>
<td>Continuous Performance Test</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>CVLT</td>
<td>California Verbal Learning Test</td>
</tr>
<tr>
<td>DAOA</td>
<td>D-amino oxidase activator</td>
</tr>
<tr>
<td>DISC1</td>
<td>Disrupted-in-Schizophrenia-1</td>
</tr>
<tr>
<td>DSM-IV/III-R</td>
<td>Diagnostic and Statistical manual of Mental disorders, 4th edition/revised 3rd edition</td>
</tr>
<tr>
<td>DTNBP1</td>
<td>Dysbindin</td>
</tr>
<tr>
<td>FGFR2</td>
<td>fibroblast growth factor receptor</td>
</tr>
<tr>
<td>GRM3</td>
<td>glutamate receptor, metabotropic 3</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome Wide Association Study</td>
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<tr>
<td>sMRI</td>
<td>structural magnetic resonance imaging</td>
</tr>
<tr>
<td>HGP</td>
<td>Human Genome Project</td>
</tr>
<tr>
<td>HNK-1</td>
<td>Human Natural Killer-1</td>
</tr>
<tr>
<td>HUBIN</td>
<td>Human Brain Informatics</td>
</tr>
<tr>
<td>HWE</td>
<td>Hardy Weinberg Equilibrium</td>
</tr>
<tr>
<td>ICD-10</td>
<td>International Classification of Diseases, 10th revision</td>
</tr>
<tr>
<td>ICD-10-DCR</td>
<td>ICD-10-Diagnostic Criteria for Research</td>
</tr>
<tr>
<td>KA</td>
<td>kainate</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage Disequilibrium</td>
</tr>
<tr>
<td>MAF</td>
<td>Minor Allele Frequency</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>NAAG</td>
<td>Neuropeptide N-acetylaspartylglutamate</td>
</tr>
<tr>
<td>NMDA(R)</td>
<td>N-methyl-D-aspartate (receptor)</td>
</tr>
<tr>
<td>NRG1</td>
<td>Neuregulin 1</td>
</tr>
<tr>
<td>NRXN1</td>
<td>Neurexin 1</td>
</tr>
<tr>
<td>OC</td>
<td>Obstetric Complication</td>
</tr>
<tr>
<td>OPCRIT</td>
<td>Operational Criteria Checklist for Psychotic Illness and Affective Illness</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>PANSS</td>
<td>Positive and Negative Syndrome Scale</td>
</tr>
<tr>
<td>PCP</td>
<td>phencyclidine</td>
</tr>
<tr>
<td>PGC</td>
<td>Psychiatric GWAS Consortium</td>
</tr>
<tr>
<td>PN</td>
<td>Perineuronal Net</td>
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The genes investigated in this thesis:

ASTN1     astrotactin-1
B3GAT1    beta-1,3-glucuronyltransferase 1 (glucuronosyltransferase P)
B3GAT2    beta-1,3-glucuronyltransferase 2 (glucuronosyltransferase S)
BCAN      brevican
CDC42     cell division cycle 42
CDK5      cyclin-dependent kinase 5
CDK5R1    cyclin-dependent kinase 5, regulatory subunit 1
CHST10    carbohydrate sulfotransferase 10
DLX1      distal-less homeobox 1
ENAH      enabled homolog
EVL       enah/vasp-like
FILIP1    filamin A interacting protein 1
GRIK3     glutamate receptor, ionotropic, kainate 3
HAPLN1    hyaluronan and proteoglycan link protein 1
HAPLN2    hyaluronan and proteoglycan link protein 2
HAPLN3    hyaluronan and proteoglycan link protein 3
HAPLN4    hyaluronan and proteoglycan link protein 4
ITGA3     integrin, alpha 3
MAP1B     microtubule-associated protein 1B
MDGA1     MAM domain containing glycosylphosphatidylinositol anchor 1
NCAN      neurocan
NDEL1     nudE nuclear distribution gene E homolog
PAFAH1B1  platelet-activating factor acetylhydrolase, isoform Ib, subunit 1
PAX6      paired box 6
PDE4B     phosphodiesterase 4B
RELN      reelin
SPARC1    SPARC-like 1
TNR       tenascin R
VASP      vasodilator-stimulated phosphoprotein
YWHAE     tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide
5. Introduction

5.1 Schizophrenia: phenotype, epidemiology, treatment

Schizophrenia is a severe mental disorder affecting approximately 1% of the general population, with onset in late adolescence or early adulthood. It is one of the major causes of disability worldwide, ranked as one of the most costly disorders to afflict humans [Murray, 1996]. The schizophrenia phenotype is characterized by the presence of positive symptoms (delusions, hallucinations and other distortions of reality), negative symptoms (loss of motivation, inability to experience pleasure, poverty of speech, lack of initiative, apathy, and reduced social drive), cognitive impairment, and mood symptoms [Tandon et al., 2008; Tandon et al., 2009]. The negative symptoms could be primary or secondary, the first if being fundamental to schizophrenia, and the latter when being caused by for example antipsychotic drug treatment or depression. The cognitive deficits is a core feature in schizophrenia, and domains affected are e.g. speed of processing, attention, working memory, verbal learning and memory, visual learning and memory, reasoning and problem solving, verbal comprehension, and social cognition [Nuechterlein et al., 2004]. Abnormal brain activation, as measured by functional brain imaging, have been associated with cognitive deficits [Greene et al., 2008]. The schizophrenia phenotype is complex, and the diagnosis is solely based on the symptoms presented, implemented in the main diagnosis systems Diagnostic and Statistical manual of Mental disorders, 4th edition (DSM-IV) [American Psychiatric Association, 1994] and International Classification of Diseases, 10th revision (ICD-10) [World Health Organization, 1992].

There are indications of gender difference in schizophrenia, with earlier age at onset, and a higher lifetime disease risk reported for males (male-female relative risk of 1.35) [Aleman et al., 2003]. Migrant status, urbanicity, prenatal starvation and infection, winter/spring birth, perinatal and obstetric complications (OCs), and older paternal age, are also associated with increased risk for schizophrenia [McGrath, 2007].

The antipsychotic medications used to treat schizophrenia are grouped into the so-called typical or first generation (e.g. chlorpromazine, haloperidol), and the atypical or second generation (e.g. clozapine, olanzapine) antipsychotics. Both groups have strong affinity to the dopamine D₂ receptor (see 5.2.1), which seems to be essential for the antipsychotic effect. The clinical effect is mainly similar, but the typical antipsychotics are known for extrapyramidal side effects, such as tardive dyskinesia, and elevated serum prolactin levels, while the atypicals have less motor side effects, but instead metabolic side effects such as weight gain. It has been shown that typical drugs bind to
the D₂ receptors more tightly than the atypicals, and the latter also block 5-HT₂A-
receptors [Seeman, 2002].

5.2 Schizophrenia etiology: main hypotheses

5.2.1 The dopamine hypothesis
Abnormal dopamine neurotransmission has long been implicated in schizophrenia etiology [Carlsson and Lindqvist, 1963; Carlsson et al., 1957], and the dopamine hypothesis was further based on data showing that the treatment outcome of antipsychotic drugs depended on their dopamine receptor affinity [Creese et al., 1976; Seeman and Lee, 1975], and the ability of the dopamine agonist amphetamine to induce psychosis. The main view was that a general increase in dopaminergic neurotransmission was involved in schizophrenia pathology. In a revised hypothesis, a co-occurrence of both increased and decreased dopamine activity was suggested, namely prefrontal hypodopaminergia and subcortical hyperdopaminergia, causing negative and positive symptoms, respectively [Davis et al., 1991].

In a recent review, the main findings the last two decades in support of the dopamine hypothesis are discussed, and five main domains are highlighted, pointing towards a focus on presynaptic striatal hyperdopaminergia in schizophrenia pathology [Howes and Kapur, 2009]. Firstly, results from neurochemical imaging have revealed several abnormalities in schizophrenia patients, such as: increased striatal presynaptic dopamine synthesis, consistently reported in all studies investigating patients during acute psychosis; increased striatal dopamine release upon neuronal challenge, as well as dopamine baseline occupancy of D₂ receptors; increased level of striatal D₂/₃ receptor density, as well as an association between prefrontal cortical D₁ receptor intensities with cognitive impairment and negative symptoms, independent of antipsychotic medication. Also, all licensed antipsychotic drugs are striatal D₂ receptor blockers. Secondly, environmental factors, such as lack of close friends, urban upbringing, migration, complications during pregnancy and birth, as well as psychoactive substance abuse have been associated with increased risk of schizophrenia, and an overactivity of the dopamine system in response to the same factors or linked experiences has been reported in animal studies. Thirdly, increased risk of schizophrenia has been associated with interactions between gene variants involved in the dopamine system and environmental factors; the interaction between a variant of the cathechol-O-methyltransferase (COMT) gene, an enzyme involved in dopamine degradation, and cannabis use, was associated with increased risk for
psychosis. Fourthly, it has been reported that individuals at-risk for psychosis, such as those in the prodromal state or family members of patients with schizophrenia, show dopaminergic abnormalities and brain structural changes in areas linked to striatal dopaminergic function in animals. Finally, variants of genes involved in the dopamine system have been implicated in schizophrenia etiology [Glatt and Jonsson, 2006; Talkowski et al., 2008].

5.2.2 The glutamate hypothesis

The amino acid glutamate is the major excitatory neurotransmitter of the brain, and its receptor signaling is essential for excitatory neurotransmission, neuronal development and synaptic plasticity. The glutamate actions are mediated through three ionotropic receptor classes, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), and kainate (KA), and three groups (I, II, III) of metabotropic glutamate receptors [Kew and Kemp, 2005]. Glutamate can also exert receptor-mediated excitotoxicity leading to neuronal damage in the central nervous system [Greenamyre, 1986].

The hypothesis that hypofunction of the glutamate transmitter system is involved in the etiology of schizophrenia was first suggested almost three decades ago, when low levels of glutamate in cerebrospinal fluid (CSF) from patients with schizophrenia was reported [Kim et al., 1980]. Although later CSF findings have been inconsistent [McCullumsmith et al., 2004], other subsequent discoveries have provided strength to the glutamate hypothesis of schizophrenia [Coyle, 2006; Olney and Farber, 1995]. Specifically, hypofunction of the NMDA receptor has been suggested, which might be due to for example increased presence of endogenous NMDA antagonists or reduced release of glutamate. The exposure to NMDA antagonists, such as the dissociative anaesthetics phencyclidine (PCP) and ketamine, have been shown to trigger schizophrenia-like positive and negative symptoms in normal human subjects [Javitt and Zukin, 1991; Krystal et al., 1994; Luby et al., 1962], and increase psychotic symptoms in patients with schizophrenia [Lahti et al., 2001], which is not sufficiently explained by their dopamine dysregulation [Javitt and Zukin, 1991]. Also, NMDA antagonists cause permanent structural changes in several cerebrocortical and limbic regions in rats [Ellison, 1994; Olney et al., 1989], and the vulnerability to the neurotoxic effects is age-dependent, with susceptibility onset, or markedly increased susceptibility, in late adolescence of rats [Farber et al., 1995] and humans [Sussman, 1974], respectively, similar to the age of schizophrenia onset in late adolescence and early adulthood. The endogenous NMDAR antagonist neuropeptide N-
acetylaspartylglutamate (NAAG) was increased in the hippocampus of postmortem brains of schizophrenia patients, compared with both neuroleptic-treated, and non-treated, controls [Tsai et al., 1995]. NAAG is catabolized into glutamate and NAA, and the activity of the responsible peptidase was reduced in the same study, both in schizophrenia hippocampus and prefrontal cortex, accompanied by reductions of glutamate in the same regions. These findings are in line with the NMDAR hypofunction hypothesis of schizophrenia.

To move beyond the NMDA receptors, the above mentioned NAAG also selectively activates the metabotropic receptor mGluR3 [Wroblewska et al., 1997]. Another agonist for mGluR3/4 (the group II of metabotropic glutamate receptors) was shown to reverse the cognitive and behavioral abnormalities in the PCP-animal model of schizophrenia, without reducing dopamine release [Moghaddam and Adams, 1998]. More recent clinical evidence suggests that an mGluR3 agonist is an effective new approach in the treatment of schizophrenia [Patil et al., 2007]. The gene encoding mGluR3, GRM3 (alias MGLUR3), has been implicated in schizophrenia susceptibility and cognitive function [Egan et al., 2004; Harrison and Weinberger, 2005]. In addition, aberrant copy-number of three loci were found in two small samples of brain tissue from patients with schizophrenia and bipolar disorder, but not in controls [Wilson et al., 2006]. These loci included three genes involved in glutamate signaling, e.g. the KA receptor subunit gene glutamate receptor, ionotropic, kainate 3 (GRIK3) [Wilson et al., 2006]. Changed GRIK3 expression was shown in cortical and hippocampal regions of postmortem brains from schizophrenia patients compared with controls [Benes et al., 2001; Sokolov, 1998].

5.2.3 The neurodevelopmental hypothesis

The evidence for a neurodevelopmental etiology of schizophrenia is based on several domains, such as cognitive deficits in children who later develop the disease, and abnormalities in brain structure and neuronal distributions in schizophrenia patients compared with controls [Arnold et al., 2005; Harrison and Weinberger, 2005]. There is a lack of astrogliosis in post-mortem brain tissue [Falkai et al., 1999], which is present in neurodegenerative disorders [Rodriguez et al., 2009]. Background details for some of the main supportive areas are given below.

**Macroscopic neuropathology**

Already in the seventies, enlarged ventricles were shown to be associated with both schizophrenia and increased cognitive impairment among patients [Johnstone et al., 1976]. A high number of brain imaging studies of schizophrenia patients have been
performed since then, and ventricle enlargement is among the most frequent structural findings [Vita et al., 1997; Vita et al., 2000]. In a review of 193 structural magnetic resonance imaging (sMRI) studies, several abnormalities of the brains of schizophrenia patients are reported, with the most robust findings being: lateral and third ventricular enlargement, medial temporal lobe reductions (including amygdala, hippocampus, and parahippocampal gyrus), superior temporal gyrus (STG) gray matter reductions, and enlarged cavum septi pelucidum, with all present in first-episode patients [Shenton et al., 2001]. Two recent meta-analyses reported reduced whole brain and hippocampus volumes, and increased ventricular volumes, in first-episode schizophrenia [Steen et al., 2006; Vita et al., 2006]. Another meta-analysis showed reduced hippocampal and total grey matter volumes, as well as enlarged third ventricular volume, in non-psychotic relatives of patients with schizophrenia [Boos et al., 2007]. The cortical structure has been investigated with sMRI for a subset of Swedish and Norwegian schizophrenia spectrum patients and controls included in the present thesis, and thinner prefrontal and temporal cortex was observed among patients compared with controls [Nesvag et al., 2008; Rimol et al., submitted], which was also shown in another study of patients who were off medication at least two weeks prior to the sMRI scan [Hazlett et al., 2000].

Important to note is that the brain structural abnormalities reported are subtle and not present in all patients with schizophrenia, rather are based on group means with substantial overlap between patients and controls [Shenton et al., 2001]. In addition, the potential confounding of antipsychotic medication, substance abuse, and chronic illness, on brain structural abnormalities, is a concern. However, the presence of structural abnormalities in first-episode schizophrenia patients [Steen et al., 2006; Vita et al., 2006], in relatives of patients with schizophrenia [Boos et al., 2007], and in antipsychotic-naïve patients [Szeszko et al., 2003], is in line with a potential genetically determined neurodevelopmental origin.

**Microscopic neuropathology**

Abnormal cytoarchitecture of neurons in the entorhinal cortex, such as heterotopic displacements of cortical layer II neurons, is a histological finding reported in several studies of post-mortem brains from schizophrenia patients [Arnold et al., 1991; Arnold et al., 1997; Jakob and Beckmann, 1986; Kovalenko et al., 2003], but which was not found by others [Akil and Lewis, 1997; Bernstein et al., 1998]. Changed neuronal distribution in the prefrontal cortex was reported [Kalus et al., 1997]. In addition, subcortical interstitial neurons in prefrontal white matter were shown to have a changed distribution, with reduced densities in the superficial, but increased densities in the deeper white matter, in a subgroup of schizophrenia patients but in none of the
controls [Akbarian et al., 1993; Akbarian et al., 1996], which was not replicated in a subsequent study [Beasley et al., 2002]. Others found higher overall neuronal densities in prefrontal white matter tissue of schizophrenia individuals, which was most apparent in the more superficial parts [Anderson et al., 1996]. Taken together, results are conflicting and cannot yet be either confirmed or rejected. A limiting factor in these studies is the use of post-mortem tissue, where many factors not specific to disease development can influence the results. If the cytoarchitectural abnormalities are indeed causative, these imply an involvement of early neurodevelopment in schizophrenia etiology, affecting neuronal migration and brain connectivity. Interstitial neurons represent remaining early neurons of the subplate, which is important for the cortical development and connectivity [Kanold, 2004]. Other examples of cytoarchitectural aberrancies reported in post-mortem brain tissue of schizophrenia patients are smaller size of hippocampal neurons [Arnold et al., 1995; Benes et al., 1991; Zaidel et al., 1997], and synaptic abnormalities in a number of hippocampal studies [Harrison and Eastwood, 2001]. These early brain abnormalities could be genetically determined, but also caused by other factors such as OCs and toxic agents.

**Premorbid cognitive and behaviour findings**

Cognitive deficits in childhood have consistently been associated with increased risk for schizophrenia [Maccabe, 2008]. Using large birth cohorts, childhood abnormalities in cognitive function, language and behaviour of both individuals who later develop schizophrenia and their unaffected siblings have been reported at higher rates, compared with non-psychiatric control subjects [Bearden et al., 2000; Cannon et al., 2000; Jones et al., 1994; Niendam et al., 2003]. Overall cognitive functioning, as measured by full intelligence scales, was shown to be compromised both in future schizophrenia cases and their unaffected siblings, as early as at the age of 4 and 7 years [Cannon et al., 2000]. The findings were not significantly associated with OCs or birth weight. Also, early childhood focal deviant behaviour (e.g. meaningless laughter, excessive crying, echolalia), and poor expressive language ability, predicted the outcome as schizophrenia patient and unaffected sibling, whereas social maladjustment was associated with schizophrenia outcome only [Bearden et al., 2000]. Moreover, delayed childhood motor development was associated with schizophrenia later in life, and with adult cognitive function [Murray et al., 2006]. Lower adolescent IQ has also been associated with higher risk for schizophrenia-spectrum disorders when investigating army conscripts [Reichenberg et al., 2006]. The findings that higher numbers of children who later develop schizophrenia, and their unaffected siblings, show developmental abnormalities early in life, suggest that these deficits are susceptibility phenotypes for schizophrenia. The fact that also unaffected siblings show similar patterns of impairment, also imply that these domains are heritable.
phenotypes which are associated with an increased risk for schizophrenia but not causal themselves. That this instead should be the cause of shared environment is less likely, since several of the studies corrected for parental education and socioeconomic status [Bearden et al., 2000; Cannon et al., 2000; Niendam et al., 2003].

**Neurodevelopmental genes and susceptibility to schizophrenia**

Some of the genes which have emerged as strong schizophrenia susceptibility candidates, are involved in neurodevelopmental processes, and initially implicated in studies with no prior mechanistic hypotheses. In a study of a balanced translocation in a large Scottish family, segregating with severe mental illness such as schizophrenia, the gene Disrupted-in-Schizophrenia-1 (DISC1) was found to be disrupted [Millar et al., 2000]. Neuregulin 1 (NRG1) was originally implicated in schizophrenia after LD mapping across the linked region 6p24-22 [Stefansson et al., 2002]. DISC1 and NRG1 have both important neurodevelopmental functions and have been extensively studied in the field of schizophrenia genetics [Chubb et al., 2008; Mei and Xiong, 2008]. Altered expression of genes involved in neuronal development and myelination have also been reported [Hakak et al., 2001].

**Glutamate and neurodevelopment**

Glutamate receptors have an important role during neurodevelopment, with evidence of being critical for neuronal migration [Manent and Represa, 2007], corticogenesis [Furuta and Martin, 1999] and synaptogenesis [Yen et al., 1993], as examples. The three ionotropic glutamate receptor subtypes were shown to have distinct subunit binding patterns in cortical and subcortical regions during the second trimester [Lee and Choi, 1992], and in fetal cortex already in the first trimester [Ritter et al., 2001].

5.3 The main domains of neurodevelopment and plasticity in this thesis

**5.3.1 Neuronal migration**

Neuronal migration is the process where neurons move to their final destination in the cortex and other areas of the central nervous system. This movement occurs either through radial or tangential migration [Corbin et al., 2001]. None of the cortical neurons are generated within the cortex itself during cortical development, but instead pyramidal cells originate in the proliferative ventricular and subventricular zones, from where they migrate with radial glial cells leading their way [Rakic, 1988], and most interneurons move tangentially from the lateral ganglionic eminence of the ventral
forebrain [Rakic and Zecelic, 2003]. The six-layered neocortex is arranged in an inside-out manner, with the formation of inner layers first, and neurons destined to outer layers passing through those layers already formed [Rakic, 2002].

Neuronal migration is dependent on extracellular signals which regulates cell movement, modulates the migrational speed, determines the direction of migration, and finally participates in ending migration when the proper position has been reached. The extracellular signals affect cell motility by regulating the cytoskeleton through intracellular signaling pathways, and cell adhesion molecules mediate cell-cell recognition and adhesion [Sobeih & Corfas, 2002]. The final position of the neuron affects its function, morphology, and formation of synaptic connections [Rakic, 1990]. Aberrant neuronal migration is suggested to be involved in the neurodevelopmental pathophysiology of schizophrenia, indicated from previously mentioned cytoarchitectural abnormalities found in brain tissue from schizophrenia patients. The reelin gene (RELN) is important for neuronal migration, and show decreased expression in several regions of the brain in tissue from schizophrenia patients compared with controls [Impagnatiello et al., 1998]. Also, DISC1 has been shown to be important for neuronal migration [Kamiya et al., 2005].

5.3.2 Perineuronal networks and the HNK-1 pathway

In the adult brain, lattice-like extracellular matrix structures called Perineuronal Nets (PNs) are condensed around a subset of both inhibitory interneurons and excitatory neurons, and their proximal dendrites [Bruckner et al., 2000; Celio and Blumcke, 1994; Viapiano and Matthews, 2006]. These net structures may regulate synaptic transmission and plasticity [Dityatev and Schachner, 2003] through their activity-dependent deposition around successful synaptic connections [Dityatev et al., 2007; McRae et al., 2007], and inhibition of experience-dependent plasticity [Berardi et al., 2004; Pizzorusso et al., 2002]. They could also potentially contribute to the synaptic overpruning suggested to occur in schizophrenia patients [Karlsgodt et al., 2008]. During neurodevelopment and in synaptic plasticity, carbohydrate-carrying molecules create diversity on cell surfaces and in the extracellular matrix, thereby mediating cell recognition [Kleene and Schachner, 2004]. The Human Natural Killer-1 (HNK-1) carbohydrate is among the most characteristic glycoepitopes in the nervous system, involved in neural crest cell migration, neurite outgrowth, neuronal cell adhesion, and synaptic plasticity [Kleene and Schachner, 2004; Morita et al., 2008]. One of the enzymes involved in the biosynthesis of HNK-1, Glucuronosyltransferase-P (GlcAT-P) (encoded by beta-1,3-glucuronyltransferase 1, B3GAT1), has been suggested as a candidate gene for severe psychiatric phenotypes, including schizophrenia and...
schizoaffective disorder, in a family study of a balanced chromosomal translocation [Jeffries et al., 2003].

5.4 Human genetics and disease mapping

5.4.1 The human genome and genetic variation

The first draft of the human genome sequence was announced in June 2000, and simultaneously published in February 2001, by the publicly funded Human Genome Project (HGP) [Lander et al., 2001] and the private company Celera Genomics [Venter et al., 2001]. A more comprehensive and high quality version of the human genome, including 99% of the gene-containing sequence, was made available in public databases (http://genome.ucsc.edu/; http://www.ncbi.nlm.nih.gov/) by HGP in spring 2003 [Collins et al., 2003a; Collins et al., 2003b], fifty years after the discovery of the DNA structure [Watson and Crick, 1953]. These milestones formed the basis for a new era in the field of genetics research [Collins et al., 2003a], and one of the major challenges of the new millennium is to investigate on a larger scale how genetic variation contribute to complex disease. Even though humans share most of their DNA sequence, there are also millions of base pairs that differ between individuals.

Human genome variants can be divided into two major classes, structural and single nucleotide variants, with the former including variation such as inversions, insertion-deletions, and Copy Number Variants (CNVs) [Frazer et al., 2009]. A Minor Allele Frequency (MAF) of 1% defines a genetic variant as a polymorphism, in contrast to rare variants. Polymorphisms can be called common variants [Frazer et al., 2009], even though variants with a MAF above 5-10% are those often referred to as common [Owen et al., 2009]. The most prevalent variation in the human genome is the Single Nucleotide Polymorphism (SNP), with an estimated total number of about 11 million [Kruglyak and Nickerson, 2001]. In parallel with the public deposition of the first human draft sequence, a first systematic SNP Map became open source in the dbSNP Database, comprising of 1.4 million SNPs, mostly discovered by The SNP Consortium (TSC) and the HGP [Sachidanandam et al., 2001; Sherry et al., 2001]. Since then, SNP data mainly from TSC, the Perlegen SNP genotyping initiative and the HapMap project (described below) has been continuously added, and in 2006 the dbSNP contained almost 10.5 million human SNPs [Phillips, 2007].

A high number of studies have used SNPs in their quest for the genetic determinants in heritable complex or monogenic disorders, either by a direct or indirect approach. In the first, there is a hypothesis that the actual SNP is functional, for example by changing protein composition, transcription control or splicing pattern. Using the latter
approach, the SNPs investigated are chosen based on their property to capture surrounding SNP information, based on the correlation structure present in the genome.

In all papers (I-IV) of this thesis, an indirect approach investigating the association between SNPs and psychiatric disease has been used. The two central concepts making this possible are Linkage Disequilibrium (LD), and tagSNPs. Information of the LD structure is assembled in the so called International HapMap Project, with the possibility to select tagSNPs as a project output. These three topics are described in detail below.

5.4.2 Linkage disequilibrium

Virtually all variable loci are caused by a single historical mutation event, which in the case of SNPs have increased in frequency to the level where the MAF ≥1% in the population. The new allele is surrounded by a distinct set of genetic variants present on the ancestral chromosome on which the mutation arose, and the set of alleles along the chromosome is called a haplotype. New haplotypes can form when new mutations or recombination events occur, and the coinheritance of SNP alleles of the same original haplotype is affected by the local recombination pattern. LD is the term for non-random association of alleles at two or more loci, i.e. the situation when the frequency of certain combinations of alleles in a population differ from what would be expected if the alleles were randomly combined based on their frequencies. There are several LD measures that are all related to the quantity of D, which is calculated as:

\[ D_{AB} = P_{AB} - P_A \cdot P_B, \]

where \( D_{AB} \) represents the difference in frequency of gametes carrying both allele A at one locus and allele B at another locus \( (P_{AB}) \) and the product of the allele frequencies of A and B \( (P_A, P_B) \). In the case of perfect linkage equilibrium the D measure will be equal to zero. Since D varies not only with the extent of LD, but also with the allele frequencies, the normalized D’ variable \( (0 \leq D' \leq 1) \) was introduced [Lewontin, 1964]:
An additional common LD measure, the squared correlation coefficient \( r^2 \) (0 \( \leq r^2 \leq 1 \)), incorporates the four allele frequencies when estimating LD between two SNPs [Hill and Robertson, 1968; Pritchard and Przeworski, 2001]:

\[
D' = \begin{cases} 
\frac{|D_{AB}|}{\min(P_A \cdot (1 - P_B), P_B \cdot (1 - P_A))} & \text{if } D > 0 \\
\frac{|D_{AB}|}{\min(P_A \cdot P_B, (1 - P_A) \cdot (1 - P_B))} & \text{if } D < 0 
\end{cases}
\]

The \( r^2 \) measure is always equal to, or lower than, \( D' \), and determines how well a locus can serve as a proxy for another locus. In a region with no historical recombination \( D' = 1 \) between loci, \( r^2 \leq 1 \), depending on the historical time points when the SNPs arose. When using LD in the search for disease causing loci the basis is that investigated markers are potentially linked to disease causing alleles on the same chromosome. LD is affected by factors such as recombination, mutation, changes in population size (especially population bottlenecks), genetic drift, and natural selection [Slatkin, 2008].

### 5.4.3 The international HapMap project and tag SNPs

Because of the presence of LD, one can screen specific genes, chromosomal regions, or the whole genome, for disease causing loci, using non-redundant sets of so called tag SNPs, which serve as proxies for untyped correlated SNPs. To enable such large-scale investigation of the contribution of common variation to phenotypic diversity, the International HapMap Project was initiated, with the goal to identify SNPs, investigate the pattern of LD, and make the data publicly available at their website (www.hapmap.org) [The et al., 2003; Thorisson et al., 2005]. 270 DNA samples from four geographically diverse populations were included: 30 trios (two parents and an adult child) from Utah, USA, with northern and western European ancestry (CEU), previously included in the Centre d’Etude du Polymorphisme Humain (CEPH) panel [Dausset et al., 1990], 90 Yoruba people in Ibadan, Nigeria (30 trios) (YRI), 45 unrelated Japanese in Tokyo, Japan (JPT), and 45 unrelated Han Chinese in Beijing, China (CHB). About 1.2 million SNPs were successfully genotyped in Phase I of the project [The et al., 2005], and data analysis verified the generality of the previously
reported presence of recombination hotspots, long stretches of high LD, limited haplotype diversity, and widespread redundancy among nearby SNPs [Daly et al., 2001; Gabriel et al., 2002]. Methods of picking tag SNPs was provided on the project website, of which the pairwise tagging option was used for all papers (I-IV) included in this thesis (discussed further in Material and Methods and Discussion sections). The HapMap data set has been steadily increasing (twelve data releases between June 2005 and February 2009), with a total of over 3.1 million SNPs in Phase II [Frazer et al., 2007], and 1.6 million SNPs genotyped in 1,115 samples from 11 populations in Phase III.

5.5 Association study design

The power and genetic resolution is greater in association studies, compared with linkage [Risch and Merikangas, 1996], making such design the more common choice when searching for genetic determinants of complex disease today. Association studies can be family- or population-based; the former mainly using trios (cases and their parents), and the latter using unrelated cases and controls [Laird and Lange, 2006]. In turn, population-based association studies can either be case-control or case-cohort studies; the former considering cases with a disease of interest and controls without this disease drawn from the same population, and the latter include cases and controls both sampled from a cohort study. Since the four association studies included in the present thesis are case-control association studies, such design will be the focus of this thesis. A protocol for a proper design of case-control association studies have been published in the *Nature protocols* journal [Zondervan and Cardon, 2007], and the important steps highlighted were:

a) Define the phenotype in adequate detail  
b) Check the heritability of the disease of interest  
c) Consider whether a population-based study is suitable  
d) Select the controls appropriately  
e) Calculate the required sample size  
f) Consider whether it is a replication or de novo study

In schizophrenia genetics, the first step (a) is not a trivial issue since the schizophrenia phenotype is defined according to a set of symptoms rather than using objective biological measures, as for e.g. Type 2 diabetes. The disease phenotype as well as other alternative phenotypes used in this thesis will be further described and discussed in the “Materials & Methods” and “Discussion” sections. Step (b) is already at hand
when studying schizophrenia, which has been shown to be a highly heritable disorder [Sullivan et al., 2003]. When considering step (c) regarding this thesis, the so called Common Disease-Common Variant (CDCV) hypothesis [Schork et al., 2009] was the basis for the design of Papers I-IV. We wanted to investigate if common variants of specific candidate genes were associated with the fairly common disorder schizophrenia, and the case-control design is in such a case appropriate. The selection of controls (d), as well as the power of our study with the used sample size (e), will be illuminated in the “Discussion” section. As for step (f), a true replication study should involve the genotyping of the exact same markers (or markers in high LD), in an ethnically similar population, as in the original study. The initial effect size needs to be properly assessed for appropriate power calculations. In the present thesis, most of the 21 genes investigated are themselves de novo in the field of schizophrenia genetics, even though several have relations to other studied candidates, e.g. through biological interaction.

One fundamental additional step in the design of association studies is the choice of the region of interest and subsequently the genetic markers, which is mentioned below. The above steps are equally important in all scenarios, from single markers, to candidate genes or the whole genome.

5.5.1 Candidate genes or the whole genome?

The region of interest in an association study can be single genetic variations, believed to exhibit a functional impact on disease [Fan et al., 2005], candidate genes (Paper I-IV), chromosomal regions of interest implicated by linkage [Stefansson et al., 2002; Thomson et al., 2007] and cytogenetic studies [Callicott et al., 2005; Millar et al., 2000], or the whole genome [O'Donovan et al., 2008]. The last is referred to as a Genome Wide Association Study (GWAS), a design which has become increasingly popular in parallel with decreasing genotyping costs. An advantage with GWAS is that you are able to screen the whole genome without being limited to predefined biological hypotheses. The same hypothesis-independent benefit is present when studying so called positional candidate genes, which have been highlighted because of its location in linkage regions, or in regions with chromosomal abnormalities which segregate with disease in families. This enables researchers to discover new biological mechanisms which might never otherwise have been suggested, based on current knowledge. However, GWAS are still not totally unbiased, because of the non-equal genome coverage of the genotyping chips, giving results which will be biased toward the best covered genes and regions. For six commonly used genotyping chips, the number of genes with 0% coverage (only considering genes with 5 HapMap common
SNPs), and global coverage, both given for the CEU population, ranged from 8-575 genes and 64-93%, respectively [Li et al., 2008]. Depending on the genotyping success rate, the final coverage can be markedly lower than in theory [O’Donovan et al., 2008]. Therefore, candidate gene-based association studies are not yet outperformed by the GWASs, and are still an important complement for well-covered gene- or region-wide studies of potential susceptibility loci suggested based on solid hypotheses.

5.6 Genetics and Schizophrenia

5.6.1 A complex disorder with high heritability

Adoption, twin and family studies of schizophrenia have consistently suggested an important genetic component [Owen, 2000], but the underlying biological mechanisms remain elusive. Schizophrenia is regarded as a complex disorder, where genetic variation as well as environmental effects are involved [Cowan et al., 2002; Hyman, 2000]. The estimated heritability is approximately 80% [Cardno and Gottesman, 2000; Sullivan et al., 2003], clearly reflecting the importance of trying to elucidate which genes or other genetic loci are involved in causing schizophrenia. Even though it is apparent from genetic epidemiology that schizophrenia is a complex disease, the number of susceptibility loci, the extent of genetic heterogeneity, the disease risk conferred by each locus, and the degree of interaction among susceptibility loci are still unknown. A combination of common alleles with small effect and rare alleles with larger effects are probable [Owen et al., 2009] (Figure 1).

![Figure 1](image.png)

**Figure 1.** An overview on the relationship between allele frequency, effect size, and the genetic studies available in common complex disease [modified after Owen et al., 2009].
A high number of studies, and study designs, have been performed and published in
the search for clues on schizophrenia aetiology, reflected by almost 6000 hits from a
gross medical literature search on articles with “schizophrenia” in the title and
including “genetics” (http://www.ncbi.nlm.nih.gov/sites/entrez, accessed 23rd of April
2009). An overview of the main findings is given below.

5.6.2 Linkage studies, positional candidates, chromosomal
abnormalities

By studying inheritance patterns in pedigrees, certain genome regions can be linked to
a disease. If individuals with a disorder of interest inherit certain regions significantly
more often than expected by chance, those are called linkage regions and might
harbour susceptibility loci. A high number of linkage studies have been performed
within the field of schizophrenia genetics, and even though the replication of these has
been difficult, some potential regions of linkage have emerged [Owen et al., 2005].

Three promising candidate genes for schizophrenia, dysbindin (DTNBP1), NRG1, and
D-amino oxidase activator (DAOA), were initially implicated after LD mapping across
the linked regions 6p24-22, 8p21-22, and 13q34, respectively [Chumakov et al., 2002;
Stefansson et al., 2002; Straub et al., 2002]. The three regions 8p, 22q, and 13q were
suggestively linked with schizophrenia in a meta-analysis of genome-wide linkage
scans, and the first two were highlighted as candidate linkage regions in a subsequent
meta-analysis [Badner and Gershon, 2002; Lewis et al., 2003]. Recently, an extended
version of the study by Lewis et al. (2003) was published, with almost three times
more pedigrees, based on 32 independent genome-wide linkage scans [Ng et al.,
2008]. In the primary analysis, suggestive evidence for linkage was found on
chromosomes 5q and 2q when including all genome scans, and 8p when including the
22 European-ancestry scans only. A difficulty with linkage studies is that methods that
are designed to detect a single major gene have been used. This may be a weak
strategy for complex disorders, where family and linkage data suggest that multiple
genes of small effect are much more likely [Risch, 2000]. However, even though
single variants of weak effects are impossible to detect with linkage, the pooled effects
of several rare or common copy number variants or SNPs, in a gene or chromosomal
region, could produce linkage signals. In this aspect, linkage studies are flexible to
allelic heterogeneity, and linkage regions suggested across several family sets might be
of interest for in-depth studies [Holmans et al., 2009].

By studying chromosomal abnormalities segregating with psychiatric disease in
families, several potential susceptibility genes have emerged, such as DISC1 and
phosphodiesterase 4B (PDE4B) [Millar et al., 2004; Millar et al., 2005].

5.6.3 Candidate based association studies

Several reviews on the topic of candidate genes and schizophrenia are at hand, providing overviews of suggested susceptibility genes and their genetic evidence for being true causative genes [Harrison and Weinberger, 2005; Straub and Weinberger, 2006; Williams et al., 2009]. A vast number of genetic association studies have been performed to elucidate the potential true involvement of genes of interest, and the field is hampered with inconsistent findings which are difficult to evaluate and interpret. For a constantly evolving overview of these schizophrenia genetic association studies, the SZGene database was created [Allen et al., 2008] (www.schizophreniaforum.org/res/sczgene), providing a platform for navigation of studied candidate genes and a basis for systematic meta-analyses. The database included 1439 studies of 7094 polymorphisms in 787 genes, and the gene in first place on the top results list was DISC1 (by 17th of May, 2009). Numerous association studies on DISC1 has been performed, one including the Scandinavian Collaboration on Psychiatric Etiology (SCOPE) sample used in this thesis [Saetre et al., 2008], and strong evidence for the involvement of DISC1 in schizophrenia etiology has emerged [Porteous et al., 2006].

The inconsistency in numerous association studies on schizophrenia may be due to both false positives and negatives, and true variability in associations across different populations. An important trend in association studies today is the progress from using small sample sizes (e.g. 200 cases & 200 controls), and the genotyping of a few markers, towards gene-wide genotyping of candidate regions using tagSNPs, in larger samples. Several collaborative efforts have evolved in order to achieve larger samples, e.g. the SGENE consortium (www.sgene.eu), and SCOPE, providing large multi-center samples which are both used in this thesis. Through association analysis in ten independent case-control samples a SNP in the vicinity of the gene encoding fibroblast growth factor receptor 2 (FGFR2) was suggested as a potential susceptibility gene for schizophrenia [O'Donovan et al., 2009]. The first study to systematically select SNPs for LD-based gene-wide genotyping of the strong candidate gene NRG1, found a new gene region to be associated with schizophrenia [Thomson et al., 2007].

5.6.4 Genome wide association studies (GWAS)

It is estimated that approximately 16,000 genes are expressed in the brain [Insel and Collins, 2003], but only a fraction of these genes have been investigated so far. Instead of trying to choose candidates among this large brain-related gene pool, based on own
hypotheses limited by the current knowledge, GWAS has been introduced in the field of psychiatry. So far, six schizophrenia GWAS has been published, three based on DNA pooled samples and three on individual genotyping, without any findings reaching strict genome-wide significance ($P=5\cdot10^{-8}$) [Owen et al., 2009]. However, one of the studies investigated all hits below a threshold of $P=5\cdot10^{-5}$ in a second follow-up sample, and three out of twelve loci were associated with $P<5\cdot10^{-4}$ in the second run. The best hit from meta-analysis was a locus nearby a putative transcription regulator ($\text{zinc finger protein 804A, ZNF804A}$) ($P=1.61\cdot10^{-7}$), and when including about 2,000 extra bipolar disorder samples the association was strengthened ($P=9.96\cdot10^{-9}$) [O'Donovan et al., 2008]. One of the studies based on DNA pooling found suggestive evidence for a marker in $\text{RELN}$ only in women. This female-specific effect was tested in four additional samples: the marker was nominally associated in one of the samples, with non-significant effects in the same direction in the other three, and a meta-analysis $P=8.8\cdot10^{-7}$ [Shifman et al., 2008].

A large collaboration effort has been initiated, Psychiatric GWAS Consortium (PGC; http://pgc.unc.edu), with the aim to perform meta-analyses of GWAS for five psychiatric disorders (Attention Deficit Hyperactivity Disorder (ADHD), Autism, Bipolar disorder, Major depressive disorder, Schizophrenia) [PGC, 2009]. Eleven schizophrenia studies have been included, with a total of 9,588 cases and 13,500 controls, as well as 650 trios. In a near future, PGC will be able to provide potentially revolutionizing new data for the community of psychiatric research. Whatever outcome is ahead, such large combined GWAS will be informative in how to proceed with future schizophrenia genetic studies, and will provide guidance when it comes to questions such as: is the common disease-common variant hypothesis valid and important for schizophrenia etiology?

### 5.6.5 Studies of copy number variations (CNVs)

It is now apparent that the presence of CNVs in the human genome is extensive [McCarroll et al., 2008]. CNVs have been associated with severe neurodevelopmental disorders such as autism [Sebat et al., 2007], and mental retardation [Sharp et al., 2008]. The association between a 22q11.2 microdeletion and psychiatric disease has been reported since the 1990s, and the so called velo-cardio-facial syndrome (22q11.2 deletion syndrome) is associated with a dramatically increased risk for schizophrenia [Bassett and Chow, 2008]. Recently, several publications have provided additional evidence for the involvement of CNVs in schizophrenia etiology. The total burden of duplications and deletions was shown to be markedly increased in schizophrenia
patients [International Schizophrenia Consortium, 2008; Walsh et al., 2008; Xu et al., 2008]. CNVs within the neurexin 1 gene (NRXN1), has been independently reported in two affected siblings and their mother [Walsh et al., 2008], in identical twins concordant for childhood-onset schizophrenia [Kirov et al., 2008], to a higher extent in schizophrenia patients compared with controls in a large multicenter European case-control sample [Rujescu et al., 2009], and in patients with autism [Szatmari et al., 2007]. NRXN1 is a synaptic cell adhesion molecule, implicated in synaptogenesis. In addition, two large rare deletions (15q13.3 and 1q21.1) were independently shown to associate with schizophrenia, [International Schizophrenia Consortium, 2008; Stefansson et al., 2008], and mental retardation [Sharp et al., 2008]. Although the deletions were present in only 0.17% (15q13.3) and 0.23% (1q21.1) of the schizophrenia cases in the study by Stefansson et al., when compared with control frequencies, they conferred a highly increased risk for disease, with odds ratio (OR) of 11.5 and 14.8, respectively [Stefansson et al., 2008].

5.6.6 Genetic studies using endophenotypes and clinical features

Schizophrenia is a heterogeneous disorder, lacking characteristic biological markers or laboratory tests for diagnostics. One approach to assess a phenotype with a potentially closer and less complex link to specific underlying biological mechanisms, with presumably fewer genes involved, is to define so called endophenotypes (intermediate phenotypes). The endophenotype is a quantifiable trait, and five criteria have been suggested to be useful in the identification of such traits: the endophenotype should be heritable, associated with illness in the population, primarily state-independent, co-segregating with illness in families, and found in nonaffected family members at a higher rate than in the general population [Gottesman and Gould, 2003]. The first step to find schizophrenia endophenotypes is commonly to study deficits which are associated with the disease, followed by heritability estimates [Braff et al., 2007]. Such an endophenotype could be neurophysiological, biochemical, endocrinological, neuroanatomical, cognitive, or neuropsychological. About six years ago, a large consortium was initiated, with the goal to investigate the occurrence and genetic architecture of quantitative endophenotypes associated with schizophrenia [Calkins et al., 2007]. The following endophenotypes, with reported deficits both in schizophrenia patients and their first-degree relatives, were included and subsequently shown to be heritable: three neurophysiological measures (P50 event-related potential suppression, prepulse inhibition of the startle response, and the antisaccade task for eye movements), as well as three neurocognitive measures (the Continuous Performance Test (CPT), the California Verbal Learning Test, second edition (CVLT-II), and the Letter-Number-Sequencing test (LNS)) [Greenwood et al., 2007]. In addition, brain
morphometric abnormalities were suggested by others to be useful endophenotypes [Keshavan et al., 2007]. Studies of endophenotypes and genetic variation have reported associations, such as between DISC1 variation and both cognitive function [Porteous et al., 2006], and reduced prefrontal gray matter [Cannon et al., 2005], as well as for dopamine D2 receptor (DRD2) gene variants and working memory performance and brain activity in the prefrontal cortex and striatum [Bertolino et al., 2009].

Subtypes of schizophrenia defined by core symptoms or clinical characteristics, such as age at onset, has also been suggested to better represent the underlying genetic mechanisms [Fanous and Kendler, 2008]. The age of onset of psychosis in schizophrenia have recently been shown to be heritable [Hare et al., 2009], and age at onset was associated with Brain derived neurotrophic factor (BDNF) variants [Numata et al., 2006]. Based on symptom measures using the Positive and Negative Syndrome Scale (PANSS), a DTNBP1 haplotype was associated with hostility/excitement symptoms [Corvin et al., 2008], and a DAO SNP allele with depression/anxiety [Corvin et al., 2007]. DTNBP1 haplotypes were previously associated with negative symptoms using other rating scales [DeRosse et al., 2006; Fanous et al., 2005].

5.7 Genetic overlap of bipolar disorder and schizophrenia

In current psychiatric diagnostic systems (DSM-IV and ICD-10), schizophrenia and bipolar disorder are treated as distinct disease entities, in line with the dichotomous classification by Emil Kraepelin from the early 19th century. Schizophrenia and bipolar disorder were divided into what he then called dementia praecox, and manic-depressive insanity, respectively, but he later doubted this separation [Jablensky, 1999]. Whether these disorders are in fact part of the same psychiatric continuum, with shared underlying biological mechanisms is currently debated [Craddock and Owen, 2007; Crow, 2008]. Convincing evidence that bipolar disorder and schizophrenia have a partially shared genetic cause was recently presented in a large Swedish population-based study [Lichtenstein et al., 2009]. Relatives of probands with bipolar disorder, including adopted away children of bipolar disorder biological parents, had an increased risk for schizophrenia. Common causative risk factors for schizophrenia and bipolar disorder has additionally been suggested based on other findings, such as potential endophenotype measures associated with both schizophrenia and bipolar disorder, e.g. reduced white matter densities [McIntosh et al., 2005; Sussmann et al., 2009] and reduced anterior thalamic gray matter [McIntosh et al., 2004], common candidate susceptibility genes [Craddock et al., 2006], gene expression abnormalities
in post mortem brain tissue of both disorders [Guidotti et al., 2000], common epidemiological risk factors, e.g. winter/spring birth [Torrey, 1999], and similarities in cognitive impairment, especially in schizophrenia and psychotic bipolar disorder [Simonsen et al., 2009]. Recent evidence also suggests that antipsychotic drugs are effective in both disorders, and several atypical antipsychotics are now approved for bipolar disorder.
5.8 Aims of the study

The overall aim of this thesis was to gain more knowledge about the molecular genetic basis of schizophrenia, by performing case-control candidate gene-based studies using common SNP genotypes, and with special emphasis on genes important for neurodevelopmental processes, such as neuronal migration.

Specifically, the aims were to:

1) investigate if variants of 18 candidate genes involved in neuronal migration are associated with schizophrenia
   (Paper I)

2) assess the potential involvement of DISC1 interactor PDE4B in schizophrenia etiology, and a) test if there is a difference in association results between men and women as previously reported, b) investigate if variants of the PDE4B gene could be part of a genetic overlap with bipolar disorder susceptibility, c) investigate if PDE4B gene variants are associated with the extent of positive and negative symptoms in schizophrenia and bipolar disorder patients
   (Paper II)

3) explore if variants of 10 key genes in the HNK-1 pathway and PNs are associated with schizophrenia, and a) study potential effects on age at onset and the extent of positive and negative symptoms in schizophrenia patients, as well as b) effects on learning and memory function, and IQ, in schizophrenia patients and controls
   (Paper III)

4) Test the potential association between GRIK3 variants and schizophrenia, as well as with the extent of positive and negative symptoms
   (Paper IV)
6. Materials and methods

6.1 Study samples

The following three case-control samples have been used in this thesis:

- **SCOPE schizophrenia sample** [≈ 850 cases & 1600 controls]
  Schizophrenia spectrum cases (schizophrenia, schizoaffective, and schizophreniform disorder) and controls from Norway, Sweden and Denmark.

- **SCOPE bipolar disorder sample** [≈ 600 cases & 1400 controls]
  Bipolar disorder cases and controls from Norway and Denmark.

- **SGENE-plus sample** [≈ 2,700 cases and 13,500 controls]
  Schizophrenia cases and controls from Iceland, Scotland, Germany, England, Italy, and Finland

6.1.1 SCOPE schizophrenia sample description

The SCOPE sample builds on and extends the Thematically Organized Psychoses (TOP) study in Norway, the Human Brain Informatics (HUBIN) study in Sweden, and the Danish Psychiatric Biobank in Denmark. The Norwegian and Danish studies are continuously including new patients and controls.

Patients with schizophrenia, schizoaffective or schizophreniform disorder, and age 16-65 years, are included in the TOP study, HUBIN and Danish Psychiatric Biobank, from the departments of the University Hospitals of Oslo, from psychiatric clinics in northwestern Stockholm County, and from the psychiatric departments at the six hospitals in the Copenhagen region, respectively. The Norwegian, Swedish, and Danish schizophrenia patients are diagnosed with the Structural Clinical Interview for DSM-IV (SCID), DSM-III-R/DSM-IV based on interviews and record reviews, or ICD-10, respectively. The protocol for the SCOPE studies is approved by the local Committees for Medical Research Ethics and the Data Inspectorates.

*The Norwegian sample*

The Norwegian patients had been recruited to the TOP study from all the psychiatric hospitals in the Oslo area, and clinically diagnosed with schizophrenia,
schizoaffective, or schizophreniform disorder. Two clinical professors continuously trained and supervised a group of research fellows in order to secure the quality of the clinical assessments. Reliability of the diagnosis has recently been tested, and the percentage of agreement was 82%, and Kappa 0.77 (95% CI: 0.60–0.94).

The healthy Norwegian control subjects were randomly selected from the same catchment area as the patient groups, of Caucasian origin and born in Norway. Moreover, all participants had to have Norwegian as their first language or have received their compulsory schooling in Norway. The control subjects were screened by interview and with the Primary Care Evaluation of Mental Disorders (PRIME-MD). None of the control subjects had a history of moderate/severe head injury, neurological disorder, mental retardation or an age outside the range of 18-60 years. Healthy subjects were excluded if they or any of their close relatives had a lifetime history of a severe psychiatric disorder (schizophrenia, bipolar disorder and major depression), if themselves have a history of medical problems thought to interfere with brain function (hypothyroidism, uncontrolled hypertension and diabetes), or significant illicit drug use.

The Danish sample
All patients had been clinically diagnosed with schizophrenia (F20) or schizoaffective disorder (F25), without ever having received a diagnosis of mania or bipolar illness (F30-31). An experienced research- and consultant psychiatrist verified high reliability of the clinical diagnoses [Jakobsen et al., 2005] using Operational Criteria Checklist for Psychotic Illness and Affective Illness (OPCRIT) semi-structured interviews. The majority (85%) of the patients were ethnical Danish, i.e. the patients and both parents were born in Denmark, while in a minor fraction of the cases (15%) one parent was Caucasian and born outside Denmark in another North-western European country, primarily in Sweden or Norway, but also some in Germany, the Netherlands, England or France.

The Danish controls were randomly selected out of a population of 15,000 blood donors from the Danish Blood Donor Corps in the Copenhagen area, which includes >5% of the Danish population who donate blood on a voluntary and unpaid basis. Apparent behavioral abnormality was an exclusion criterion and all individuals stated that they felt completely healthy with a possibility to discuss any health related issues with a physician. Two unrelated healthy control subjects of Danish Caucasian origin were matched to each patient on gender, year of birth and month of birth, with matching ethnicity.
The Swedish sample
Swedish patients had been recruited from psychiatric clinics in northwestern Stockholm County, and clinically diagnosed with schizophrenia, schizoaffective, or schizophreniform disorder. All patients were Caucasian. Based on the birth country of the grandparents or greater grandparents, 79%, 12% and 9% of the patients were estimated to be of Swedish, Finnish or other European origin, respectively.

The Swedish control subjects were recruited among subjects previous participating in biological psychiatric research at the Karolinska Institute or drawn from a representative register of the population in Stockholm County. All controls were Caucasian and 86%, 6%, and 8% were estimated to be of Swedish, Finnish or other European origin, respectively. The mean age was 40.5 (+/−9.8) years when entering the study. None of the controls suffered from schizophrenia.

6.1.2 SCOPE bipolar disorder sample description
The Norwegian sample
The Norwegian bipolar disorder patients were recruited to the TOP study as the schizophrenia patients, and were diagnosed with bipolar disorder type I, bipolar disorder type II, and bipolar disorder not otherwise specified, according to DSM-IV using SCID.

The criteria for inclusion of the control subjects are the same as stated above for the Norwegian controls in the SCOPE sample. A subset (n=152) of the controls in the bipolar sample in Paper II, are overlapping with controls in the schizophrenia case-control sample.

The Danish sample
The Danish patients had been included all over Denmark (1996-1998), or in the Copenhagen area by the Danish Psychiatric Biobank (2002-2007). The first patient group had been diagnosed with Schedules for Clinical Assessment in Neuropsychiatry (SCAN) [Wing et al., 1998] interviews fulfilling a best estimate diagnosis of bipolar affective disorder and BPI, according to the ICD-10-DCR [WHO, 1993] and the DSM-IV, respectively. The latter group was clinically diagnosed with bipolar affective disorder according to ICD-10-DCR.

The Danish controls were distinct from those in the schizophrenia case-control sample, but recruited as previously described above, or included as selected controls screened for psychiatric disease in a previous study [Mellerup et al., 2001].
6.1.3 SGENE-plus sample description
Genotypes of SNPs serving as proxies for six tagSNPs genotyped in SCOPE in Paper III, were drawn from a genome-wide data set of a multi-national European sample. This replication sample included 1,321 patients affected with schizophrenia and 12,277 control individuals from Iceland, Scotland, Germany, England, Italy, and Finland (the SGENE sample, www.SGENE.eu); as well as 1,342 schizophrenia patients and 1,221 control individuals from Scotland and Germany (the SGENE-plus sample). Ethical approval was obtained from the local Ethical Committees. All participants have given written informed consent prior to inclusion into the project.

6.1.4 Clinical features and endophenotypes in the TOP study
At the time of inclusion, Norwegian patients’ symptoms are assessed using a number of different tests, such as the PANSS. At a later occasion the neurocognitive performance of both patients and controls is measured with a comprehensive test battery (see below). In Paper II and IV, we searched for susceptibility-modifier variants in PDE4B and GRIK3, respectively, for association with PANSS positive and negative sum scores. In Paper III we included neurocognitive measures of learning and memory, as well as IQ, in addition to PANSS scores and age at onset, and searched for both susceptibility-modifier and pure modifier variants, based on all tagSNPs in the three genes of the HNK-1 pathway, including B3GAT2.

Neurocognitive tests
A unit for neurocognitive testing is established in the TOP group in collaboration with the Institute of Psychology, UiO (Prof. K. Sundet), and all Norwegian patients go through the following tests: Grooved Pegboard (Motor function), Coding/Trail Making (Psychomotoric tempo), Number memory (Attention), Paced Auditory Serial Addition Task (PASAT) (Working Memory), CVLT-II (Executive function), Wechsler Abbreviated Scale of Intelligence (WASI), National Adult Reading Test (NART) (Premorbid level). In the follow-up study, the following tests are also included: CPT (Attention /Vigilance), N-Back (Working Memory), Continous Visual Memory Test (CVMT), Wisconsin Card Sorting Test (WCST) (Executive function), Face/Voice Emotion (Social cognition), Assessment of Interpersonal Problem Solving Skills (AIPSS), WCST +/- help (Learning potential), The Social Functioning Scale (Daily Life function).

Positive and negative syndrome scale (PANSS)
PANSS is an instrument for measurement of the severity of symptoms, based on 30 psychiatric parameters, which are part of a positive scale (delusions, conceptual
disorganization, hallucinatory behavior, excitement, grandiosity, suspiciousness, hostility), a negative scale (blunted affect, emotional withdrawal, poor rapport, passive-apathetic social withdrawal, difficulty in abstract thinking, lack of spontaneity & flow of conversation, stereotyped thinking), and the 16 remaining items are part of a general psychopathology scale. For each parameter the minimum and maximum scores are 1 and 7, respectively (1=absent, 2=minimal, 3=mild, 4=moderate, 5=moderate-severe, 6=severe, and 7=extreme), which means that the potential ranges for the positive and negative symptom scales are 7-49 each.

At the time of inclusion, the patients’ symptoms are assessed with PANSS scores, reflecting the status during the last week before inclusion in the TOP study. All raters participated in a structured training course, and reliability was measured based on videotaped actual case interviews. Inter-rater reliability was good, with an intra-class correlation coefficient (1.1) of 0.73, 0.73 and 0.71 for PANSS positive, negative and general subscales, respectively.

6.1.5 Genetic features of the TOP sample

Based on GWAS data from the Affymetrix Genome-Wide Human SNP Array 6.0 on the Norwegian TOP sample [Athanasiu et al., manuscript], the overall genetic structure for this sample has been compared with HapMap samples representing ten different populations (Figure 2). Norwegians cluster mainly with the CEU sample, comprised of Americans with north-western European ancestry. When visualizing the Norwegian TOP data set alone, to compare the schizophrenia spectrum and bipolar disorder patients, as well as controls, it is evident that the three groups are very similar in the overall genetic structure (Figure 3). The TOP dataset in Figure 2 and 3 contain 747 samples, including 89% of the Norwegian schizophrenia data sets used in Paper I, II, IV, and 83% of the data set in Paper III.
Figure 2. Multidimensional scaling (MDS) based on 535,000 SNPs genotyped in the Norwegian TOP sample (n=747) and ten separate HapMap samples. On top, the first component (x-axis) is plotted against the second (y-axis), and below, the first component (x-axis) is plotted against the third (y-axis). TOP QC1: TOP sample after main quality control; ASW: African ancestry in Southwest USA; CEU: Utah residents with Northern and Western European ancestry from the CEPH collection; CHB: Han Chinese in Beijing, China; GIH: Gujarati Indians in Houston, Texas; JPT: Japanese in Tokyo, Japan; LWK: Luhya in Webuye, Kenya; MEX: Mexican ancestry in Los Angeles, California; MKK: Maasai in Kinyawa, Kenya; TSI: Toscans in Italy; YRI: Yoruban in Ibadan, Nigeria.
Figure 3. Principal components analysis (PCA) on 535,000 SNPs genotyped in the Norwegian TOP sample (n=747), including schizophrenia spectrum patients, bipolar patients, and controls. Principal component 2 (PCA2, x-axis) is plotted against principal component 1 (PCA1, y-axis), showing a complete overlap between the three groups. This overlap is similar in the corresponding PCA1-PCA3 plot (not shown).

6.2 The candidate genes studied

In total, 30 genes have been investigated in Paper I-IV. An overview of all genes, with their chromosomal localization, is shown in Table 1. Genes involved in the process of neuronal migration, the formation of perineuronal nets during brain maturation, the HNK-1 biosynthesis, and glutamate signaling were included, some of which had been associated with schizophrenia previously. PDE4B was included mainly because of its direct interaction with DISC1, which regulates its cAMP hydrolysis function, and the gene was also independently associated with increased risk for schizophrenia in a family with a balanced translocation [Millar et al., 2005]. Several of the genes encode proteins that interact with DISC1 directly (PAFAH1B1 [LIS1], NDEL1, YWHAE) [Camargo et al., 2007], or with a DISC1 interactor (CDK5,
CDK5R, MAP1B, CDC42) [Jimenez-Mateos et al., 2005; Kholmanskikh et al., 2006; Niethammer et al., 2000].

Table 1. The candidate genes.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Chromosomal Position</th>
<th>Biological Process of Interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASTN1</td>
<td>1q25.2</td>
<td>Neuronal migration – adhesion</td>
</tr>
<tr>
<td>MDGA1</td>
<td>6p21.2</td>
<td>Neuronal migration – adhesion</td>
</tr>
<tr>
<td>SPARCL1</td>
<td>4q22-q25</td>
<td>Neuronal migration – adhesion</td>
</tr>
<tr>
<td>ITGA3</td>
<td>17q21.33</td>
<td>Neuronal migration - adhesion &amp; motility</td>
</tr>
<tr>
<td>RELN</td>
<td>7q22.1</td>
<td>Neuronal migration - adhesion &amp; motility</td>
</tr>
<tr>
<td>CDC42</td>
<td>1p36.12</td>
<td>Neuronal migration – motility</td>
</tr>
<tr>
<td>CDK5</td>
<td>7q36</td>
<td>Neuronal migration – motility</td>
</tr>
<tr>
<td>CDK5R1</td>
<td>17q12</td>
<td>Neuronal migration – motility</td>
</tr>
<tr>
<td>ENAH</td>
<td>1q42.12</td>
<td>Neuronal migration – motility</td>
</tr>
<tr>
<td>EVL</td>
<td>14q32.32</td>
<td>Neuronal migration – motility</td>
</tr>
<tr>
<td>FILIP1</td>
<td>6q14.1</td>
<td>Neuronal migration – motility</td>
</tr>
<tr>
<td>MAP1B</td>
<td>5q13.2</td>
<td>Neuronal migration – motility</td>
</tr>
<tr>
<td>NDEL1</td>
<td>17p13.1</td>
<td>Neuronal migration – motility</td>
</tr>
<tr>
<td>PAFAH1B1 (LIS1)</td>
<td>17p13.3</td>
<td>Neuronal migration – motility</td>
</tr>
<tr>
<td>VASP</td>
<td>19q13.2-13.3</td>
<td>Neuronal migration – motility</td>
</tr>
<tr>
<td>YWHAE</td>
<td>17p13.3</td>
<td>Neuronal migration – motility</td>
</tr>
<tr>
<td>DLX1</td>
<td>2q31.1</td>
<td>Neuronal migration - transcription</td>
</tr>
<tr>
<td>PAX6</td>
<td>11p13</td>
<td>Neuronal migration - transcription</td>
</tr>
<tr>
<td>PDE4B</td>
<td>1p31</td>
<td>cAMP signalling</td>
</tr>
<tr>
<td>BCAN</td>
<td>1q31</td>
<td>Perineuronal net formation</td>
</tr>
<tr>
<td>HAPLN1</td>
<td>5q14.3</td>
<td>Perineuronal net formation</td>
</tr>
<tr>
<td>HAPLN2</td>
<td>1q23.1</td>
<td>Perineuronal net formation</td>
</tr>
<tr>
<td>HAPLN3</td>
<td>15q26.1</td>
<td>Perineuronal net formation</td>
</tr>
<tr>
<td>HAPLN4</td>
<td>19p13.1</td>
<td>Perineuronal net formation</td>
</tr>
<tr>
<td>NCAN</td>
<td>19p12</td>
<td>Perineuronal net formation</td>
</tr>
<tr>
<td>TNR</td>
<td>1q24</td>
<td>Perineuronal net formation</td>
</tr>
<tr>
<td>B3GAT1</td>
<td>11q25</td>
<td>HNK-1 biosynthesis</td>
</tr>
<tr>
<td>B3GAT2</td>
<td>6q13</td>
<td>HNK-1 biosynthesis</td>
</tr>
<tr>
<td>CHST10</td>
<td>2q11.2</td>
<td>HNK-1 biosynthesis</td>
</tr>
<tr>
<td>GRIK3</td>
<td>1p34-33</td>
<td>Glutamate signalling</td>
</tr>
</tbody>
</table>

6.3 Genotyping technologies

The SNPlex™ Genotyping System

Thirty-one tagSNPs in six of the genes in Paper I, as well as 103 tagSNPs in the ten genes of Paper III, were genotyped with the 48-plex SNPlex technology (Applied Biosystems, Foster City, CA, USA), at the Department of Medical Genetics (Oslo
University Hospital-Ulleval, Oslo, Norway). The assay was performed in a 384-plate format, with four equal control CEPH-samples and four buffer samples on each plate, in addition to eight positive and eight negative controls included in the SN Plex kit. All pipetting steps were performed using Biomek Fx robots (Beckman Coulter Inc., Fullerton, CA, USA). DNA concentration was measured with the Quant-iT DNA Assay Kit (Invitrogen Corporation, Carlsbad, CA, USA), and diluted to around 40 ng/μl. In the first step, 3 μl of genomic DNA was dispensed into a chilled 384-well plate and placed in a thermal cycler for fragmentation, 1 minute at 4°C, 5 minutes at 99°C, followed by cooling to 4°C. The SN Plex oligonucleotide ligation assay (OLA) reaction mix was prepared, composed of allele- and locus-specific probes, universal linkers, enzymes promoting 5’-end phosphorylation of probes and linkers, as well as ligase for ligation with the genomic DNA. 3 μl was added to the 384-well plate with DNA, and the ligation reaction was run using a thermal cycler. The subsequent steps were performed according to the protocol provided by the manufacturer (Applied Biosystems). In brief, first, the ligation products from the previous step were purified using two exonucleases to digest parts of the ligated reaction products, unligated oligonucleotides and genomic DNA. Second, the purified ligation product was PCR amplified, the biotinylated PCR product were bound to streptavidin covered plate wells. Allele calling was performed automatically using the GeneMapper software 4.0 (Applied Biosystems), with 384 samples clustered simultaneously using the “Rules” clustering algorithm. The automatic allele calling was manually evaluated, and samples appearing among negative control samples in the polar plots were excluded from further analyses.

**The GoldenGate® Assay**

Two hundred fifty-eight tagSNPs in 12 of the genes in Paper I, 40 and 72 PDE4B tagSNPs in the schizophrenia and bipolar disorder sample of Paper II, respectively, as well as the 30 GRIK3 tagSNPs of Paper IV, were genotyped with the 1536-plex GoldenGate assay (Illumina, Inc., San Diego, CA) on Illumina BeadStation 500GX at the SNP Technology Platform, Uppsala University, Sweden (www.genotyping.se), accredited by the Swedish accreditation agency SWEDAC, and approved according to a quality system based on the international SS-EN ISO/IEC 17025 standard.

**The TaqMan® SNP Genotyping Assay**

Six tagSNPs (Paper III) were genotyped by the single-plex Taqman assay (Applied Biosystems, Foster City, CA, USA), because they failed to be genotyped by the SN Plex technology. The genotyping was performed at the Department of Medical Genetics (Oslo University Hospital-Ulleval, Oslo, Norway).
6.4 Statistics

6.4.1 Statistical tests and methods

*Hardy Weinberg equilibrium*
The exact $\chi^2$ test was used to test for departure from Hardy Weinberg Equilibrium (HWE) in controls. In **Paper I**, we corrected the significance threshold with the number of genes tested ($P=0.05/18=0.0028$), being conservative but at the same time taking into account the high number of genes tested. In **Paper IV**, with only one gene tested, we used the uncorrected nominal threshold of 0.05 as exclusion criteria. In **Paper II** and **III**, the default threshold 0.001, in the software PLINK, was used.

*The fixation index*
To estimate the genetic heterogeneity between the three Scandinavian samples, we calculated the overall fixation index, $F_{ST}$, using the controls. An $F_{ST}$ was calculated for each gene with at least three SNPs genotyped, and then the weighted mean over all gene-based $F_{ST}$ was the overall $F_{ST}$, with the SNP number in each gene used as weights.

*Standard and stratified singlemarker association tests*
The most common test for genetic association is the standard $\chi^2$ test, based on a $2 \times 2$ (Table 2) or $2 \times 3$ (Table 3) table, depending on if testing for independence of the phenotype from alleles or genotypes, respectively. The null hypothesis of no association is rejected if the $\chi^2$ test statistic is large enough.

**Table 2.** $2 \times 2$ contingency table for SNP alleles.

<table>
<thead>
<tr>
<th>Allele</th>
<th>A</th>
<th>a</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency/Cases</td>
<td>A1</td>
<td>A2</td>
<td>Atot</td>
</tr>
<tr>
<td>Allele count Controls</td>
<td>C1</td>
<td>C2</td>
<td>Ctot</td>
</tr>
<tr>
<td>Total</td>
<td>A</td>
<td>a</td>
<td>N</td>
</tr>
</tbody>
</table>

**Table 3.** $2 \times 3$ contingency table for SNP genotypes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>AA</th>
<th>Aa</th>
<th>aa</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency/Cases</td>
<td>A1</td>
<td>A2</td>
<td>A3</td>
<td>Atot</td>
</tr>
<tr>
<td>Allele count Controls</td>
<td>C1</td>
<td>C2</td>
<td>C3</td>
<td>Ctot</td>
</tr>
<tr>
<td>Total</td>
<td>AA</td>
<td>Aa</td>
<td>aa</td>
<td>N</td>
</tr>
</tbody>
</table>
The allele-based test statistic is calculated as follows (with each included factor given in Table 2):

\[ \chi^2_{\text{allele}} = N \frac{(A2 \cdot C1 - A1 \cdot C2)^2}{Atot \cdot Ctot \cdot A \cdot a} \]

When using the standard \( \chi^2 \) test to compare genotype frequencies between cases and controls, the observed frequencies are compared with those expected under the null hypothesis. The test statistic is calculated as follows (with each included factor given in Table 3):

\[ \chi^2_{\text{geno}} = \frac{(A1 - AA \cdot A_{tot}^2)}{AA \cdot A_{tot}^2} + \frac{(A2 - Aa \cdot A_{tot}^2)}{Aa \cdot A_{tot}^2} + \frac{(A3 - aa \cdot A_{tot}^2)}{aa \cdot A_{tot}^2} + \frac{(C1 - AA \cdot C_{tot}^2)}{AA \cdot C_{tot}^2} + \frac{(C2 - Aa \cdot C_{tot}^2)}{Aa \cdot C_{tot}^2} + \frac{(C3 - aa \cdot C_{tot}^2)}{aa \cdot C_{tot}^2} \]

In the present thesis, three multi-national case-control samples were used, combining several independent subsamples, from three Scandinavian countries (Paper I-IV), as well as the SGENE-plus North-European countries (Paper III). In all initial association tests, the covariate “sample origin” has been included, to correct for potential differences between the independent subsamples. Standard \( \chi^2 \)-tests were used to compare effects sizes between the separate subsamples (Paper I, II, IV).

A commonly used statistical method for stratified association analysis is the Cochran-Mantel-Haenszel (CMH) \( \chi^2 \) test for independence of i x j x k contingency tables, where k is the number of strata (=number of subsamples in this case). We used both allele-based 2 x 2 x k (Paper I, III, IV), and genotype-based 2 x 3 x k (Paper I, IV) tests. To evaluate the effect size of the potential risk conferred by a certain genetic marker studied, the OR is the statistic conventionally calculated. For a SNP with alleles A and a, the OR is the ratio between the odds of being a case when having the A allele and the odds of being a case when having the a allele. More specifically, for a 2 x 2 contingency table (Table 2), where A1, A2, C1, and C2 are the allele frequencies or allele counts, the \( \text{OR} = (A1/C1)/(A2/C2) = A1 \cdot C2/C1 \cdot A2 \). The OR is thus calculated by comparing the allele frequencies between cases and controls for each of the two alleles. The squared OR is also the numerator in the allele-based \( \chi^2 \) test statistic calculation. To determine the uncertainty of the calculated risk measure, a 95% confidence interval is commonly accompanying the OR. The Breslow day test (BD) measures the heterogeneity of the ORs between strata defined in the CMH test, which
in our case are groups based on “sample origin” (Paper I, III, IV), or gender (Paper IV). In Paper II, the software UNPHASED (described below) were used for both allele- and genotype-based single marker, as well as haplotype, analysis. The software uses a retrospective likelihood, and to account for possible population stratification, we included country of origin as a confounder factor in the analysis.

To test an additive model, with the covariate sample origin, we used logistic regression in Paper III. In this paper, the non-stratified trend and standard $\chi^2$ tests were performed, with the only intention to compare with the stratified results and not to find potential new associations. When investigating the effects of SNP genotypes on cognitive raw test scores and IQ in the same paper, linear regression analysis, with age and sex as covariates, was used. Three inheritance models: additive, recessive, and dominant, were tested for. For SNPs nominally associated for the same measure in both cases and controls, the interaction between case control status and genotypic effect on the phenotype was investigated.

The effect of genotypes on PANSS positive and negative sum scores were assessed using non-parametric Kruskal Wallis (Paper II and III) and Mann-Whitney tests (Paper III), the first including the three genotypes for each tagSNP as grouping variable, and the latter was performed after re-coding of the genotypes into two groups, according to a recessive or a dominant inheritance model. In Paper IV, the PANSS scores were tested for association with tagSNPs through independent sample t-tests (Paper IV).

Survival analysis with the Cox proportional hazards regression model, with sex and country of origin as covariates, was used to investigate the effects of genotype on AAO. The difference of each of the three genotypes, as well as dominant and recessive inheritance models, was tested for.

Haplotype block estimation and haplotype analysis
Through-out the genome, there are regions of high and low LD, and segments with high LD are referred to as LD blocks, within which there is limited haplotype diversity [Patil et al., 2001]. In Paper I, we estimated haplotype blocks using the method by Gabriel et al. [Gabriel et al., 2002], implemented in Haploview. The estimation was based on the genotyped tagSNPs in our sample, which might be limited. As comparison, I have now retrospectively used the MDGAI genotypes for the HapMap CEU sample, and estimated LD blocks based on the solid spine method implemented in Haploview (D’>0.8) (as in Paper III), and by joining adjacent blocks when multiallelic D’>0.95. The estimated LD blocks are the same as previously, with the
exception of the most 5’ block analyzed for haplotype association, which was increased in size including also the first seven 5’ tagSNPs. In Paper II, the solid spine block estimation was used based on the tagSNP genotypes in the bipolar disorder sample.

Potentially, combinations of tagSNPs into haplotypes might better pick up association signals, if a disease variant is in stronger LD with a certain haplotype than with any single tagSNP. For all papers, haplotype analysis was performed using the software UNPHASED, testing for both global and individual haplotype associations including sample origin as a confounder. In Paper I, all 2-4 marker combinations within all estimated blocks harboring tagSNPs with a P≤0.1 from single markers tests, were tested for association with schizophrenia. In Paper II and IV, the whole gene regions were assessed testing 2-, 3-, and 4-marker sliding windows for association. In Paper III, the haplotype analysis was restricted to one haplotype block, including three tagSNPs nominally associated in the initial analysis, as well as a forth tagSNP. All 2-4 combinations were tested for association.

**Correction for multiple testing**

In Paper I and IV, a so called Nyholt-corrected threshold was presented [Nyholt, 2004], for the single SNP association tests, which implements the LD structure between the markers tested, to take into account their potential non-independency, in order to estimate the number of independent tests. Using this estimated number, a regular Bonferroni correction can be applied. In the thesis papers we used the recommended better estimate of the effective number of markers [Li and Ji, 2005]. Permutation-based correction was used for the single SNP tests in Paper II and III, as well as for all haplotype analyses, shuffling the case control status 1,000-100,000 times. For the haplotype analyses the permutations were performed separately for each sliding window (Paper II and IV), for each specific single marker combination chosen based on the best initial single marker results (Paper I and IV), within each of the separate blocks tested (Paper I and III), and for each of the 2-4 windows within blocks with more than 4 markers (Paper I). A non-combined permutation-correction might not be ideal, but on the other hand, a lot of non-independent tests were included in the haplotype analyses.

**6.4.2 The statistical softwares**

All of the softwares used in this thesis, except for SPSS, are open-source tools and free to download at their respective web pages.
PLINK
PLINK is a toolset designed for whole genome genetic association analysis (pngu.mgh.harvard.edu/purcell/plink/) [Purcell et al., 2007], and the Windows/ MSDOS command line application was utilized for single marker association analysis in three of the papers (Paper I, III, IV), and for the HWE test in two papers (Paper III, IV), using versions 0.99q (Paper I, IV), 1.04 (Paper II), and 1.05 (Paper III).

Unphased
UNPHASED is an application for performing genetic association analysis, which implements maximum-likelihood inference of haplotypes and genetic effects while allowing for uncertain phase and missing genotypes (www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased) [Dudbridge, 2008]. The graphical user interface (GUI) of UNPHASED was used in all four papers for haplotype analysis, and also single marker association tests in Paper II. It was run locally or at the University of Oslo Bioportal (www.bioportal.uio.no/), the latter being a web-based service platform providing access to a large computational cluster, using versions 3.0.8 and 3.0.9 (Paper I), 3.0.10 (Paper IV), and 3.0.13 (Paper II, III).

Haploview
Haploview is an analysis platform that was used for calculation and visualization of LD, as well as haplotype block estimation, in the present thesis (www.broad.mit.edu/haploview/haploview) [Barrett et al., 2005]. The GUI for Windows of versions 3.32 (Paper I, IV), and 4.1 (Paper II, III) was used.

R
The R platform is a software environment for statistical computing and graphics (www.r-project.org/), which was used for data management, as well as genotype-based single marker association tests and the exact HWE test implemented in the Genetics package (Paper I, IV).

Arlequin
The software Arlequin is a GUI-based tool for population genetics data analysis that was used to calculate the Fixation index, F_{ST}, in all four papers, using version 3.1 (cmpg.unibe.ch/software/arlequin3/) [Excoffier et al., 2005].

Single Nucleotide Polymorphism Spectral Decomposition (SNPSpD)
The software SNPSpD is a web-based tool (gump.qimr.edu.au/general/daleN/ SNPSpD) implemented by D. Nyholt [Nyholt, 2004] for a multiple testing correction method. The effective number of markers, M_{eff}, taking into account the LD structure
between markers, can be estimated, as well as the recommended more accurate estimation, $M_{\text{effL}}$ [Li and Ji, 2005].

**SPSS**

One of the products from SPSS Inc. (www.spss.com/) is the statistical program *SPSS Statistics* (renamed into *Predictive Analytics Software (PASW) Statistics*), which was used for the PANSS analyses.
7. Summary of results

All four papers included in this thesis are candidate gene-based association studies of schizophrenia, investigating 18 (Paper I), one (Paper II), 10 (Paper III), and one (Paper IV) gene(s). In each paper the markers have been chosen using pair-wise tagging, with the aim of covering most of the known common variation in the genes. In Paper II, tagSNPs were also genotyped in bipolar disorder patients. The results of each paper are summarized below.

In the sections below, schizophrenia spectrum cases, including patients diagnosed with schizophrenia, schizoaffective disorder, and schizophreniform disorder, are referred to as “schizophrenia”. Patient groups with solely the schizophrenia diagnosis are referred to as “strict schizophrenia”. In all combined analyses of the three Scandinavian samples, the sample origin is included as a covariate.

Paper I: Association analysis of schizophrenia on 18 genes involved in neuronal migration: MDGA1 as a new susceptibility gene.

To investigate if tagSNPs in the candidate genes are associated with schizophrenia, we used a sample of 839 schizophrenia cases + 1,473 controls for 12 genes, and 758 schizophrenia cases + 1,293 controls for 6 genes. The difference in numbers is due to the use of two separate genotyping occasions as well as methods. The overall fixation index F_{ST} was 0.00071 based on the Norwegian, Danish and Swedish control populations, indicating general allele frequency homogeneity between samples. The final coverage of common HapMap SNPs by the genotyped tagSNPs (r^2≥0.8; MAF≥5%; MAF≥20% for RELN), using an updated HapMap data release (Data Rel Jan07), was ≥90% for 11 genes, between 64 and 84% for 6 genes, and only 25% for CDK5. Twenty tagSNPs (out of 289, 6.9%) located in six genes, attained nominal significant P-values (P≤0.05) in genotypic and/or allelic association tests, when comparing schizophrenia cases with controls. When using strict schizophrenia in the follow-up analysis of these 20 tagSNPs, 15 were still nominally significant, despite the reduction in case sample size with over 12%. The stongest association was found for tagSNP rs9462341, located in MDGA1 (schizophrenia: P_{genotypic}=0.00095, P_{allelic}=0.010; strict schizophrenia: P_{genotypic}=0.00068, P_{allelic}=0.0054), together with five other nominally associated tagSNPs (17% of the MDGA1 tagSNPs). The ORs for those five MDGA1 tagSNPs nominally associated in the allelic test were calculated in the combined and three Scandinavian sample(s) separately, showing a risk effect for the same allele in all groups tested. Nine of the RELN tagSNPs (13%) were nominally
associated, five in both genotypic and allelic tests, and two out of six OR calculations showing a risk effect for the same allele in all groups tested. Two out of the three DLX1 tagSNPs assessed were nominally associated in the genotypic test. One out of the eight ITGA3 tagSNPs assessed was nominally associated in the allelic test, as well as in the genotypic test for strict schizophrenia only. The same ITGA3 tagSNP allele conferred a risk effect in the combined as well as the three separate samples. The one ASTN1 and SPARCL1 tagSNP nominally associated, had $P_{HWE}=0.014$ and $P_{BD}=0.05$, respectively. The best finding was above the Nyholt-corrected significance threshold of $P=0.00027$, and no association tests with 2- to 4-marker haplotypes achieved higher significance levels.

To note: For one of the 21 tagSNPs given in the Table III of Paper I, there was only three minor control homozygotes present, and since it was only nominally associated in the genotype test this result was not reliable. It is therefore not included in the mentioned result figures above.

**Paper II: Association study of PDE4B Gene Variants in Scandinavian Schizophrenia and Bipolar Disorder multicenter case-control samples**

To assess the potential involvement of DISC1 interactor PDE4B in schizophrenia and bipolar etiology, we examined the PDE4B gene in a sample of 837 schizophrenia cases + 1,473 controls, as well as 594 bipolar disorder cases + 1,421 partly overlapping (10.7%) controls. Forty and 72 tagSNPs were genotyped and statistically analyzed in the former and latter sample, respectively, with the 40 giving a final coverage of 86% ($r^2 \geq 0.8$, MAF $\geq 20\%$, 318 HapMap SNPs in total), and the 72 a final coverage of 92% ($r^2 \geq 0.8$, MAF $\geq 5\%$, 449 HapMap SNPs in total). The gene-based $F_{ST}$s were 0.00004 and -0.00008 for PDE4B, in the comparison of the Norwegian, Danish, and Swedish controls in the schizophrenia sample, and the Norwegian and Danish controls in the bipolar disorder sample, respectively. No association findings were statistically significant after correction for multiple testing (Best $P \geq 0.17$), but hypothesis-generating nominal associations ($P \leq 0.05$) are summarized.

Two independent tagSNPs ($r^2 = 0.04$) were nominally associated with schizophrenia, and with ORs $\geq 1$ for the major allele in the combined, as well as separate Norwegian, Danish, and Swedish sample. When genders were analyzed separately, there were nominally significant associations only in females, between four tagSNPs and schizophrenia. The major alleles and major allele homozygotes had OR $\geq 1$ in the combined (OR: 1.18-1.48), as well as separate Norwegian, Danish, and Swedish sample.
Eleven tagSNPs were nominally associated with bipolar disorder, two of which are in complete LD ($r^2 = 1.0$). The same allele had OR≥1 in the combined, as well as separate Norwegian, Danish, and Swedish sample. For the tagSNP with the strongest association, the best fitting model was recessive (homozygote minor allele: OR(95%CI) = 4.43(1.78-11.02), $P = 0.00052$). The gender-specific associations were not as consistent as for schizophrenia, with tagSNPs being nominally associated with both females (two) and males (three).

All of the six nominally associated tagSNPs in the schizophrenia sample have either been genotyped themselves or by proxies in the bipolar disorder sample. Two of the proxies for tagSNPs nominally associated in women, were nominally associated with bipolar disorder in the total sample. All four tagSNPs were present in a 48 kb region spanning the $PDE4B3$ splice site. However, increased risk for schizophrenia was associated with being homozygous for the major alleles, while in contrast the homozygotes for the minor alleles displayed increased risk for bipolar disorder.

The potential association between tagSNPs and positive and negative symptom sum scores were tested in Norwegian schizophrenia (n=153; five tests) and bipolar disorder (n=128; twelve tests) patients. One schizophrenia tagSNP was associated with positive symptoms in the total sample ($P = 0.003$), and all of the three tagSNPs that were analyzed in females were associated with positive symptoms ($0.001 \leq P \leq 0.004$). Two of those three tagSNPs have proxies in the bipolar disorder sample, and the proxy tagSNPs were nominally associated with negative symptoms in females with bipolar disorder. This association was stronger in a subsample (n = 36) of women with a history of at least one psychotic event ($P = 0.002$ and $P = 0.007$).

**Paper III: A Study of Ten Genes in the HNK-1 Pathway and Perineuronal Nets: B3GAT2 is associated with schizophrenia in two large European Multi-Center Case Control Samples**

To investigate if the candidate genes are associated with schizophrenia we used a total genotyped sample of 849 schizophrenia cases and 1602 controls, but some samples were only genotyped for a few tagSNPs using the Taqman assay, and the numbers for the best SNP are therefore 823 cases and 1532 controls. The final coverage of common HapMap SNPs by the genotyped tagSNPs ($r^2 \geq 0.8$; MAF$\geq 5\%$), using an updated HapMap data release (Data Rel Jan07), was $\geq 90\%$ for 9 genes, and $80\%$ for $B3GAT1$. The overall $F_{ST}$ was 0.0002, and the gene-based $F_{ST}$s between -0.00003 and 0.0009, indicating general allele frequency homogeneity between samples. Five of the 104 tagSNPs analyzed were nominally associated with schizophrenia in both the allele-
based test (0.005≤P≤0.05), as well as when testing the SNPs for additive effects in a logistic regression model (0.004≤P≤0.05). These SNPs were all located in a region of high D’-based LD in the first intron of B3GAT2. The nominally associated SNPs are still point-wise significant after calculating the empirical P-values by permuting the case control status, but none of the SNPs remain significant after correction for multiple testing.

As a follow up we attempted to replicate the findings using already present GWAS data in the SGENE-plus sample, comprising of 2,663 cases and 13,498 controls. The optimal proxy SNPs were searched for in the B3GAT2 gene, and in an estimated LD block spanning the 5’ start site (approximately 17 kb upstream). All five initial tagSNPs were represented by proxy SNPs with HapMap-based r²=0.61-0.95. Three of the five nominally associated markers were represented by the same proxy SNP, which was associated with schizophrenia in the SGENE-plus sample (P=0.044). These four SNPs were located in the same estimated LD block (based on HapMap CEU), together with a fourth SNP genotyped in our Scandinavian sample, with a proxy SNP also nominally associated in SGENE-plus (P=0.014). The major alleles conferred a risk effect for the four initial tagSNPs and the two proxy SNPs. None of the other 27 SNPs genotyped by us were in higher r²-based LD with the two associated SGENE-plus SNPs, than those nominally associated. The combined P-value for the most associated SNP and its SGENE-plus proxy was 0.002.

Forty-two SNPs were tested for association with IQ, measures of learning and memory, and PANSS positive and negative sum scores in Norwegian patients (n=116; n=145 for the PANSS analysis) and the first two endophenotypes also in controls (n=273). Forty-four SNPs were tested for association with AAO, in a larger Scandinavian case sample (n=801). Each SNP was tested under a recessive, dominant and additive disease model. None of the SNPs associated with schizophrenia in both the SCOPE and SGENE-plus sample were associated with any of the endophenotypes/clinical subtypes in cases, but the risk allele homozygote of one of the other two findings in SCOPE was nominally associated with lower scores in the two WMS tests in controls (measuring learning and memory), compared with the heterozygotes and major allele homozygotes. The same homozygotes have non-significantly lower scores in the smaller case sample. None of the other tagSNPs were significantly associated if corrected for the tests performed. Thirteen SNPs were nominally associated with more than one subphenotype, or in both cases and controls for the cognitive measures. One B3GAT2 tagSNP was associated with four out of five cognitive test scores in controls (IQ, verbal memory, visual memory, verbal learning), and with IQ in patients. Another tagSNP was also associated with IQ in both cases and
controls, and there was an interaction effect between case control status and genotype for both tagSNPs (P=0.003). These markers are in moderate LD (r²=0.53) and located in the second intron of B3GAT2, in an estimated LD block 3’ of the one harboring the SNPs associated with schizophrenia.

**Paper IV: A possible association between schizophrenia and GRIK3 polymorphisms in a multicenter sample of Scandinavian origin (SCOPE)**

To test potential association between tagSNPs in GRIK3 and schizophrenia, as well as with the extent of positive and negative symptoms, we used a sample of 839 schizophrenia cases + 1,473 controls. Thirty tagSNPs were successfully genotyped and their final coverage of common HapMap SNPs (r²≥0.8; MAF≥5%; Data Release Jan07) was 72%. Four tagSNPs were nominally associated with schizophrenia in both allelic and genotypic tests (P≤0.05), and for three of these the ORs pointed in the same direction in the combined and three Scandinavian sample(s) separately. The Nyholt-corrected significance threshold was P=0.0022, and one of the tagSNPs lead to a P-value of 0.001 in the combined sample and P=0.00009 in the Swedish sub sample. When combining multiple alleles using 2-, 3-, and 4-marker sliding window haplotype analysis over the whole gene region, none of the obtained associations were significant after permutation-based correction for multiple testing. When combining alleles of the four most associated tagSNPs, the strongest finding was a 2-marker haplotype of the major alleles (P=1.02·10⁻⁵, OR(95%CI)=1.50(1.25-1.81)). To investigate if the tagSNPs associated with schizophrenia diagnosis also exert effects on the degree of positive or negative symptoms, PANSS measures in a Norwegian patient sub sample (n=129) were used. There was no association between risk tagSNPs and the PANSS positive sum scores. In contrast, there were nominal associations between two of the tagSNPs and PANSS negative sum scores, but this was not significant after correcting for all tests performed.
8. Discussion

8.1 Findings and interpretations

The four papers that form the basis of this thesis are all genetic association studies aimed at investigating the role of common variants in schizophrenia susceptibility, with special focus on neurodevelopment. Genes involved in neuronal migration and the formation of perineuronal networks, a glutamate receptor subunit gene, and an interactor of DISC1, are included in Paper I-IV. In total, tagSNPs in 30 different genes have been tested for association with schizophrenia, in a homogenous Scandinavian sample set.

The strongest finding was the association of a 2-marker haplotype of common alleles in GRIK3 (Paper IV), and one of the tagSNPs in the associated haplotype was associated in the single marker allele-based test after correcting for the 30 tagSNPs assessed. Other results are at the nominal association level ($P \leq 0.05$), and did not remain significant after correction for multiple testing within each of the four papers. However, for the association with tagSNPs in B3GAT2 (Paper III), two proxy SNPs were also associated in a large multi-national European sample, which strengthens this finding. The nominal tagSNP associations in the remaining papers should be viewed as findings in need of replication, albeit generated in a homogenous population, with potential value in creating new hypotheses (see sections 8.1.2, 8.1.3, 8.1.4) or for future meta-analyses. The lowest nominal $P$-values in Paper I were for tagSNPs in neuronal cell adhesion molecule gene MDGA1, and tagSNPs in PDE4B were nominally associated with both schizophrenia and bipolar disorder in Paper II. In addition, nominal associations were present for DLX1, RELN, ITGA3, SPARCL1, and ASTN1 (Paper I). Furthermore, totally negative findings are thereby presented for the other 21 genes investigated, although we cannot exclude that there are common variants of weak effect in these genes, due to power limitations (see section 8.2.3).

8.1.1 Major risk alleles in B3GAT2 and GRIK3

The initial nominal association findings for B3GAT2 were replicated in an independent sample set. Specifically, three of the five nominally associated tagSNPs were represented by the same proxy SNP, which was associated with schizophrenia in the large multi-center replication sample. These four SNPs were located in the same estimated LD block (based on HapMap CEU), together with a fourth SNP genotyped in our Scandinavian sample, with a proxy SNP also nominally associated in the
replication sample. The initial best hit and its proxy SNP are only in moderate HapMap-based LD (r²=0.64), which could explain why the combined meta-analysis P-value was just slightly lower than half the initial P-value. The potential functional variant might be more efficiently tagged by the initial than the replication SNP, resulting in a weaker replication association despite the follow-up sample being much larger.

The major alleles conferred a risk effect for the four initial tagSNPs and the two proxy SNPs, and are therefore frequent also in the general population, which have been reported previously for other loci associated with schizophrenia [Ingason et al., 2007], and bipolar disorder [Wellcome Trust Case Control Consortium, 2007]. Since the genetic architecture in psychiatric illness is complex, with potential common functional alleles exerting a modest effect, healthy subjects having some of the risk alleles would then not carry detrimental combinations of additional risk variants potentially present in the psychiatric patients.

**B3GAT2** encodes one of the two key enzymes regulating the biosynthesis of the carbohydrate neural epitope HNK-1, which is involved in processes such as neuronal cell adhesion, and synaptic plasticity [Kleene and Schachner, 2004; Morita et al., 2008]. **B3GAT2** was highly expressed in several regions of mouse brain, such as the CA2/CA3 hippocampal subfield, neocortical layers V and VI, and several nuclei of the thalamus [Inoue et al., 2007]. In mice deficient for the **B3GAT1** gene, which is also involved in HNK-1 biosynthesis, the widely distributed HNK-1 brain expression was nearly completely lost [Yamamoto et al., 2002]. However, smaller amounts of HNK-1 were expressed on neurons in the cerebral cortex and hippocampus, and specifically on neocortical neurons in PNs, probably synthesized by B3GAT2. Highly speculative, **B3GAT2** SNPs associated with schizophrenia in our study might be linked to functional variants affecting B3GAT2-dependent HNK-1 biosynthesis in PNs. In turn, HNK-1 perturbations might influence its main carrier TNR, which was shown to be important for PN composition [Bruckner et al., 2000]. Abnormal PN formation could lead to excessive synaptic pruning during brain maturation, which has been suggested as a susceptibility factor for schizophrenia [Karlgodt et al., 2008], in line with the reduced cortical thickness and dendritic spine density reported for schizophrenia patients [Glantz and Lewis, 2000]

The haplotype combination of the major alleles of two tagSNPs in **GRIK3**, were significantly associated with schizophrenia (Table 4). If being conservative, 430 tests between genetic variation and the schizophrenia phenotype, have been performed in **Paper IV** (30 tagSNPs in the combined sample x 2 tests, 90 x 2 tests in the three
countries separately, 84 sliding window haplotype tests, 11 multimarker combinations of the four best tagSNPs. However, all these tests are not independent, and tagSNPs only associated in one of the country-based sub samples have not been a discovery focus. The corrected $P$-value for the best 2-marker combination, with the 430 tests, is 0.0044.

By combining alleles of SNPs in haplotype tests, specific haplotypes might more efficiently tag a potential functional variant, compared with the single SNPs alone. The two mentioned $GRIK3$ tagSNPs are in low LD ($D^*=0.38$, $r^2=0.10$), and present in distinct LD blocks, which were estimated using the solid spine method and SNP genotypes for the HapMap CEU population. The haplotype association implies that having both the common T and C alleles of the two tagSNPs is related to an increased risk for schizophrenia. The estimated T-C haplotype is present in 89% of the cases and 85% of the controls. The combined risk allele frequencies in cases, controls, and in those HapMap populations where the SNPs are polymorphic, are given in Table 4. The test for homogeneity of ORs between the three country strata for tagSNP rs6671364 was nominally significant, reflecting the fact that the OR for the major allele was larger in the Swedish sub sample, compared with the other two, although the same alleles conferred “risk” in all three groups.

Table 4. Major allele frequencies and allele- and haplotype-based association results for the two tagSNPs in the significantly associated $GRIK3$ 2-marker haplotype

<table>
<thead>
<tr>
<th>tagSNP</th>
<th>Risk allele</th>
<th>Minor Allele</th>
<th>Cases</th>
<th>Controls</th>
<th>HapMap Rel 27(^\dagger)</th>
<th>Rel 24(^\dagger)</th>
<th>Allelic Assoc</th>
<th>Haplotype Assoc</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs6671364</td>
<td>T</td>
<td>C</td>
<td>0.920</td>
<td>0.887</td>
<td>0.833</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs17461259</td>
<td>C</td>
<td>T</td>
<td>0.943</td>
<td>0.920</td>
<td>0.920 0.940 0.881</td>
<td>0.006 1.0·10⁻⁵</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CEU, Utah residents with Northern and Western European ancestry from the CEPH collection; MEX, Mexican ancestry in Los Angeles, California; TSI, Toscans in Italy

\(^\dagger\)HapMap release 27 represents Phase II+III, and release 24 is part of Phase II.

$GRIK3$ is one out of five KA ionotropic receptor subunits, and was one out of two KA subunits predominantly expressed in fetal cortex already in the first trimester [Ritter et al., 2001], suggesting an important role in neurodevelopment. Also, the three ionotropic glutamate receptor subtypes were shown to have distinct subunit binding patterns in cortical and subcortical regions during the second trimester [Lee and Choi, 1992]. $GRIK3$ mRNA expression was lower in the frontal cortex of postmortem brain tissue from neuroleptic-free (longer than 6 months) schizophrenia patients, compared with controls and schizophrenia patients receiving antipsychotic medication, and the expression level was shown to be negatively correlated with time without treatment before death [Sokolov, 1998]. In an additional post mortem study, the $GRIK1/2/3$
immunoreactivity was reduced in hippocampal regions from schizophrenia patients compared with controls and bipolar disorder patients [Benes et al., 2001].

As can be seen in the Table 4, GRIK3 allele frequencies in controls are similar or equal in the HapMap CEU population, and like the B3GAT2 SNPs, the major alleles are conferring the risk effect. The six B3GAT2 SNPs are located in the first intron, and the two GRIK3 tagSNPs are located in introns 13 and 2. The potential functionality of these SNPs is not known and they have not been associated with schizophrenia previously.

8.1.2 Schizophrenia and neuronal migration

Out of 18 genes investigated in Paper I, six nominally associated tagSNPs, including the strongest finding, were located in MAM domain containing glycosylphosphatidylinositol anchor 1 (MDGA1), which encodes a cell adhesion molecule shown to be important for radial glial guided neuronal migration [Takeuchi and O’Leary, 2006]. Five out of six MDGA1 tagSNPs were nominally associated in the allele-based test, and the same alleles conferred “risk” in the combined and each separate Scandinavian sample, which strengthens the uncertain finding.

The genes included in Paper I are encoding proteins either involved in the adhesion between radial glial fibers and migrating neurons (four genes, 102 tagSNPs), affecting the rearrangements of the cytoskeleton to influence cell polarity or motility during migration (eleven genes, 110 tagSNPs), or both of these domains (RELN, 71 tagSNPs), as well as two transcription factors affecting neuronal migration (7 tagSNPs). All of the five genes (MDGA1, RELN, ITGA3, ASTN1, SPARCL1) involved in neuron-glial adhesion have at least one nominally associated tagSNP in the present association study. Potentially could the process of cell adhesion during neuronal migration be of interest for further studies within schizophrenia genetics. However, the best finding for MDGA1 was above the Nyholt-corrected significance threshold, and no association tests with 2- to 4-marker haplotypes achieved higher significance levels, making such an implication highly speculative.

In addition to the five mentioned genes, two out of only three genotyped tagSNPs in transcription factor DLX1 were nominally associated with schizophrenia, a gene important for the tangential migration of GABAergic interneurons [Anderson et al., 1997]. Interestingly, the number of DLX-1 expressing neurons was previously shown to be reduced in the mediodorsal nucleus of the thalamus of psychosis patients.
[Kromkamp et al., 2003], and reduced total neuron count has been reported in the same brain region of schizophrenia patients [Popken et al., 2000].

8.1.3 Common genetics for schizophrenia and bipolar disorder

In Paper II, tagSNPs in PDE4B were genotyped in both a schizophrenia and bipolar disorder case-control sample, to investigate if PDE4B might be part of the suggested genetic overlap between the two disorders [Lichtenstein et al., 2009]. PDE4B interacts with DISC1, which is an indicated risk factor for severe psychiatric illness both involving schizophrenia, schizoaffective, and bipolar disorder [Porteous et al., 2006]. The segregation of a balanced translocation directly disrupting PDE4B, with psychotic disorder in a Scottish family, implicated PDE4B as a schizophrenia risk factor [Millar et al., 2005]. PDE4B is a phosphodiesterase that hydrolyze the second messenger cAMP [Houslay and Adams, 2003], and its interaction with DISC1 was shown to be regulated by cAMP levels, with PDE4B release from DISC1 in response to cAMP elevation [Millar et al., 2005]. PDE4 genes are orthologous to the dunce gene in Drosophila melanogaster, and dunce mutants show impaired learning and memory [Davis et al., 1995]. The selective PDE4-inhibitor Rolipram has antidepressant effects in humans [Zhu et al., 2001], as well as antipsychotic-like behavioural effects in mice [Kanes et al., 2007] and rats [Siuciak et al., 2007].

There were tagSNPs nominally associated with both schizophrenia and bipolar disorder in our combined sample and gender-stratified subsamples. The subdivision based on gender was performed based on a previous study reporting an association between PDE4B and schizophrenia, restricted to females [Pickard et al., 2007]. One drawback with the study is the fact that most tagSNPs genotyped in the two samples are non-overlapping, since they were selected and genotyped at different time points. As a first screen of the large gene region, tagSNPs in the schizophrenia sample was chosen with a MAF cut-off of 20%, and in the bipolar disorder sample this was increased to include also tagSNPs with a MAF ≥ 5%. However, all the nominally associated tagSNPs in the schizophrenia sample have either been genotyped themselves or by proxies in the bipolar disorder sample.

Two of the tagSNPs nominally associated in schizophrenia females had proxies which were nominally associated in the total bipolar disorder sample. Based on LD block estimation using the HapMap CEU population and the solid spine method (D’>0.8; adjacent blocks with multiallelic D’ values>0.95 were joined), the four SNPs were located in the same block, surrounding the splice site for the PDE4B isoform 3. In a recent screen for regulatory regions in schizophrenia candidate genes, including
PDE4B, with a chromatin immunoprecipitation-based method, brain tissue from two second-trimester fetuses was used to capture epigenetic events during brain development [Pedrosa et al., 2009]. In the analysis, the isoform 1 and 3 promoters were detected, with the latter being located in between the two markers in each of the nominally associated schizophrenia tagSNP-bipolar proxy SNP pairs, suggesting that these SNPs might tag functional variants affecting promoter function. Decreased PDE4B3 expression in cerebellar post mortem brain tissue from patients with bipolar disorder compared with controls has previously been reported [Fatemi et al., 2008]. Based on the opposing directions of association in our study, with major and minor allele homozygotes conferring a risk effect for schizophrenia and bipolar disorder, respectively, functional variants in the PDE4B3 might exert a balancing role. Although highly speculative, depending on the genetic make-up on other susceptibility loci, one functional genetic effect could predispose to the psychiatric phenotype of schizophrenia, and the opposing effect to bipolar disorder.

In a markedly smaller subset of Norwegian patients, the same two schizophrenia tagSNPs were associated with positive symptom scores, and the two bipolar disorder proxy SNPs nominally associated with negative symptom scores if investigated in females only. The latter association was more prominent in females with a history of at least one psychotic event, but this group only consisted of 36 women.

One of the three highlighted putative regulatory regions reported in the study by Pedrosa et al. described above, is encompassed by a two-marker haplotype associated with schizophrenia in another case-control study [Fatemi et al., 2008]. One of these two markers is genotyped in both our schizophrenia and bipolar disorder, but is only nominally associated with bipolar disorder.

### 8.1.4 Gender-specific associations of PDE4B tagSNPs with schizophrenia

Before we statistically analyzed the PDE4B tagSNP genotypes in the SCOPE sample, a gender-specific association of an individual three-marker haplotype within intron 3 of PDE4B was published [Pickard et al., 2007]. The specific haplotype conferred a protective effect against schizophrenia in a Scottish case-control sample, but only in females. Therefore, we tested the PDE4B tagSNPs for association both in our total sample, and in women and men, separately. In line with the previous report, four tagSNPs were exclusively nominally associated in women, compared with none in only men, in addition to two nominally significant tagSNPs in the total sample.
Associations only in female subjects, between gene variants and both schizophrenia and bipolar disorder, have previously been reported for other loci [Hennah et al., 2003; Thomson et al., 2005a; Thomson et al., 2005b]. A haplotype in the DISC1 gene was significantly associated with schizophrenia [Hennah et al., 2003], and another DISC1 haplotype was associated with bipolar disorder [Thomson et al., 2005a], both only in women. Based on five populations, there was a female-specific association between an intronic SNP in RELN and schizophrenia, with a significant gene-sex interaction [Shifman et al., 2008]. We did not test for such a formal interaction, and the gender-specific effect of PDE4B based on associations in females only, should be interpreted with caution [Patsopoulos et al., 2007].

On the schizophrenia phenotype level, there seems to be differences between males and females, which might reflect underlying genetic differences [Leung and Chue, 2000]. Although still controversial due to methodological problems, the schizophrenia incidence was shown to be increased in men in a recent comprehensive meta-analysis, with a risk ratio of 1.35 relative to women, when controlling for the possible bias from the factors age, diagnosis criterion, and inpatient/outpatient sample inclusion [Aleman et al., 2003].

8.1.5 Associated genes and regional schizophrenia linkage

In this section, the genetic regions highlighted in the thesis are placed on the chromosomal map of schizophrenia linkage. Linkage data on the same or surrounding genetic sections, as those harboring the main nominal/significant findings in Paper I-III, are put forward, specifically this includes: B3GAT2 (6q13; Paper III), PDE4B (1p31.3; Paper II), and MDGA1 (6p21.2; Paper I).

The first suggestive evidence for the involvement of the 6q genome region in schizophrenia, was based on a genome scan of chromosome 6 showing an excess of identity by descent allele sharing for microsatellite markers on 6q13-q26, in both an initial and a replication multiplex family sample set [Cao et al., 1997]. The most centromeric marker on 6q was located in 6q13, about 52 kb upstream (towards the centromere) of the B3GAT2 gene. When excluding families without genotypes for both parents, all maximum likelihood scores (MLSs) in 6q (6q13-6q27) were greater than 0.97, and in the replication sample the region with MLSs ≥ 1 started in the 6q13-q16.1 interval and reached 6q22.31. However, the actual MLS peaks were at 6q21-22 and 6q16, in the initial and replication set, respectively, which might reflect a difficulty in locating the causative locus [Roberts et al., 1999]. Combining the latter sample with an additional replication set gave rise to significant linkage to 6q16 (MLS=3.82;
A balanced 6;11 chromosomal translocation with the breakpoint at 6q14.2 was partially co-segregating with schizophrenia spectrum disorder in a family [Holland and Gosden, 1990], and another translocation t(5;6) (p13;q15) was present in a patient with acute paranoid psychosis [Axelsson and Wahlstrom, 1984], both implicating regions centromeric of the 6q16 linkage peak, in schizophrenia susceptibility. Genome-wide suggestive linkage was also reported for 6q12 and suicide attempts in major depression [Zubenko et al., 2004].

In a recent meta-analysis of 32 whole-genome linkage scans of schizophrenia, one of the ten genome bins which met the empirical “aggregate” criteria for genome-wide significant linkage, was 1p32.2-p31.1, containing PDE4B (1p31.3) [Ng et al., 2008], but the nearby region including GRIK3 (1p34-33) has to our knowledge not been linked with schizophrenia.

Initial linkage reports of 6p, implicated a susceptibility locus for schizophrenia on 6p24-22 [Schwab et al., 1995; Straub et al., 1995; Wang et al., 1995], harboring the DTNBP1 candidate gene on 6p22.3 [Straub et al., 2002]. The marker closest to MDGA1 (6p21.2), which was genotyped in the more comprehensive study by Straub et al. (including the pedigrees used by Wang et al.) as well as an additional investigation [Schwab et al., 1995], was non-significant and positioned 1.2 Mb away on 6p21.31. However, in a subsequent study, potential linkage was suggested based on the same marker, in a two-stage genome-wide screen [Moises et al., 1995]. In a large meta-analysis of genome-wide linkage scans, the linkage region 6p22.3-p21.1 was proposed [Lewis et al., 2003], encompassing MDGA1. Also, significant and suggestive linkage between the eye tracking dysfunction schizophrenia endophenotype, and 6p21.1, was shown [Arolt et al., 1999], supported by others [Matthysse et al., 2004].

8.1.6 Genetic effects on symptoms, neurocognition, and age at onset

The diagnosis of schizophrenia embraces a heterogeneous group of patients, with varying degree of positive and negative symptom severity, and cognitive performance. To genetically assess a potentially more homogenous phenotype, endophenotypes and clinical characteristics have been used in three of the thesis papers. It has been suggested that besides genes conferring disease susceptibility, additional gene variants may only exert their function on the clinical expression of the phenotype, so called
modifier genes, and others might be both susceptibility and modifier variants [Fanous and Kendler, 2005]. In the present work we investigated if the genes could be both susceptibility and modifier variants.

In Paper II-IV, we searched for gene variants with a potential effect on the extent of positive and negative symptoms in schizophrenia patients, as measured by the PANSS. **Paper II** includes both schizophrenia and bipolar disorder patients, and those results were therefore integrated in a combined discussion of data on both disorders in section 8.1.3. The negative, but not positive, symptom dimension was previously shown to be associated with cognitive dysfunction, as measured with a number of different tests of learning and memory [O'Leary et al., 2000]. Therefore, genetic variants involved in cognitive deficits might also be associated with more severe negative symptom scores. However, there were no significant associations between tagSNPs and symptom scores for GRIK3 (Paper IV) or B3GAT2 (Paper III).

In Paper III, we also investigated age at onset and neurocognitive measures of learning and memory, the latter in both cases and controls. Two tagSNPs, in distinct LD blocks and separate introns of B3GAT1, were nominally associated with symptom sum scores as well as one of the cognitive measures in the patients. Major allele homozygotes had lower IQ and higher positive symptom sum scores for one tagSNP, and heterozygotes had higher measures of verbal learning (CVLT) and lower negative sum scores, as compared with both homozygotes, for the other tagSNP. The verbal learning measure was similar to the test most significantly correlated with the negative symptom dimension [O'Leary et al., 2000]. Two B3GAT2 tagSNPs was nominally associated with IQ scores in both cases and controls, with one of them also associated with three additional cognitive test scores in controls, suggesting an effect of B3GAT2 on cognitive function in both groups. There was an interaction effect between case control status and genotype for both tagSNPs, reflecting gradually higher IQ scores in controls in contrast to lower IQ scores in cases, with the number of minor alleles (for the tagSNP with sufficient numbers in each genotype group).

There are several limitations of our analyses of quantitative measures and gene variants. Regarding the use of the PANSS scores as clinical subtypes of schizophrenia and bipolar disorder patients, there is an uncertainty to what extent these scores are a true reflection of the clinical picture caused by intrinsic factors. The scores vary substantially over time and with treatment, especially the positive but also negative symptoms [Lindenmayer et al., 2007]. Other studies have shown genetic association to symptom scores in schizophrenia, for genes such as DAO and DTNBP1 [Corvin et al., 2007; Corvin et al., 2008; Fanous et al., 2005]. However, the symptom measures have
differed slightly between studies: one group [Corvin et al., 2007] used PANSS-derived symptom factors assessed on the basis of the worst documented episode of illness, compared to the reflection of the last week in our studies, and another group [Fanous et al., 2005] used life-time ratings of clinical features according to the Operational Criteria checklist for psychotic illness. Therefore, a comparable measure reflecting a longer time-frame would have been optimal also in our study, although the included individuals are mainly from out-patient clinics, where patients are treated in stable phases. It is possible that persisting high PANSS levels in this population can reflect treatment resistant subjects, and specific sub-groups of patients. However, only a restricted number of patients have symptom scale scores and neurocognitive measures, which imply that the nominal association findings found by us are preliminary and might represent type I error.

8.2 General methodological issues

8.2.1 The schizophrenia and bipolar disorder phenotype

The diagnosis of schizophrenia and bipolar disorder are both based on descriptive criteria on the basis of clinical symptoms, without objective biological measures for validation, since the pathogenic mechanisms are largely unknown. However, both clinically diagnosed schizophrenia and bipolar disorder has a high estimated heritability, providing evidence for an important biological basis. If this biological etiology is largely shared within the separate schizophrenia and bipolar disorder patient groups, if there is a common background spanning these two groups and also major depression and autism at the far ends, and/or if there are several “schizophrenias” and “bipolar disorders” caused by numerous distinct biological mechanisms, remains to be answered. There is new research in favour of the presence of a psychiatric continuum including both shared and unique biology. Increased knowledge will guide both future diagnostic tools and development of new medications. Today’s psychiatric diagnoses are based on two main diagnostic systems, the DSM-IV and ICD-10, with both being represented in this thesis.

It has been shown that there is high concordance between the ICD and DSM systems (pairwise concordance rate (CR)>0.70, χ>0.70) [Jakobsen et al., 2006]. The vast majority of the patients (96%) who fulfilled the ICD-10 criteria of schizophrenia also complied with the corresponding DSM-IV standards. The main difference between the schizophrenia diagnoses in the two systems is the time factor, where symptoms should have been present for 6 months in DSM-IV/III-R and one month in ICD-10. The
diagnosis of schizophreniform disorder which is given to patients with symptoms of schizophrenia that do not reach the 6 month-criteria in DSM-IV/III-R, is to a high extent included in the schizophrenia F20 diagnosis in ICD-10. Potential differences in the patient phenotype in our studies, due to differences between the three Scandinavian countries, was corrected for by including the covariate “sample origin” in all main analyses.

In the initial genetic association tests in Paper I-IV, a defined broad diagnosis of schizophrenia has been implemented, including patients with schizophrenia, schizoaffective disorder, and schizophreniform disorder. In the case of association tests with bipolar disorder, bipolar disorder type I, II, and NOS have been included. Depending on if the biological target mechanisms evaluated in a hypothesis-driven candidate gene study, are in fact involved in a pathway with importance only for a subset of patients with strict schizophrenia diagnosis, or in a pathway affecting the propensity to develop psychosis, the choice of either strict schizophrenia or a broader schizophrenia spectrum disorder phenotype would affect the results. Since there are several studies showing strengthened findings with a broad phenotype, both including schizophrenia, schizoaffective, and schizophreniform disorder, as well as bipolar affective disorder [O'Donovan et al., 2009], we focused on a broad schizophrenia group to maximize the sample size.

In Paper I, we re-analyzed all tagSNPs nominally associated with broad schizophrenia, using a narrow schizophrenia group, only including patients with the strict schizophrenia diagnosis. Despite the decrease in sample size (N_broad=839; N_narrow=736), the results were similar.

8.2.2 Strategies for picking tagSNPs

The international HapMap project was launched with a goal to identify SNPs, and to investigate the pattern of LD, throughout the human genome. The knowledge of the LD pattern between gene variants can subsequently be used for gene-wide assessment of candidates in association studies. To reduce marker redundancy, a set of tagSNPs can be genotyped, which capture information on other non-genotyped common variants in high LD. The tagSNPs can be selected based on public projects such as HapMap or a subset of individuals from the study in question. Hopefully the tagSNPs capture both known variants, as well as potential unknown functional loci in the candidate region. There are three commonly used methods for picking tagSNPs, namely pairwise, multimarker, and haplotype-based tagSNP selection [Goode et al., 2007]. In more detail, the first is based on correlating each known SNP with a SNP to
be genotyped, the second on correlating known SNPs with a single SNP or a combination of SNPs to be genotyped, and the third is based on representing each estimated haplotype of interest with a set of SNPs to genotype. If the functional variant is actually one of the genotyped tagSNPs, or a SNP in strong LD with one of the tagSNPs, using pairwise and multimarker methods would be the optimal strategy. On the other hand, if the haplotype tagging SNPs define a haplotype which carry the functional allele, haplotype-based methods would be optimal. At the design phase of an investigation, it is therefore difficult to know which method would be the best one in that particular association study. In Paper I-IV, pairwise tagging based on HapMap data was implemented, which was the method presented in a recent publication on marker selection in *Nature protocols* [Pettersson et al., 2009]. Each tagSNP represents either only itself, if not in LD with any other known HapMap SNP, or a tagged bin, which includes a set of covered markers ranging from one and up to several tens.

8.2.3 Power, control selection, and population stratification

The power of a genetic association study is obviously based on the size of the sample, but is also highly influenced by several additional known as well as unknown factors [McGinnis et al., 2002]. In the publicly available Genetic Power Calculator (pngu.mgh.harvard.edu/~purcell/gpc/cc2.html), the power calculations are based on the following features: disease-causing allele frequency, heterozygote OR, risk homozygote OR, D’ between marker and disease allele, marker allele frequency, number of cases and controls, disease prevalence, and whether the controls are unselected or not. The sample size was shown to be minimized when the marker and disease allele frequencies are equal and 0.5, when the mode of inheritance is dominant or recessive (either one or the other depending on OR and disease allele frequency), and if the linkage disequilibrium between disease and marker polymorphism is complete [McGinnis et al., 2002]. The power of the sample used in Paper I (including the highest number of genes out of the four thesis papers) for a set of different conditions is given in Figure 4. The power is calculated for a range of ORs for four different MAFs, at the nominal significance level of $P=0.05$. The mean MAF in Paper I was 26%. As can be seen the power for SNPs with allele frequencies in the lower frequency range is not sufficiently high, which can result in type II errors. The Power when $OR_{homozygote}=1.5$ (additive inheritance model) was 39, 63, 88, and 91 % for MAFs 5, 10, 25, and 50%, respectively. For the Nyholt-corrected threshold given in Paper I ($P=0.00027$), the power is substantially lower, being 37% for $OR_{homozygote}=1.5$ and MAF=25%, as an example.
Figure 4. Power calculations based on the 839 cases and 1473 controls in Paper I using Genetic Power Calculator. The following settings are fixed: $r^2=1$, $P=0.05$, and additive inheritance model. The power is given for the allelic association test.

Association studies can be based on genotyping data of either families or unrelated individuals. The studies in Paper I-IV are all based on the latter, with a case-control design. When using the same number of cases and controls in such a study, roughly the same number of affected child and parent trios is required for equal power in the family-based design, which means that the family-based sample will need to be 50% larger than the case-control sample [McGinnis et al., 2002]. Case-control studies are therefore more cost effective. In addition, to boost the power without including more cases, the number of unrelated controls can be increased, an approach used in Paper I-IV, mostly accounted for by the additional Danish controls. The control:case ratio in Paper I is 1.76.

In contrast to the parents serving as controls when using trios and the transmission-disequilibrium test (TDT), the optimal selection of controls for case-control studies is more complex. In contrast to family-based association studies, the main concern using unrelated cases and controls is the risk for so called population stratification, meaning that the two comparison groups have allele frequency differences unrelated to the studied phenotype, instead caused by diversity in background population [Cardon and
Palmer, 2003]. Therefore, population stratification can cause spurious association findings. The general criteria for suitable controls are that these should be derived from the same population as the cases, in order to serve as representatives of genetic make ups not related to the disease under study. For population stratification to be problematic in genetic association studies, both differences in disease prevalence and allele frequency must be present between cases and controls [Wacholder et al., 2000]. Of course, if the presence of different ethnicities between cases and controls is due to selection bias, only a difference in allele frequency between ethnicities could cause false positive findings. Due to limited evidence showing the actual effects of population stratification on type I errors and non-replications, the fear for stratification has been viewed as exaggerated [Cardon and Palmer, 2003]. The risk for population stratification might mostly be a problem in populations with recent admixture, such as African Americans, and for disorders with distinct prevalence across the ancestral populations [Freedman et al., 2004]. A minority of individuals in SCOPE has one Caucasian parent born in North-Western Europe, but outside Scandinavia, but when excluding those patients with one non-Danish parent from the Danish sample this had no effect on the association results in that study [Hansen et al., 2007].

The Scandinavian sample used in Paper I-IV, is well suited for genetic studies. Scandinavians are genetically homogenous with only recent non-Caucasian immigration. In a study of 23 European populations, using SNP-based PCA (≈300,000 SNPs), it was shown that northern Europeans have higher mean LD and smaller mean heterozygosity, compared with southern Europeans [Lao et al., 2008], which is more suitable for LD-based association studies. Fifty Danish controls included in the study by Lao et al., located in PCA clusters with Norwegians and northern Germans, were randomly drawn from the same sample pool as the Danish controls included in SCOPE (the SCOPE controls are discussed further below). As a measure on how genetically similar the Danish, Norwegian, and Swedish SCOPE controls were, we estimated the Fixation index, based on 289 marker genotypes (Paper I), which showed no indication of genetic heterogeneity ($F_{ST}=0.00071$). Based on GWAS data, the Norwegian sample was shown to be genetically homogenous (Figure 2 & 3), and risk for population stratification due to substructures in Norwegian cases and controls does not seem to be a concern.

Whether optimal controls should be completely unselected of other phenotypes, or being represented by health-seeking individuals with other unrelated disorders, is under debate [Zondervan and Cardon, 2007]. In addition, the extent to which the controls should be screened for psychiatric illness in schizophrenia studies is not straightforward. If the genetic variants under study are involved in the etiology of a
broad phenotype including a range of psychiatric disorders, such as depression, schizophrenia, autism, and bipolar disorder, the power to detect disease variants would decrease if any of the phenotypes were present in controls. On the other hand, screening for sub-diagnostic depressive states, would exclude a high number of individuals and create a non-representative sample of the general population. In the SCOPE sample, the Norwegian and Swedish controls are screened for schizophrenia, whereas the Danish controls were randomly selected out of a population of 15,000 blood donors. Norwegian subjects were additionally excluded if they or any of their close relatives had a lifetime history of a severe psychiatric disorder (schizophrenia, bipolar disorder and major depression).

8.2.4 Correction for multiple testing

In the quest for gene variants involved in complex disorders, such as schizophrenia and bipolar disorder, a high number of candidate genes have been implied and tested for association (www.schizophreniaforum.org/res/sczgene), combined with a current wave of whole-genome studies. With the investigation of several genes, different markers and marker combinations within each gene, and up to over a million markers when assessing the whole genome, comes the risk of false discoveries due to multiple statistical comparisons [Sullivan, 2007]. A stringent approach is to use the so called Bonferroni corrected significance level, \( \alpha / m \), where the original threshold is \( \alpha \) and the number of independent tests is \( m \). However, most often all tests within an association study is not independent, and by the use of an overly conservative threshold true effects might be missed. A method where the LD structure between assessed SNPs is taken into account when estimating an “effective” number of independent tests was suggested [Nyholt, 2004], but also such a threshold might be too conservative [Nicodemus et al., 2005]. A proposed better estimate [Cannon et al., 2005] for the Nyholt threshold was used in Paper I and IV, where the best nominal \( P \)-values were above and below the Nyholt significance threshold, in the first and the latter paper, respectively. The golden standard for correction of multiple testing is otherwise permutation-based, where the case-control status is shuffled, although this is more time-consuming and computationally demanding. Permutation-based correction was used for single and multi-marker analyses in Paper II and III, with 10-100,000 permutations.

Independent of which correction-method is chosen, the common procedure for candidate gene association studies is to base the correction on the tests performed within the actual study, which means that the multiple testing for the whole research community is not assessed [van den Oord and Sullivan, 2003]. Also, as in the present
thesis (Paper II-IV), endophenotypes and clinical subtypes, in addition to the main disease phenotype, are commonly investigated, further increasing the risk of type I errors. However, if the probability of excluding false discoveries is maximized, the power to detect true effects if present is lowered. In Paper III, we used a non-stringent level of nominal association as a first step, and investigated markers with $P$-values below 0.05 in a second replication step in a markedly larger sample. The importance of the definition of a “true” replication was highlighted [Sullivan, 2007], and as proposed, the replication of tagSNPs in Paper III are based on the “same” SNPs (proxy SNPs), the same statistical test (allelic CMH), the same phenotype (schizophrenia), and the same direction of association (OR>1 for the same alleles).
9. Concluding remarks and future studies

During the research work in this thesis, which started in the beginning of 2006, the field of genetics has evolved rapidly. Only a few years ago, the GWASs which are presently delivering huge amounts of exciting data, were at the planning and design phase, while the high-throughput genotyping tools emerged and improved.

When designing the PhD thesis, and starting out with the first data analysis, there was a lot of focus on specific candidate gene variants as markers of interest, with hypothesized functions. We wanted to focus our hypotheses on the genes to be studied, and take advantage of the data from the HapMap project in order to search for disease association on a gene-wide level, trying to capture most of the common SNPs in the regions of interest. The question of whether the HapMap CEU sample, chosen to represent Europeans, was comparable to the individuals in today’s Northern Europe, was recently answered by Lao et al. [Lao et al., 2008], who used genome-wide genotyping data, to demonstrate that the CEU sample individuals are genetically North-Europeans.

The SNPs included in the HapMap database has constantly increased, so the selected tagSNPs in 2006 do not cover as much of the variation as those picked later on. In the present thesis work, tagSNPs in 30 candidate genes involved in neurodevelopment and plasticity, have been chosen and investigated for association with schizophrenia, as well as bipolar disorder for one gene, in Scandinavian multi-center case-control samples. The importance of large collaborative efforts within the field of genetics has the last few years been put in the spotlight, and several multi-national consortia have emerged. By taking advantage of already present GWAS data for another markedly larger European sample, the association with common variation in the B3GAT2 gene in one of the thesis projects, was replicated.

In parallel with the increased amount of SNPs deposited in public databases, the interest and importance of CNVs has been raised. For the field of schizophrenia genetics, GWAS data on both SNPs and CNVs has provided evidence for both common alleles of weak effects and rare alleles with fairly large effects, in disease etiology [Owen et al., 2009]. A main challenge for future psychiatric genetics is to elucidate the underlying functional effects, both for common SNPs and for the genes located in the large deleted regions strongly associated with schizophrenia [International Schizophrenia Consortium, 2008; Stefansson et al., 2008]. So far, most associated alleles in GWASs are located in non-exonic regions, as well as the tagSNPs
associated in this thesis, and different effects on gene expression might be a main action for common variants in complex disease [Hardy and Singleton, 2009].

About a year ago, the first individual genome sequenced by the next-generation technologies, was published [Wheeler et al., 2008], leading the way for future high-throughput personal genome sequencing, at lower costs. In the quest for functional variants in regions of interest, more efficient sequencing efforts are now possible, of importance for trying to find both possible common and rare variants. In the future, when hopefully being able to investigate the functional variation associated with schizophrenia directly, there will be a time for improved evaluations of the actual contribution of common and rare variation to disease susceptibility.
10. Conclusions

The main conclusions of this work are:

1) Gene variants related to neuronal migration are nominally associated with schizophrenia, and therefore of interest for further studies.

2) Variation in the region surrounding the $PDE4B$ isoform 3 splice site, might be involved in both schizophrenia and bipolar disorder etiology, and possibly are there variants affecting schizophrenia susceptibility only in women.

3) Gene variants in the HNK-1 pathway seem to be associated with schizophrenia, based on the replication of initial nominal findings in a large additional European sample, for SNPs in $B3GAT2$.

4) $GRIK3$ variants were significantly associated with schizophrenia, supporting the involvement of glutamate signaling in schizophrenia etiology.

The present study provides a basis to gain more knowledge about the molecular genetic mechanisms of schizophrenia, and the results suggest that gene variants related to neurodevelopment are associated with schizophrenia, thus supporting the neurodevelopmental hypothesis.
11. Errata

Paper I:
Page 1092, Section “Single-Marker Association Analysis”, first sentence:
“Nineteen markers (out of 289, 6.6%)” should be “Twenty-one markers (out of 289, 7.3%)”

Paper II:
Page 2, Section “The bipolar case-control sample”, sentence twelve, should read:
“The first patient group had been diagnosed with SCAN [Wing et al., 1998] interviews fulfilling a best estimate diagnosis of bipolar affective disorder according to the ICD-10-DCR [WHO, 1993] and BPI according to the DSM-IV.

The figure text for the supplementary figure in the supporting information published online is missing, and should be:

**Supplementary Figure 1.** Overview of the SNPs genotyped in the present (Kähler BP and Kähler SZ) and the three previously published PDE4B association studies, as well as the PDE4B gene structure. The following features are represented (from top to bottom): LD blocks estimated based on the BP case control sample; vertical bars representing each SNP genotyped (dark grey=not associated, green=associated in the total case control sample, red=associated in women, blue=associated in men, bars marked with ‘*’ (from top left to bottom right)= rs11208776, rs2196122, rs1937450, rs3009872 (two BP-SZ SNP pairs which serve as r²-based proxies for each other), rs1040716, rs910694 (the same associated SNPs in two studies)); the exon structure of the three main isoforms given by NCBI (23 december 2008) (alt 1=a unique alternative exon 1); the basepair position at chromosome 1; a heatmap based on the BP case-control sample showing D’ (color coded) and r² (figures) between SNPs (created in Haploview 4.1).

Paper IV:
Page 244, Section 2.5.3, should read:
…Cochran-Mantel-Haenzcel test for 2x2xk stratified tables in PLINK (v0.99q-2007) (Purcell, 2007; Purcell et al., 2007), on the combined sample with population as stratification factor. To evaluate the heterogeneity of population-as well as gender-based odds ratios (ORs), the Breslow-Day test was performed…

Page 245, Section 3.2, 2nd paragraph, should read:
There were no significant OR heterogeneity between the three nationalities or between genders, except for marker rs6671364 with nominally significant heterogeneity between nationality strata (P=0.027; Breslow-Day test).
The following changes of the submitted thesis have been made in the printed version:

- The reference “Sobeih & Corfas, 2002” referred to on page 19, line 10, was added to the reference list:


- On page 29, line 9, the first reference given was “2008”, but changed to: “International Schizophrenia Consortium, 2008”

- In the figure text for Figure 2, page 38, the two different pictures were referred to as “to the left” and “to the right”, which was changed to: “on top” and “below”

- In Table 2, page 42, the header and bottom row of the table was changed:

  Now: Allele A a Total
  Previously: Allele A A Total

  Now: Total A a N
  Previously: Total A A N

- In Table 3, page 42, the header and bottom row of the table was changed:

  Now: Genotype AA Aa aa Total
  Previously: Genotype AA Aa

  Now: Total AA Aa aa N
  Previously: Total AA Aa Aa N

- The reference “(Patil 2001)” referred to on page 44, line 33, was changed to “[Patil et al., 2001]”, and added to the reference list:


- Page 56, first line in section 8.1.2:
  “…in Paper I, the six nominally…” ➔ “…in Paper I, six nominally…”
12. References


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