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Genes and Symptoms of Schizophrenia: Modifiers, Networks, and Interactions in Complex Disease

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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List of Abbreviations

AA	.....	African ancestry
CFA	.....	Confirmatory factor analysis
DSM	.....	Diagnostic and Statistical Manual
EA	.....	European ancestry
EFA	.....	Exploratory factor analysis
ES	.....	Effect size
GAIN	.....	Genetic association information network
HRR	.....	Haplotype relative risk
ICCSS	.....	Irish case-control study of schizophrenia
ISHDSF	.....	Irish study of high density schizophrenia families
LDPS	.....	Lifetime dimensions of psychosis scale
MAF	.....	Minor allele frequency
OPCRIT	.....	Operational criteria checklist for psychotic illness
OR	.....	Odds ratio
RAF	.....	Risk allele frequency
SD	.....	Standard deviation
SNP	.....	Single nucleotide polymorphism
SS	.....	Sample size

Global Abstract

GENES AND SYMPTOMS IN SCHIZOPHRENIA: MODIFIERS, NETWORKS, AND INTERACTIONS IN COMPLEX DISEASE

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Understanding the genetic foundations of schizophrenia and the resultant symptom manifestations is an important step as we work toward development of new prevention and treatment strategies. This work has sought better understanding of this disease through use of three subject cohorts and two studies using simulated data exploring features of complex disease.

First, we probed the symptoms of schizophrenia in subjects of African and European ancestry drawn from the Genetic Association Information Network (GAIN) schizophrenia study and found significant differences between groups, particularly in affective symptoms.

The genetic basis of symptom variation was then explored in a selection of candidate genes in two Irish samples, the Irish Study of High Density Schizophrenia Families (ISHDSF) and Irish Case-Control Study of Schizophrenia (ICCSS). We found a significant association of PAH with delusions, GABRB3 with hallucinations, and SNAP25 with both of these symptom factors. AKT1 alleles conferred greater Schneiderian symptoms, but dysbindin, MAOB, and SLC6A4 were not related to any symptom dimensions.

Simulated data was used to probe the parameters necessary to detect susceptibility genes as modifiers in a scenario in which two disease groups with incompletely overlapping symptom profiles are examined together. The heterogeneous genetic underpinnings and variable symptom manifestation of schizophrenia make the findings from this study particularly relevant to this disease.

Convergent lines of evidence implicating myelin and synaptic dysfunction in schizophrenia prompted us to test related gene networks for association with this disease in two populations, African-ancestry and European-ancestry, from the GAIN study.

Some evidence supporting myelin-related genes in the etiology of schizophrenia was presented but only in the African-ancestry group.

Epistatic (gene-gene) interactions may confer much greater disease risk than single-gene results would indicate, but their detection is often difficult. The final study included here explored two approaches to family-based epistasis detection under a range of epistatic models. The haplotype relative risk (HRR) approach yields greater power for detection under conditions of dominance, but the Cordell approach is more powerful under most other models.

Together, these studies provide a modest advancement in our understanding of schizophrenia and the methodological avenues available for future studies of this disease.

Chapter 1: General Introduction

Schizophrenia Symptoms and Demographics:

Schizophrenia is a severe and debilitating mental disorder characterized by distorted perceptions of reality. Approximately .4% of the world population has schizophrenia (Bhugra, 2005), but the full impact of this disease on individuals, their families, and society is difficult to assess. A diagnosis of schizophrenia can be given at almost any stage of life from childhood to old age, but it most commonly appears in the late teenage or early adult years. Women generally have a later age of onset than men, but lifetime prevalence rates of schizophrenia are similar between the sexes. A somewhat lower life expectancy is related to this diagnosis owing to an elevated suicide risk and health complications often concomitant with antipsychotic-related weight gain.

Symptoms of schizophrenia generally include positive symptoms, which are experiences additional to the usual human experience. Auditory hallucinations (i.e. hearing voices) are among the most common positive symptoms, but any sensory experience for which there is not a physical cause constitutes a hallucination. Schizophrenics may experience hallucinations related to any of the five senses. Delusions are also frequently experienced positive symptoms, and these include any aberrant beliefs about the world not grounded in reality and not experienced by most other members of their culture. Schizophrenia also almost always involves negative symptoms as well. These represent diminished experiences compared to the normal population. Negative symptoms include: flattened affect (reduced emotional expression), alogia (reduced speech), or avolition (reduced

motivation). A third category of symptoms is usually mentioned, too, but it is more variable. Cognitive symptoms such as difficulty planning and working memory disturbances sometimes represent this third category. Mood symptoms including features of depression or mania are also sometimes highlighted.

The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) (American Psychiatric Association, 1994) criteria for diagnosing schizophrenia are as follows:

1) Characteristic symptoms: Two or more of the following, each present for much of the time during a one-month period (or less, if symptoms remitted with treatment).

- Delusions
- Hallucinations
- Disorganized speech (a manifestation of formal thought disorder) severe enough to impair communication
- Grossly disorganized behavior (e.g. dressing inappropriately, crying frequently) or catatonic behavior
- Negative symptoms—affective flattening, alogia, or avolition

If the delusions are considered bizarre, or auditory hallucinations involve a running commentary of the patient's actions or hearing two or more voices conversing with each other, only that symptom is required above.

2) Social/occupational dysfunction: For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care, are markedly below the level achieved prior to the onset.
3) Duration: Continuous signs of the disturbance persist for at least six months. This six-month period must include at least one month of symptoms (or less, if symptoms remitted with treatment).

These symptoms cannot be due to a mood disorder, pervasive developmental disorder, or the use of drugs.

Five clinical subtypes of schizophrenia are recognized by the DSM-IV-TR: Paranoid, Disorganized, Catatonic, Undifferentiated, and Residual. These clinically derived subtypes are not generally validated through statistical methods of symptom analysis as later chapters attest.

Etiology of Schizophrenia:

Schizophrenia is a complex genetic disease, and, as such, many genes confer some risk for illness, but no single genetic markers confer a certain diagnosis of schizophrenia. Genetic hererogeneity has complicated gene finding efforts, and although many genes have been proposed as candidate genes for schizophrenia, none have been unequivocally linked to the disease.

All complex genetic diseases also have environmental components, and several non-genetic causal factors have been implicated in schizophrenia. Pre- and perinatal factors such as gestational viral exposure (Brown, 2006) and obstetric complications have been linked to later schizophrenia diagnosis (Mittal et al, 2008). Cannabis use may also be a factor in subsequent psychosis (Moore et al, 2007). Migration or the experience of being a minority could also play a role in developing schizophrenia (Cantor-Graae et al, 2005; Fearon et al, 2006; Chakraborty and McKenzie, 2002). Environmental factors probably represent the best targets for prevention of some cases of schizophrenia, since genes are immutable and gene expression is usually difficult to modify.

The heritability of schizophrenia has been estimated at 81% by meta-analysis (Sullivan et al, 2003) which indicates a very strong genetic influence for this disease compared to most other mental illnesses. For many years, genetic studies of schizophrenia centered around linkage and candidate-gene association methods. The genes yielded from these studies have rarely been substantiated through replication without numerous contrasting reports of non-association. The advent of GWAS studies offered much promise with coverage of the entire genome instead of requiring pre-selection of genetic loci. Unfortunately, even using large samples of subjects, GWAS studies have added few uncontested candidate genes to the field of schizophrenia. A recent spate of copy number variation (CNV) studies has generated a few loci with replicated associations with schizophrenia, but these account for only a small proportion of cases.

Additional ways of analyzing SNP chip data have recently been gaining in popularity. Pathway or network analyses show promise for determining biological processes implicated in schizophrenia and identifying causal alleles. Epistatic interactions may also be responsible for some of the lack of success in unveiling single-locus genetic associations. New and more powerful methods for detecting gene-gene interactions would be useful in assessing this possibility. The recent and rapid advances in developing methods uncovering genetic variation predisposing to schizophrenia and other complex diseases give hope to a field that has long been plagued by slow progress and inconsistent associations.

Cohorts: ICCSS, ISHDSF, GAIN:

The studies presented here which used real data (as apposed to simulations) drew subjects from three cohorts: the Irish Study of High Density Schizophrenia Families (ISHDSF), the Irish Case-Control Study of Schizophrenia (ICCSS), and the Genetic Association Information Network (GAIN) schizophrenia study. Details of their ascertainment, assessment, and genotyping are included here to avoid redundant descriptions throughout this document.

Irish Study of High Density Schizophrenia Families (ISHDSF)

The ISHDSF is a collaborative effort between Queen's University, Belfast, the Health Research Board, Dublin, and Virginia Commonwealth University, Richmond, VA. Subject recruitment was conducted in Northern Ireland, the United Kingdom and the Republic of Ireland.

Ascertainment criteria, subject characteristics, and phenotypic assessments:

The full sample consisted of 1,425 individuals from 270 families ascertained on the basis of two or more members with DSM-III-R schizophrenia or poor outcome schizoaffective disorder. Families were ascertained through public psychiatric services in Ireland and Northern Ireland that together provided over 90% of all in-patient psychiatric care in the island of Ireland. Interviews were conducted between April 1987 and November 1992 by Irish psychiatrists and social scientists following informed consent.

Diagnoses were generated using modified sections of the Structured Clinical Interview for DSM-III-R (SCID) for selected Axis I disorders (Spitzer et al., 1979). All relevant diagnostic information for each individual relative was reviewed, blind to pedigree assignment and marker genotypes, independently by Kenneth S. Kendler, MD and Dermot Walsh, MB, FRCPI. Each diagnostician made up to three best estimate DSM-III-

R diagnoses. Four definitions of affection were used, as follows: narrow, including only schizophrenia and poor-outcome schizoaffective disorder; intermediate, adding also schizophreniform disorder, delusional disorder, atypical psychosis, good-outcome schizoaffective disorder, and schizotypal personality disorder; broad, further adding psychotic affective illness, and paranoid, avoidant, and schizoid personality disorders; very broad, adding to the broad definition all other psychiatric diagnoses (e.g., psychotic and non-psychotic affective disorders, anxiety disorders, alcoholism, and other non-schizophrenia spectrum personality disorders) (Fanous et al, 2004).

In the ISHDSF, the operational criteria checklist for psychotic illness (OPCRIT) (McGuffin et al, 1991) was used for phenotypic assessment. Sixty of the 75 items in the OPCRIT were selected for inclusion in a factor analysis based on their assessment of signs and symptoms rather than course or historical features of illness. This yielded five symptom factors: hallucinations, delusions, and negative, manic, and depressive symptoms. Subjects were assigned scores for these factors by summing items clustering within each factor (Fanous et al, 2005).

Genotyping:

In the initial phase of the ISHDSF genome scan, the sample was randomly divided into three family sets. Of the 684 markers used, 488 were unique to individual subsets. Markers used in the preliminary analyses presented here were all unique to one subset of

subjects and represent a 30 centimorgan scan. The majority of markers genotyped were tri- and tetra-nucleotide repeat microsatellites generated by the Cooperative Human Linkage Center (CHLC), and many are included in the Weber screening set, version 8.0. Methods used in genotyping have been previously described (Straub et al, 1993, 1999).

Irish Case-Control Study of Schizophrenia (ICCSS):

The Irish Case-Control Study of Schizophrenia (ICCSS) sample was collected in the same geographic regions as that of the ISHDSF sample, but does not include any of the same subjects.

Ascertainment criteria, subject characteristics, and phenotypic assessments:

The 1021 affected subjects were selected from in-patient and outpatient psychiatric facilities in the Republic of Ireland and Northern Ireland. Subjects were eligible for inclusion if they had a diagnosis of schizophrenia or poor-outcome schizoaffective disorder by DSM-III-R criteria, which were confirmed by a blind expert diagnostic review. Controls were recruited in Northern Ireland predominantly from volunteers donating at the Northern Ireland Blood Transfusion Service and also from the Republic from the Garda Siochana (the national police force) and the Forsa Cosanta Aituil (the army reserve). Both cases and controls were included only if they reported all four grandparents as being born in Ireland or the United Kingdom (Chen et al, 2007).

Subjects were rated for each symptom using case records and interviews independently. All subjects were assessed using a modified version of the Structured Clinical Interview for DSM-III-R (Spitzer et al. 1987), and family history was assessed using a modified version of the Family History Research Diagnostic Criteria. In addition, case notes were thoroughly reviewed and rated using our Casenote Rating Scale (Fanous et al, in preparation). These assessments were averaged and entered into a factor analysis using the statistical program MPLUS (Muthen and Muthen, 2001). This yielded three symptom factors: Positive, Negative, and Schneiderian. Sum scores were generated for each factor with eleven items used to generate Positive and Schneiderian symptom scores and eight for Negative symptoms. These were then standardized to have values ranging from one to two (Fanous et al, in preparation).

Genotyping:

Individual genes or small groups of genes were genotyped using a range of assays. Some were genotyped using the GenomeLab SNPstream platform from Beckman Coulter which is used for multiplex reactions of 12-plex or 48-plex levels. Single SNPs were genotyped using the TAQMAN system from ABI (Foster City, CA). Fluorescence polarization detection with template-directed dye-terminator incorporation (FP-TDI) (Chen and Kwok, 1997) was also used for some genotyping experiments in this sample.

Genetic Association Information Network (GAIN):

The GAIN schizophrenia study is one of six diseases under investigation by this consortium. The other diseases are: bipolar disorder, major depression, attention deficit hyperactivity disorder, neuropathy in type I diabetes, and psoriasis.

Ascertainment criteria, subject characteristics, and phenotypic assessments:

Participants were recruited from 11 sites: Chicago, IL; Irvine, CA; Denver, CO; Houston, TX; Iowa City, IA; St. Louis, MO; New York, NY; Philadelphia, PA; New Orleans, LA; Atlanta, GA; Brisbane, Australia. Subjects were all of European or African ancestry. To meet ascertainment criteria for this GAIN study, probands must have a consensus best-estimate DSM-IV (Diagnostic and Statistical Manual of Mental Disorders) diagnosis of schizophrenia or of schizoaffective disorder with at least six months' duration of the "A" criteria for schizophrenia. Furthermore, subjects must be at least 18 years of age and give informed consent for participation. Currently, the full sample includes 4591 participants (1217 EA cases, 1442 EA controls, 953 AA cases, 979 AA controls) (NCBI, Genome-Wide Association Study of Schizophrenia, August, 2009).

Subjects were interviewed by trained clinicians using the Lifetime Dimensions of Psychosis Scale (LDPS) (Levinson et al, 2002). Using the LDPS, interviewer's narrative

report, and all available psychiatric records, two experienced clinicians determined all relevant diagnoses.

Genotyping:

For the GAIN study, the Broad Institute Center for Genotyping and Analysis used the Affymetrix Human Mapping Array 6.0 gene chip. In addition to detecting 906,600 SNPs, this chip also contains more than 946,000 probes for the detection of copy number variation. Quality control procedures performed prior to data release include: removal of SNPs not in Hardy-Weinberg equilibrium ($p>.000001$), and dropping SNPs with a call rate <95%. Duplicate samples should have concordance rates of >99.5% (Foundation for the National Institutes of Health GAIN Program, 2007).

Chapter 2: Schizophrenia Symptom Characteristics in Subjects of African and European Ancestry

Abstract:

The heterogeneous presentation of schizophrenia makes symptom-level analyses particularly important in designing studies exploring the etiology and pathophysiology of this disorder. In this study, symptoms were assessed using the Lifetime Dimensions of Psychosis Scale (LDPS) in 996 subjects of African ancestry (AA) and 999 of European ancestry (EA) drawn from the Genetic Association Information Network (GAIN) schizophrenia study. Factor analysis in MPlus yielded three symptom factors in both the separate and combined AA and EA cohorts: positive symptoms, negative symptoms, and affective symptoms. However, the factor loadings and thresholds significantly differed between groups, and allowing these to vary resulted in different factor structures. Differences were also observed between demographic variables such as age of onset, duration of illness, and sex and the factors in the AA and EA samples. Earlier age of onset predicted higher scores for all factors in both groups. This did not appear to be a function of duration of illness as this variable only predicted higher affective scores in the AA sample and greater negative scores in the EA sample. Females demonstrated higher affective scores in the EA group, but no other sex differences were observed. The extent to which AA and EA populations differ in symptom expression may be a function of differential environmental exposures or genetic predispositions between groups. Subsequent analyses incorporating genome wide genotypic data will aid in distinguishing these possibilities.

Introduction:

From its original naming in 1911 by Eugen Bleuler as the "group of schizophrenias", this illness has been recognized as encompassing a wide variety of clinical symptoms. However, affected individuals almost never exhibit all of these behaviors. The heritability, or genetic contribution to the variation, of a schizophrenia diagnosis has been estimated at 81% by meta-analysis (Sullivan et al, 2003) signifying that the expression of the disease itself is predominantly mediated by genes. A growing number of genes have been identified as modifiers of schizophrenia symptoms, indicating that at least some of the variability within this illness is genetically mediated.

Investigations of the variability in schizophrenia symptoms have most often taken place within the same, generally Caucasian, population, but some studies exploring differences in rates of symptoms between ethnic groups have been conducted previously. One early study found higher ratings in African-Americans than European-Americans for nearly every symptom they examined (Adebimpe et al, 1982). First-rank symptoms such as hearing conversing voices, thought insertion or thought broadcasting are reportedly higher in African-Americans than European-Americans (Strakowski et al, 1996). This was replicated in a later study in which raters blinded to ethnicity detected higher levels of first-rank and psychotic symptoms in African-American men compared to European-American men. However, no differences were detected for symptoms in women or for a diagnosis of schizophrenia between ethnic groups (Arnold et al, 2004).

Factor analysis is one tool often employed to assess unobserved variables tapped by measured items that tend to be endorsed together. Factor analyses of schizophrenia symptoms in Caucasian samples have usually found two to five factors with most converging on a three factor solution: positive symptoms, negative symptoms, and a disorganization or cognitive symptoms factor (Andreasen et al, 1995). Some studies have also found affective factors incorporating anxiety, depression and/or manic symptoms. One factor analytic study incorporating subjects of both European (mostly British) and African (African-Caribbean) descent favored a six factor solution (Hutchinson et al, 1999). Only one factor, composed of manic items (distractability, flight of ideas, and pressure of speech) and catatonic symptoms (mannerisms and posturing, and stereotypies and tics) differed between groups with the African-Caribbean subjects having elevated factor scores.

This is the first study of schizophrenia symptoms to use a large sample from two populations that were ascertained and analyzed together. In the studies undertaken here, we compare the expression of schizophrenia symptoms in subjects with African and European ancestry.

Methods:

Subjects and Demographics:

Subjects were drawn from the GAIN schizophrenia study detailed in chapter 1. The Lifetime Dimensions of Psychosis Scale (LDPS) (Levinson et al, 2002) was used to assess the duration and severity of symptoms based on a diagnostic interview and review of medical records. Examination of the item endorsements across the 11 ascertainment cities revealed many significant differences. Since the origin of these differences could be legitimate variation by city or unintentional differences in application of the rating criteria, we chose to restrict subsequent analyses to a subset of sites with similar proportions of AA and EA subjects, thereby limiting the potential for interpretation of site differences as ancestry group differences. Six sites met our criteria for having relatively balanced proportions of AA and EA subjects (i.e. no more than 70% of subjects from either ancestry group). Subsequent analyses pooled subjects by ancestry groups across these six sites: Atlanta, GA; St. Louis, MO; New York, NY; Philadelphia, PA; Chicago, IL and New Orleans, LA.

Table 2.1. Demographic information for subjects from the six included sites.
sd = standard deviation

	Subject N	Males	Females	Age at Onset (sd)	Duration (sd)
AA	996	628	368	22.4 (7.3)	20.2 (10.8)
EA	999	662	337	22.2 (7.2)	23.0 (11.2)
Total	1995	1290	705		

Age at onset was similar for subjects in both groups ($t = .37$, $p = .714$), but EA subjects tended to have a longer duration of illness at the time they were interviewed ($t = -5.50$, $p < .0001$) (Table 2.1). Female subjects were diagnosed with schizophrenia later than male subjects (23.7 yrs, sd = 7.9 versus 21.9 yrs, sd = 6.8) in keeping with prior findings (t = -

3.40, p = .0007). T-tests were conducted for each of the LDPS items between groups using SAS software (SAS Institute, 2005).

Factor Analyses:

Fourteen items from the LDPS were selected for inclusion in the factor analyses, and scores for duration and severity were summed for each item to yield one ordinal score per item per subject. Exploratory factor analyses (EFAs) were implemented using a VARIMAX (orthogonal) rotation in MPlus (Muthen and Muthen, 2001). A scree plot of the eigenvalues suggested a three factor solution fit best for the combined and individual samples. The EFAs indicated distinct factor structures for AA and EA groups compared to the combined sample. Confirmatory factor analyses (CFAs) followed using a WLSMV estimator. To test for invariance of the factor structure across ancestry groups, a robust chi-square difference test for ordinal data available in MPlus was utilized to determine whether factor loadings and symptom thresholds could be constrained to be equal across groups. This is a restrictive test of whether the factors are equivalent in the two groups.

Relationships between the sum scores derived from the factor structures within each group and the demographic variables of sex, age of onset, and duration of illness were examined using PROC REG in SAS (SAS Institute, 2005).

Results:

EA subjects had higher ratings for all four affective items relating to depression or mania as well as blunted affect (Table 2.2). AA subjects demonstrated higher levels of hallucinations and conversing/commenting/continuous hallucinations. Positive and negative symptom items were generally endorsed at higher rates than affective symptom items for both groups. All significant item-level results maintain significance following a Bonferroni correction (p = .0035).

Table 2.2. Item level comparisons by group. Number of subjects ranged from 995-999 for each group and item comparison.

Item	AA Mean	EA Mean	difference	t	p
paranoia	7.01	7.05	-0.04	-0.45	0.65
delusions	7.38	7.48	-0.10	-1.71	0.09
hallucinations	7.12	6.81	0.30	3.64	0.0003
control delusions	3.22	3.08	0.13	0.84	0.40
conversing/commenting/cont hall	5.49	4.89	0.60	3.90	<.0001
abnormal perception of thought	2.88	2.67	0.22	1.42	0.16
blunted affect	5.08	5.46	-0.38	-3.07	0.002
poverty of speech	3.56	3.80	-0.24	-1.59	0.11
formal thought disorder	4.76	4.98	-0.22	-1.58	0.11
bizarre behavior	4.65	4.79	-0.14	-1.00	0.32
depression	2.99	3.80	-0.81	-5.78	<.0001
depression with psychotic features	0.96	1.44	-0.48	-4.22	<.0001
mania	1.04	1.74	-0.70	-6.46	<.0001
mania with psychotic features	0.62	1.07	-0.45	-4.78	<.0001

Exploratory factor analysis indicated a three factor solution for the combined sample which took the form of positive symptoms (paranoia, delusions, hallucinations, control delusions, conversing/commenting/continuous hallucinations, and abnormal perception of

thought), negative symptoms (blunted affect, poverty of speech, formal thought disorder, and bizarre behavior), and affective symptoms (depression, depression with psychotic features, mania, and mania with psychotics features) (Appendix A). Although the factor structure was fairly definitive, some items identify factors more strongly than others. Namely, the items "control delusions" and "abnormal perception of thought" have lower loadings than the other positive factor items. The difference in factor loadings is most striking for the affective symptom factor in which the depression item loadings are only about half that of the mania item loadings.

A robust chi-square difference test of full invariance of the factor loadings and symptom thresholds for the three factors across the ancestry groups was significant (chi-square = 83.4, df = 25, p-value <.0001). This finding suggests that some of the factors may not be equivalent (i.e., measuring the same latent constructs) across the AA and EA groups. The same factor structure was maintained for the EA group when analyzed separately, but in the AA subjects, the depression and depression with psychotic features items loaded most highly (and in the negative direction) with the negative symptom factor. Factor loadings for the affective items were similar in the EA and combined samples. The positive factor items had similar patterns between groups and compared to the combined sample with "control delusions" and "abnormal perception of thought" having somewhat lower loadings than the other items. "Delusions" and "paranoia" had more equivocal loadings in the EA group with high loadings on the negative factors. Negative factor items demonstrated factor loadings fairly similar to each other and across groups. The fit

indices for the confirmatory factor analyses (CFAs) varied slightly between the combined sample and the two groups separately (Table 2.3).

Table 2.3. Fit indices for the separate and combined group CFAs.

	combined	AA	EA
CFI	0.893	0.903	0.889
TLI	0.893	0.900	0.889
RMSEA	0.131	0.139	0.127

Slightly more variation was observed in the factor correlations with the AA group showing a lower positive correlation between the positive and affective factors and greater negative correlation between the negative and affective factors (Table 2.4).

Table 2.4. Factor correlations for AA, EA and combined samples.

	combined	AA	EA
positive w/negative	0.385	0.374	0.397
positive w/affective	0.179	0.125	0.221
negative w/affective	-0.051	-0.157	-0.009

Sex and duration of illness were not strong predictors of the sum scores, demonstrating a significant relationship with only one or two factors each. Age of onset, however, bore a strong negative relationship with all factors - younger ages of onset predicted higher symptom levels (Table 2.5).

Table 2.5. Regression results for each factor and group with sex, age of onset, and duration.

African Ancestry						European Ancestry					
factors	covariate	t	p-value	mean (M)	mean (F)	factors	covariate	t	p-value	mean (M)	mean (F)
positive	sex	-1.42	0.1568	33.4	32.5	positive	sex	0.98	0.3287	31.8	32.4
negative		-0.15	0.8791	22.0	21.9	negative		0.43	0.6707	19.0	19.2
affective		0.97	0.3302	1.6	1.8	affective		4.75	<.0001*	7.2	9.6
positive	age at	-2.34	0.0197*			positive	age at	-4.18	<.0001*		
negative	onset	-3.44	0.0006*			negative	onset	-3.38	0.0007*		
affective		-3.97	<.0001*			affective		-2.36	0.0186*		
positive	duration	1.29	0.1966			positive	duration	1.41	0.1591		
negative		-0.32	0.7469			negative		5.62	<.0001*		
affective		2.95	0.0033*			affective		1.88	0.0601		

Discussion:

Examination of schizophrenia symptoms in a large sample of subjects from African and European ancestral populations revealed much similarity, but several noteworthy differences. A three factor solution fit best for the combined sample and separate groups, but the factor structures significantly differed between AA and EA groups with two affective items, depression and depression with psychotic features, inversely loading with the negative symptom factor in AA subjects. Other items, such as delusions and paranoia, still loaded most strongly with the same factor as in the combined sample, but clearly show differences between groups based on the factor loadings. Formal tests of

between group differences for the factor loadings and thresholds separately will provide additional information in this regard.

Item level analyses also indicated a substantive difference in the way affective symptoms relate to schizophrenia between groups. The four LDPS items indexing affective symptoms, depression and mania alone and with psychotic features, were more commonly endorsed in the EA subjects as well as blunted affect. AA subjects, on the other hand, demonstrated more hallucinations as measured by the two LDPS items assessing these symptoms. With the exception of the conversing/commenting/continuous auditory hallucinations item, we did not detect greater first-rank symptoms in the AA subjects as has been previously reported (Strakowski et al, 1996; Arnold et al, 2004).

There are reasonable grounds for considering a genetic origin for the observed symptom differences. Linkage analyses of schizophrenia in African-American and European-American samples identified different loci between groups (Kaufmann et al, 1998; Takahashi et al, 2003; Faraone et al, 2005; Suarez et al, 2006), and Ioannidis et al (2004) showed that disease associated alleles for several complex diseases vary in frequency across "racial" boundaries. It seems plausible that symptom differences might arise from distinct genetic etiologies as well. The set of genes impacting symptom variation almost certainly overlaps with the risk alleles for schizophrenia.

Numerous aspects of the environment have also been suggested to explain differences in rates of schizophrenia between groups, and these factors may also impact symptom expression. Discrimination is one variable proposed to lead susceptible individuals toward psychosis or other mental illnesses in minority groups and migrants (Chakraborty and McKenzie, 2002). Urbanicity has also been suggested as a predisposing factor (Pedersen and Mortensen, 2001; Krabbendam and van Os, 2005). Migrant status has been repeatedly raised as a potential risk factor, but is often confounded by the aforementioned possibilities (Cantor-Graae et al, 2005; Fearon et al, 2006). These and other environmental variables merit further study.

The extent to which symptoms or subtypes cluster in families has been examined with some studies supporting the idea of familial aggregation (Onstad et al, 1991; Loftus et al, 2000; Wickham et al, 2001; McGrath et al; 2009) while others have not (DeLisi et al, 1987; Kendler et al, 1988). Even when similar forms of schizophrenia are manifest in relatives, causal ambiguity between genetic and familial environmental influences remains.

Interpretation of the results presented here should be considered in the context of several possible limitations. First, we relied on self report for ancestry information and group assignment, but incorporation of ancestry informative genotypic markers would be useful in assigning finer scale distinctions to these highly admixed populations. In addition to the numerous African and European populations that have migrated to America, several

generations of intermixing within and across these broadly defined groups have contributed to the genetic complexity present in our sample. One study of African-American admixture investigated individuals in nine American cities and detected marked variability in the range of "European DNA" from 12.2% Charleston, South Carolina to 22.8% in New Orleans, Louisiana (Parra et al, 1998). The cross-site differences detected in our study may partially result from different genetic influences or other valid sources of variance such as more severely ill patients collected at some sites. Unfortunately, these legitimate differences cannot easily be disentangled from confounding sources of difference such as variation in applying the LDPS rating guidelines across sites. Our restriction of analyses to subjects from sites with less than 70% from either ancestry group serves to enhance confidence in the results.

The difference in duration of illness between groups is of potential concern, but the relationship between this variable and the factors was rather weak compared with the influence of age of onset. Our findings indicate that early onset of schizophrenia predisposes individuals to a more severe form of the illness compared with those who become ill later.

Systematic clinician bias in schizophrenia diagnosis cannot be ruled out. If African-Americans are more likely than European-Americans to be diagnosed with schizophrenia when presenting with the same symptoms, artificial differences in symptom profiles could conceivably arise. This scenario would result in increased observed severity in the

EA population for which there is only scant evidence. Of the seven LDPS items significantly different between groups, five showed increased severity/duration in the EA group. That said, the relative increase in affective symptoms we observed in the EA group may partially underlie the findings that this population is more often given a diagnosis of bipolar disorder compared to AA individuals (Mukherjee et al, 1983; Minsky et al, 2003).

The extent to which ancestry group differences reflect clinician bias in diagnosis and assessment, sociocultural differences, or biological factors has thus far been inextricable. With this large, well-characterized sample, we finally have the opportunity to examine whether genetic factors underlie symptom differences between schizophrenic subjects of African and European ancestry. Regardless of their sources, clinician awareness of group differences in illness rates and presentation would aid in the proper diagnosis and treatment of affected individuals.

Chapter 3: Candidate Gene Investigations of Schizophrenia Symptom Modification

Chapter Abstract:

Although nearly all efforts in the field of schizophrenia genetics have centered around detection of susceptibility genes, a growing literature aims to find genetic causes of symptom variation in schizophrenia. This chapter details four studies of a total of seven genes examined for schizophrenia symptom modifier effects in two Irish samples, the ICCSS and ISHDSF. The first set of genes was investigated in the ISHDSF using UNPHASED and QTDT. We found tentative associations of PAH with delusions and GABRB3 with hallucinations, but no symptom factors were associated with SLC6A4 or MAOB. We probed the remaining genes in the ICCSS cohort. AKT1 was associated with Schneiderian symptoms using UNPHASED. Using the sequential addition method implemented in UNPHASED, dysbindin was not associated with any symptom factor, but SNAP25 demonstrated association with both hallucinations and delusions. In the absence of a cure for this illness, treatments aimed at ameliorating symptoms may be the best approach. Additional studies identifying modifier and susceptibility-modifier genes will give us a better understanding of the genes (and biological pathways) giving rise to these symptoms and will aid in the generation of more targeted treatment options.

Polymorphisms in SLC6A4, PAH, GABRB3, and MAOB and Modification of Psychotic Disorder Features

Adapted from:
Bergen SE, Fanous AH, Walsh D, O'Neill FA, Kendler KS. (2009) Polymorphisms in SLC6A4, PAH, GABRB3, and MAOB and modification of psychotic disorder features. *Schizophrenia Research* 109: 94-97.

Abstract

We tested four genes [phenylalanine hydroxylase (PAH), the serotonin transporter (SLC6A4), monoamine oxidase B (MAOB), and the gamma-aminobutyric acid A receptor β-3 subunit (GABRB3)] for their impact on five schizophrenia symptom factors: delusions, hallucinations, mania, depression, and negative symptoms. In a 90 family subset of the Irish Study of High Density Schizophrenia Families, the PAH 232 bp microsatellite allele demonstrated significant association with the delusions factor using both QTDT (F=8.0, p=.031) and QPDTPHASE (chi-square=12.54, p=.028). Also, a significant association between the GABRB3 191 bp allele and the hallucinations factor was detected using QPDTPHASE (chi-square=15.51, p=.030), but not QTDT (chi-square=2.07, p=.560).

Introduction

Schizophrenia is a complex genetic disorder with numerous reported susceptibility genes (Harrison & Owen, 2003; Tsuang et al, 1999). The substantial clinical variability noted from its earliest descriptions by Kraepelin (1921) and Bleuler (1950) through present-day DSM subtypes (McGlashan & Fenton, 1991) and the results of factor analysis (Peralta & Cuesta, 2001; Fanous et al, 2005) may arise from genetic heterogeneity. Specific patterns of symptoms resulting from differing genetic etiologies may underlie variation within the disease or ultimately result in partitioning "the schizophrenias" into distinct diagnoses.

Genes that underlie a predisposition to schizophrenia may additionally influence the manifestation of the disorder (termed susceptibility-modifier genes). However, genes unrelated to susceptibility for the illness can affect expression of symptoms following onset (termed modifier genes) (Fanous & Kendler, 2005). Several reports of associations between genetic polymorphisms and clinical symptoms of schizophrenia have now been published (Malhotra et al, 1998; Cardno et al, 1999; Kaiser et al, 2000; Zhang et al, 2000; Serretti et al, 2001; Fanous et al, 2004; Reynolds et al, 2005; Fanous et al, 2005; McClay et al, 2006, DeRosse et al, 2007).

In this study, we tested for the presence of association between five clinical features of schizophrenia (delusions, hallucinations, mania, depression, and negative symptoms) and polymorphisms in four genes: phenylalanine hydroxylase (PAH), the serotonin

transporter (SLC6A4), monoamine oxidase B (MAOB), and the gamma-aminobutyric acid A receptor β-3 subunit (GABRB3).

PAH is located at 12q22-q24.2 and is most recognized for its involvement in the recessive metabolic disorder, phenylketonuria. However, allelic variation that stops short of functional inactivation may have a markedly different clinical impact. There was one report of increased incidence of schizophrenia in parents of phenylketonuric children (Vogel, 1985), but other studies exploring the relationship between PAH and schizophrenia have yielded less positive results (Sobell et al, 1993; Chao & Richardson, 2002; Richardson et al, 2003). Tentative reports of PAH polymorphisms impacting paranoid hallucinations (Uebelhack et al, 1987) and negative symptoms (Wilcox et al, 2002) have been published. The biological plausibility of the PAH mechanism of action in the pathophysiology of schizophrenia largely rests on its ties to the dopaminergic system, since PAH converts phenylalanine to tyrosine, a dopamine precursor. PAH is also involved in serotonin synthesis, and its impairment results in lowered serotonin levels (Alcaniz & Silva, 1997).

Multiple lines of evidence have previously also implicated alterations of the serotonin system in schizophrenia (Breier, 1995), and particular symptom dimensions may be more affected than others. SLC6A4 (aka SERT), located at 17q11.1-q12, has demonstrated equivocal schizophrenia association, but a recent meta-analysis of 12 studies including the 17 bp VNTR polymorphism genotyped here found strong evidence for association

(OR 1.24, p=.00014) (Fan & Sklar, 2005). Additionally, SLC6A4 has been linked to depressive symptoms in schizophrenia (Golimbet et al, 2004), the Psychopathic deviance, Paranoia, and Schizophrenia subscales of the MMPI in patients with affective disorders (Golimbet et al, 2003), and hallucinations in patients with schizophrenia or schizoaffective disorder (Malhotra et al, 1998).

The MAOB gene, located on the X-chromosome at Xp11.23, functions in the catabolism of catecholamines such as dopamine, epinephrine, norepinepherine, and phenylethylamine (a phenylalanine metabolite). A study of MAOB polymorphisms and aggression in subjects with schizophrenia failed to find a relationship between the two (Zammit et al, 2004), but there has been tentative support for MAOB in liability to schizophrenia (Dann et al, 1997; Wei & Hemmings, 1999; Carrera et al, 2008) and bipolar disorder (Lin et al, 2000).

Examinations of GABAergic system pharmacology and neuroanatomy in subjects with schizophrenia have revealed numerous changes (Wassef et al, 2003). The only study exploring polymorphisms in the β3 subunit gene showed no linkage with schizophrenia (Byerley et al, 1995). However, GABRB3, located at 15q11.2-q12, has been implicated in autism susceptibility (Cook et al, 1998; Craddock et al, 1999). Autism and schizophrenia share a few common features, and some evidence exists relating the catatonic symptoms of both to the GABAergic system (Dhossche, 2004).

Although these genes have not often been investigated with regard to schizophrenia, ample evidence exists for the involvement of neurotransmitter systems linked to these genes in this disorder to merit their examination as potential modifier genes.

Methods

Subjects and Assessment

Subjects were drawn from the Irish Study of High Density Schizophrenia Families (ISHDSF) more fully described in chapter 1. The full sample consisted of 1,425 individuals from 270 families ascertained on the basis of two or more members with DSM-III-R schizophrenia or poor outcome schizoaffective disorder, but results presented here were derived from a 90 family subset with available genotypes.

Diagnoses were generated using modified sections of the Structured Interview for DSM-III-R (SCID) for selected Axis I disorders (Spitzer et al., 1979). Four definitions of affection were used, as follows: narrow, including only schizophrenia and poor-outcome schizoaffective disorder; intermediate, adding also schizophreniform disorder, delusional disorder, atypical psychosis, good-outcome schizoaffective disorder, and schizotypal personality disorder; broad, further adding psychotic affective illness, and paranoid, avoidant, and schizoid personality disorders; very broad, adding to the broad definition all other psychiatric diagnoses (e.g., psychotic and non-psychotic affective disorders, anxiety disorders, alcoholism, and other non-schizophrenia spectrum personality disorders).

Factor analysis of the operational criteria checklist for psychotic illness (OPCRIT) (McGuffin et al, 1991) yielded five symptom factors: hallucinations, delusions, and negative, manic, and depressive symptoms (Fanous et al, 2005). Subjects were assigned scores for these factors by summing items clustering within each factor.

Genotyping

In the initial phase of the ISHDSF genome scan, the sample was randomly divided into three sets of 90 families each. Of the 684 markers used, 488 were unique to individual subsets. Markers used in the analyses presented here were all unique to one subset of subjects. The primer sequences used to amplify the 6 MAOB microsatellite alleles, 9 PAH microsatellite alleles and 14 GABRB3 microsatellite alleles were reported by Grimsby et al (1992), Goltsov et al (1993) and Beckmann et al (1993), respectively. The SLC6A4 marker genotyped here was a 17 bp VNTR with three alleles flanked by primers reported by Ogilvie et al (1996). Methods used in genotyping have been previously described (Straub et al, 1993, 1999).

Statistical Analyses

Tests of association between all genes and the diagnostic categories were performed using PDTPHASE, which is part of the UNPHASED package (Dudbridge, 2003). Rare microsatellite alleles, defined as comprising less than 3% of the sample, were dropped from analyses.

Association tests between the gene polymorphisms and each of the five symptom factors were performed using QTDT (Abecasis et al, 2000) and QPDTPHASE, another facet of UNPHASED (Dudbridge, 2003), for all genes except MAOB. Instead, this gene was only analyzed using QPDTPHASE, since QTDT is not designed to analyze X-chromosome markers. For each QTDT marker by factor test, a Bonferroni correction was calculated to account for the number of alleles tested and the most significant marker is reported. UNPHASED results are global tests of significance.

Results and Discussion

Significant associations of MAOB with intermediate (chi-square=10.43, p=.034) and broad (chi-square=9.94, p=.041) diagnostic categories, driven by the 201 bp allele, were the only gene-diagnosis relationships observed (Table 3.1). However, two genes showed a significant relationship with symptom factors (Table 3.2). The PAH 232 bp allele demonstrated a significant association with the delusions factor using both QTDT (F=8.0, p=.031) and QPDTPHASE (chi-square=12.54, p=.028). Also, a significant association between the GABRB3 191 bp allele and the hallucinations factor was detected using QPDTPHASE (chi-square=15.51, p=.030), but not QTDT (chi-square=2.07, p=.560).

Our results support the tentative implication of MAOB in the etiology of psychotic disorders but not specifically schizophrenia, since the narrow diagnostic category did not demonstrate association. These data also provide some evidence for the

impact of PAH and GABRB3 on the respective factors of delusions and hallucinations. However, these results should be interpreted with caution since no corrections for multiple testing across genes and factors were implemented due to the correlated nature of the tests. Nevertheless, neurobiological links between the GABAergic system and psychosis have been drawn (Keverne, 1999). Furthermore, dopaminergic modulation of delusions has also been demonstrated (Krieckhaus et al, 1992), lending credence to a potential PAH-delusions association. Additional scrutiny of the relationships between these genes and factors in an independent sample is warranted.

Table 3.1 PDTPHASE association test results for four genes and four diagnostic categories

	PAH		SERT		MAOB		GABRB3	
Diagnostic Categories	chi-square	p-value	chi-square	p-value	chi-square	p-value	chi-square	p-value
Narrow	6.834	0.233	0.338	0.845	6.591	0.159	6.930	0.327
Intermediate	5.838	0.322	0.529	0.768	10.430	0.034*	3.944	0.684
Broad	6.533	0.258	0.997	0.608	9.938	0.041*	6.067	0.416
Very Broad	2.221	0.818	1.447	0.485	4.561	0.335	6.389	0.381

Table 3.2a QTDT results for markers from three genes and five clinical factors

	PAH			SERT			GABRB3		
Clinical Factors	F	p-value	Bonferroni p-value	F	p-value	Bonferroni p-value	F	p-value	Bonferroni p-value
Negative Symptoms	1.070	0.303	0.886	0.680	0.411	0.653	2.530	0.114	0.452
Delusions	7.970	0.005	0.031*	0.020	0.884	0.987	1.390	0.240	0.747
Hallucinations	3.310	0.070	0.354	0.019	0.891	0.988	2.070	0.152	0.560
Manic Symptoms	4.950	0.027	0.152	0.357	0.551	0.798	2.060	0.153	0.564
Depressive Symptoms	2.400	0.123	0.544	0.460	0.500	0.750	1.950	0.164	0.591

Table 3.2b QPDTPHASE results for markers from four genes and five clinical factors

	PAH		SERT		MAOB		GABRB3	
Clinical Factors	chi-square	p-value	chi-square	p-value	chi-square	p-value	chi-square	p-value
Negative Symptoms	2.148	0.828	0.186	0.911	1.564	0.815	6.408	0.493
Delusions	12.540	0.028*	0.675	0.713	1.660	0.798	11.170	0.132
Hallucinations	6.434	0.266	0.151	0.927	2.705	0.608	15.510	0.030*
Manic Symptoms	3.443	0.632	0.385	0.825	4.287	0.369	4.666	0.701
Depressive Symptoms	3.889	0.566	2.166	0.339	1.851	0.763	3.310	0.855

Association of AKT1 with Schneiderian Symptoms in the Irish Case-Control Study of Schizophrenia

Adapted from:
DL Thiselton, SE Bergen, Wormley B, McMichael O, O'Neill FA, Walsh D, Vladimirov V, Fanous AH, Kendler KS, Riley BP. AKT1 is associated with schizophrenia in the Irish case-control study of schizophrenia (ICCSS): Follow-up analysis of specific phenotypic factors. (in preparation)

Abstract:

Several lines of evidence, including a number of association studies, suggest a role for AKT1 in the pathophysiology of schizophrenia. This research group previously demonstrated an AKT1 association with schizophrenia in an Irish family sample and also association of the risk haplotype with hallucination symptoms in that sample (Thiselton et al, 2008). Prior tests of association between AKT1 and schizophrenia in the Irish Case–Control Study of Schizophrenia (ICCSS) sample revealed a positive result for one of the seven SNPs tested, rs10149779 (chi-sq=7.041, empirical p=0.045). A two-marker haplotype, rs10149779-rs10138227, demonstrated even stronger association with schizophrenia (chi-sq=21.79, p=3.04E-06) (Thiselton et al, in preparation). In this study, we explored whether the risk haplotype in the ICCSS also predisposes subjects to particular symptom dimensions. Interviews and medical records provided phenotypic information which was entered into a factor analysis and revealed three symptom factors: Positive, Negative, and Schneiderian (Fanous et al, in preparation). In analyses using individual SNPs, the same SNP demonstrating positive association with schizophrenia, rs10149779, was also positively associated with the Schneiderian symptom factor (chi-square=5.3, p=.021). Mirroring the schizophrenia association results, the risk haplotype demonstrated an even stronger association with this factor (chi-square=8.1, p=.004). Our finding of AKT1 association with Schneiderian symptoms in combination with prior work showing association with hallucinations indicates some variants of this gene may predispose individuals to a particular form of schizophrenia with high levels of positive symptoms.

Introduction:

The AKT1 gene, also known as v-akt murine thymoma viral oncogene homolog 1, is located at 14q32.32 and encodes a serine/threonine protein kinase involved in intracellular signaling pathways that impact cell survival, synaptic transmission and neuronal plasticity (Grimes and Jope, 2001). Multiple lines of evidence have implicated AKT1 in schizophrenia pathophysiology. AKT1 is a vital part of the normal dopaminergic transmission pathway and acts downstream of the DRD2 receptor, a target of many antipsychotic drugs (Beaulieu et al, 2004). AKT1 protein levels showed a 68% decrease in the brains of individuals with schizophrenia by one report (Emamian et al, 2004). Decreased expression of AKT1 mRNA has been reported in the dorsolateral prefrontal cortex of individuals with schizophrenia, a brain region frequently implicated in schizophrenia neuropathology (Thiselton et al, 2008). Additionally, it has been suggested that dysbindin promotes neuronal viability through AKT signaling, providing a link between AKT and another putative susceptibility gene (Numakawa et al, 2004).

Since altered mRNA and protein levels could result from primary genetic changes or be downstream effects of other altered processes, genetic association tests are informative. Seven reports of positive association of AKT1 and schizophrenia have been published (Emamian et al, 2004; Ikeda et al, 2004; Schwab et al, 2005; Bajestan et al, 2006; Norton et al, 2007; Xu et al, 2007) including one from this research group using the Irish High Density Study of Schizophrenia Families (ISHDSF) (Thiselton et al, 2008). However,

six negative findings of association have also surfaced (Ohtsuki et al, 2004; Ide et al, 2006; Liu et al, 2006; Turunen et al, 2007; Sanders et al, 2008; Liu et al, 2008). With about half of the published association tests of this gene with schizophrenia reporting positive results, tentative support for a primary role of AKT1 in schizophrenia etiology has been presented.

In the previous study of AKT1 from this group, relationships between this gene and symptom factors of schizophrenia were explored, and the hallucinations factor showed the strongest association (Thiselton et al, 2008). One other exploration of AKT1 and symptoms of schizophrenia was conducted, but no significant relationships with positive, negative, or general psychopathology were found (Liu et al, 2008).

In the present study, association analysis of single SNPs in the whole sample revealed a significant association with schizophrenia for rs10149779 (chi-sq = 7.041, p = 0.008) and trends towards significance for rs3730358 (chi-sq = 3.197, p = 0.074) and rs12878684 (chi-sq = 3.567, p = 0.059). In each case the major allele was over-represented in cases relative to controls. Only the association with rs10149779 survived a 100,000 permutation test (chi-sq = 7.041, empirical p = 0.045) (Thiselton et al, in preparation).

Given the linkage disequilibrium relationships between the markers tested, a haplotypic association test between the rs10149779-rs10138227 block and schizophrenia was conducted. A stronger association was observed between this haplotypic combination and

disease compared to the individual SNPs (G-C; chi-sq = 21.79, p = 3.04E-06). There was an under-representation in cases of the rare-common haplotype (Thiselton et al, in preparation).

Based on the aforementioned results, we embarked on this study exploring whether genetic variation in AKT1 was related to schizophrenia symptom dimensions in the ICCSS sample.

Methods:

The subjects examined in this study were drawn from the Irish Case–Control Study of Schizophrenia (ICCSS) for which an in depth exposition can be found in chapter 1. The full sample includes 1021 affected individuals, and seven AKT1 SNPs were genotyped using validated Taqman SNP genotyping assays from ABI (www.appliedbiosystems.com). They were: rs3803300, rs12878684, rs1130214, rs10138227, rs10149779, rs3730358 and rs2498799.

Subjects were rated for each symptom using case records and interviews independently. These assessments were averaged and entered into a factor analysis using the statistical program MPLUS (Muthen and Muthen, 2001). This yielded three symptom factors: Positive, Negative, and Schneiderian (Fanous et al, in preparation). Sum scores were

generated for each factor with eleven items contributing to the Positive and Schneiderian symptom scores and eight for Negative symptoms. These sum scores were merged with the AKT1 genotypes for each subject using SAS 9.1 (SAS Institute, 2005) and analyzed using Unphased (Dudbridge, 2003). All three symptom factors were tested separately against each marker as well as the high risk haplotype consisting of rs10138227 and rs10149779.

Results:

Significant associations were observed between the same SNP showing schizophrenia association, rs10149779, and the Schneiderian symptom factor (chi-square=5.3, p=.021) (Table 3.4). The risk haplotype also demonstrated significant association with the Schneiderian factor (chi-square=8.1, p=.004).

Table 3.4. Association results for AKT1 and schizophrenia and three symptom factors.

	Schizophrenia		Negative		Positive		Schneiderian	
Marker	chi-square	p-value	chi-square	p-value	chi-square	p-value	chi-square	p-value
SNP1 rs3803300	0.436	0.509	2.152	0.142	1.616	0.204	0.530	0.467
SNP2 rs12878684	3.701	0.054	0.908	0.341	1.780	0.182	1.035	0.309
SNP3 rs1130214	0.512	0.474	0.139	0.710	0.084	0.772	0.444	0.505
SNP4 rs10138227	2.088	0.148	1.034	0.309	1.009	0.315	0.007	0.932
SNP5 rs10149779	7.296	0.007*	3.665	0.056	2.277	0.131	5.345	0.021*
SNP6 rs3730358	3.044	0.081	3.691	0.055	0.218	0.641	0.724	0.395
SNP7 rs2498799	0.815	0.367	1.113	0.291	0.011	0.918	0.284	0.594

Discussion:

Our investigations of AKT1 and symptom factors of schizophrenia revealed that the same risk SNP and haplotype predisposing individuals to the illness itself are also related to increased Schneiderian symptoms. Although the prior study of AKT1 and schizophrenia symptoms in an Irish family sample found association with hallucinations, several possibilities may explain this difference. One reason for the apparent discrepancy between the findings in the ICCSS and ISHDSF could be the risk allele differences between groups. The different AKT1 risk alleles may predispose to slightly different symptoms. Also, Schneiderian symptoms and hallucinations are not mutually exclusive. Schneiderian symptoms were initially thought to be specific to schizophrenia but may appear in related disorders, and they include several types of hallucinations such as audible thoughts, voices conversing, or a voice commenting on one's actions. Some delusions are also Schneiderian symptoms, and these include: external control of thoughts, thought broadcasting or insertion, or delusional perceptions. All hallucinations and Schneiderian symptoms are positive symptoms which may have similar underlying biological mechanisms including alterations of the AKT1 gene.

We would also like to mention that caution is warranted in interpreting these results given the number of statistical tests implemented and the absence of multiple testing corrections. Nevertheless, sufficient evidence exists to merit further research to

understand the relationship between AKT1 and positive symptoms of schizophrenia and the mechanisms by which this may occur.

SNAP25 Modification of Hallucinations and Delusions Symptoms

Adapted from:
Fanous AH, van den Oord ECG, Thiselton DL, Bergen SE, Wormley BT, Amdur RL, O'Neill FA, Walsh D, Kendler KS, Riley BP. Association study of SNAP25 and schizophrenia in Irish family and case-control samples. *Neuropsychiatric Genetics* (accepted)

Abstract:

Alterations at the synaptic level have been found in numerous studies of schizophrenia using a variety of methods. SNAP25, one of the vesicular docking proteins, has specifically been implicated in protein and functional studies as well as some association studies. Support for the involvement of this gene in schizophrenia was deemed sufficient to warrant additional genetic studies, and we tested for association of SNAP25 with this illness in the Irish Study of High Density Schizophrenia Families (ISHDSF). Eighteen SNPs (five haplotype blocks) were genotyped and tested using FBAT and PDTphase. The block five haplotype, consisting of two SNPs, rs362988 and rs6039820, was significantly associated with schizophrenia for all four concentric diagnostic categories and both statistical software packages. Consequently, this haplotype was selected for association tests with the symptom factors. The sequential addition method, implemented using UNPHASED, found this haplotoype of SNAP25 to be preferentially overtransmitted to subjects in the upper ~60% of the hallucinations (empirical P=.006) and delusions factors (empirical P=.01). These results suggest SNAP25 is a schizophrenia susceptibility gene which predisposes individuals to a form of illness with high rates of positive symptoms.

Introduction:

Synaptosomal-Associated Protein of 25 kDa (SNAP25) is a component of the soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) complex which mediates the exocytosis of neurotransmitters from the presynaptic neuronal membrane. Alterations of presynaptic proteins have been implicated in schizophrenia through a variety of studies (thoroughly detailed in chapter 5). SNAP25 specifically has been the focus of several studies of schizophrenia. An association between SNAP25 and decreased pre-pulse inhibition, a schizophrenia-related sensory gating defect, was found in mice (Jeans et al., 2007). Reductions in the SNAP25 protein have been reported in the hippocampus (Fatemi et al., 2001; Thompson et al., 2003a; Young et al., 1998; Thompson et al., 1998), prefrontal cortex (Karson et al., 1999; Thompson et al., 1998), temporal cortex (Thompson et al., 1998), and cingulate cortex (Gabriel et al., 1997), as well as increased levels of CSF SNAP25 (Thompson et al., 2003b; Thompson et al., 1998; Thompson et al., 1999) in schizophrenia.

The initial association study of SNAP25 in schizophrenia using a single-marker was negative (Tachikawa et al., 2001) as were two later studies (Wong et al, 2003; Musil et al, 2008). However, the two largest and most recent studies yielded more promising results. Kawashima et al (2008) found support for SNAP25 association in the first stage of a two-stage association design but not in the second stage, and Caroll et al (2009) reported a

positive association of this gene with schizophrenia. In the Irish Study of High Density Schizophrenia Families (ISHDSF), significant associations were observed for 9 of the 18 SNPs examined in at least one of the four diagnostic categories using one or both of the software packages, FBAT and PDTphase. Only one haplotype block, block 5 (rs362988 and rs6039820), was used for subsequent phenotypic analyses as it was the only one that was significant across all four concentric diagnostic groups and in both analysis packages.

Evidence that SNAP25 is altered in schizophrenia has arisen from multiple methodologies and prompted additional interest at the genetic level. Association of SNAP25 with this disease in the ISHDSF led us to examine this gene with respect to symptom dimensions of schizophrenia in this sample.

Methods:

Subjects and genotyping:

Subjects were drawn from the Irish Study of High Density Schizophrenia Families (ISHDSF) detailed in Chapter 1. Phenotypic measures were obtained for the 755 subjects used in these analyses using the operational criteria checklist for psychotic illness

(OPCRIT) (McGuffin et al, 1991). Factor analysis of items from the OPCRIT yielded five factors: hallucinations, delusions, and negative, manic, and depressive symptoms.

Eighteen single nucleotide polymorphisms (SNPs) tagging haplotypes in the SNAP25 gene were genotyped. Some were genotyped by template-directed dye-terminator incorporation with fluorescence polarization detection (FP-TDI) using the relevant AcycloPrime FP SNP detection kit (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer's instructions, and an automated allele scoring platform (Van Den Oord et al., 2003). Multiplex genotyping of additional SNPs was conducted on the GenomeLab SNPstream (Beckman Coulter, Fullerton, CA).

Statistical analyses:

We tested the associated haplotoype, rs362988 and rs6039820, for effects on the clinical phenotype using UNPHASED. To determine whether a subset of the subjects was driving the observed association between SNAP25 and schizophrenia, all cases were rank ordered according to each of five symptom factors previously extracted from the OPCRIT in this sample: negative symptoms, depressive symptoms, manic symptoms, delusions, and hallucinations (Fanous et al., 2005). The sequential addition method (Macgregor et al. 2006) was used as follows: first, a chi-square test was performed for all cases and controls. Next, for each factor, cases with the highest scale scores were compared with all control subjects using a chi-square test. Cases with incrementally

lower scores were progressively included until the chi-square value met or exceeded that of the full case sample. To assess the probability of a factor-haplotype association under the null hypothesis, permutations were carried out in which the same proportion of cases achieving a significant association was randomly sampled from the total case population using SAS 9.1.3 (SAS Institute, 2005) and compared to all controls using UNPHASED (Dudbridge, 2003) software suite v.2.404. Empirical p-values were determined from these permutations by the formula $P=d+1/n+1$, where d is the number of chi-square statistics observed in n=5000 permutations that exceeded the chi-square observed in the full sample.

Results:

Analysis of clinical covariates using sequential addition yielded non-significant results for the negative, manic and depressive factors of the OPCRIT. However, for the delusions factor, inclusion of 58% of the cases with the highest symptom levels resulted in a chi-square value exceeding that of the full sample (7.40 compared to 5.46, empirical P=.01). Including 59% of the cases with the highest hallucination scores resulted in a chi-square value of 8.21 (compared to 5.46 in the full sample, empirical P=.006).

Discussion:

We found that the most significantly associated haplotoype of SNAP25 was preferentially overtransmitted to subjects in the upper ~60% of the hallucinations and delusions factors. These factors are correlated in this sample, as would be expected due to their frequent co-occurrence as "positive symptoms". If the marker-disease associations observed in this sample are true, this would suggest that SNAP25 is what we have termed a Susceptibility-Modifier gene. This class of genes is one that predisposes to more or less specific clinical subtypes of illness, and may affect the distribution of clinical features in a population (Fanous and Kendler, 2005; Fanous and Kendler, 2008).

SNAP25 has been linked to schizophrenia through a variety of research methods, and this is the first report of association of this gene with the positive symptoms of hallucinations and delusions. Additional studies to clarify the mechanisms by which this gene could facilitate a diagnosis of schizophrenia or impact the manifestation of symptoms would be useful in elucidating the role of SNAP25 in this disease.

No Association of Dysbindin with Symptom Factors of Schizophrenia

Adapted from:
Bergen SE, Fanous AH, Riley BP, Kuo PH, Wormley BK, O'Neill FA, Walsh D, Kendler KS. No association of dysbindin with symptom factors of schizophrenia in an Irish case-control sample. *Neuropsychiatric Genetics* (accepted)

Abstract

Robust associations between the dysbindin gene (DTNBP1) and schizophrenia have been demonstrated in many but not all samples, and evidence that this gene particularly predisposes to negative symptoms in this illness has been presented. The current study sought to replicate the previously reported negative symptom associations in an Irish case-control sample. Association between dysbindin and schizophrenia has been established in this cohort, and a factor analysis of the assessed symptoms yielded three factors, Positive, Negative and Schneiderian. The sequential addition method was applied using UNPHASED to assess the relationship between these symptom factors and the high risk haplotype. No associations were detected for any of the symptom factors indicating that the dysbindin risk haplotype does not predispose to a particular group of symptoms in this sample. Several possibilities, such as differing risk haplotypes, may explain this finding.

Introduction

Schizophrenia is a severe neuropsychiatric disorder affecting approximately 1% of the global population. Despite decades of research, conclusive facts regarding the etiology of schizophrenia have remained elusive. However, a substantial genetic underpinning is indicated by a meta-analysis-derived heritability estimate of 81% (Sullivan et al, 2003), and evidence is mounting for some susceptibility genes. Dysbindin (dystrobrevin binding protein 1; DTNBP1) in particular is one of the most promising candidate genes for schizophrenia to date (Sun et al, 2008) with numerous positive associations reported (Straub et al, 2002; Van Den Oord, 2003a; Schwab et al, 2003; Kirov et al, 2004; Funke et al, 2004; Williams et al, 2004; Fallin et al, 2005; Li et al, 2005; Tochigi et al, 2006; Tosato et al, 2007; Vilella et al, 2007; Duan et al, 2007) but several negative reports, as well (Morris et al, 2003; Hall et al, 2004; Li et al, 2005; Holliday et al, 2006; Joo et al, 2006; Pedrosa et al, 2007; Wood et al, 2007; Bakker et al, 2007; Datta et al, 2007; Liu et al, 2007; Zinkstok et al, 2007; Turunen et al, 2007).

Of the many studies reporting associations between schizophrenia and dysbindin, most have genotyped somewhat different markers, and considerable heterogeneity in risk haplotypes has been reported. Variability in study design has hampered attempts at cross-study interpretation. One meta-analysis failed to find allelic heterogeneity (Mutsuddi et al, 2006), while another more recent meta-analysis did uncover substantial

allelic heterogeneity for the DTNBP1-schizophrenia association (Maher et al, in press Human Heredity). Marker rs2619538 (aka SNP A) did attain overall significance in this report (Maher et al, in press Human Heredity), and another meta-analysis of associations reported in the SzGene database found SNP rs1011313 to be significantly associated in Caucasian populations (Allen et al, 2008).

Some aspects of the dysbindin protein functions have been elucidated including its role in the biogenesis of organelles such as lysosomes, melanosomes, and platelet dense granules (Dell'Angelica, 2004). The mouse homologue of dysbindin binds to alpha and beta-dystrobrevins in muscle tissues (Benson et al, 2001), and dysbindin has also been localized to glutamatergic presynaptic terminals in humans (Talbot et al, 2004). The mechanism by which dysbindin polymorphisms impact the liability to schizophrenia is unknown, but affected subjects have shown reduced protein and mRNA expression in brain regions implicated in this disorder such as the hippocampus (Talbot et al, 2004; Weickert et al, 2008), the prefrontal cortex, and a non-significant reduction in the midbrain (Weickert et al, 2004). A study examining prefrontal and hippocampal dysbindin expression levels in mice treated with common antipsychotic medications revealed unchanged mRNA levels compared to an untreated group suggesting that the changes observed in human populations are unlikely to be due to the effects of medication (Chiba et al, 2006).

Prior reports have linked dysbindin with negative symptoms in schizophrenia. Fanous et al (2005) examined the relationship of dysbindin with clinical features of schizophrenia in a sample of 270 multiply affected Irish families, containing no overlapping individuals with the present cohort. Dysbindin's role as a susceptibility gene in this sample had been previously established (Straub et al, 2002), and further examination revealed that subjects in the upper 40th percentile for negative symptoms were significantly more likely to have the high-risk haplotype. This was in contrast to the other four symptom factors, hallucinations, delusions, manic, and depressive symptoms, which showed no association with the haplotype of interest.

DeRosse et al (2006) also reported association of a dysbindin risk haplotype (Table 1) and negative symptoms in 181 Caucasian men and women with schizophrenia, covarying for neurocognitive functioning. This cohort (and healthy volunteers) had also shown association between this haplotype and neurocognitive dysfunction (Burdick et al, 2006). Although decreased intellectual functioning often accompanies negative symptoms, these studies suggest there may be at least partially separable effects. Another relevant study of 262 Irish patients with schizophrenia found lower scores on a "hostility/excitability" factor and a trend for higher negative symptom scores in subjects with a "CAT" dysbindin risk haplotype (Table 3.5; Corvin et al, 2008).

In the Irish Case-Control Study of Schizophrenia (ICCSS), an association between the dysbindin haplotype GCACTT (Table 3.5; Fanous et al, 2005) and schizophrenia has

been found (case frequency: .454, control frequency: .388, $X^2 = 13.561$, $p = .0002$) (Riley et al, submitted to *Schizophrenia Research*). The current study explores the relationship between this haplotype and the clinical symptoms of schizophrenia in the same subjects.

Table 1. Dysbindin risk haplotypes with phenotypic associations by study.

	rs2619538	rs909706	rs1474605	rs1018381	rs2619522	rs760761	rs2005976	rs2619528	rs1011313	rs3213207	rs2619539
present study	T		T	C	A	C	G				
Fanous et al, 2005			G	C	G	T	A	A	G	G	
DeRosse et al, 2006		G		T	G	T		A	G		
Corvin et al, 2008	T									A	C
Tosato et al, 2007	A	A									

Methods

Cases from the ICCSS were collected from in-patient and out-patient psychiatric facilities in the Republic of Ireland and Northern Ireland. Subjects were eligible for inclusion if they had a diagnosis of schizophrenia or poor-outcome schizoaffective disorder by DSM-III-R criteria. Subjects were eligible for inclusion if they had a diagnosis of schizophrenia or poor-outcome schizoaffective disorder by DSM-III-R criteria, which was confirmed by a blind expert diagnostic review. Control subjects, selected from several sources, including blood donation centers, were included if they denied a lifetime history of schizophrenia. Both cases and controls were included only if they reported all four grandparents as being born in Ireland or the United Kingdom (Chen et al, 2007). In this study, 625 control subjects were used in conjunction with 730, 868, and 675 affected subjects for the Positive, Negative, and Schneiderian symptom factor analyses respectively.

Subjects were rated for each symptom using interviews, derived from the expanded psychosis section of the Structured Clinical Interview for DSM-III-R (SCID) (Spitzer and Williams, 1985), and case records independently. These two assessments were averaged and entered into a factor analysis using the statistical program MPlus (Muthen and Muthen, 2001). This yielded three symptom factors: Positive, Negative, and Schneiderian. Sum scores were generated for each factor with eleven items used to generate Positive and Schneiderian symptom scores and eight for Negative symptoms

(Fanous, in preparation). The items were scaled using different numbers of levels (eg 1-4 or 1-5) then combined and standardized to have values ranging from one to two. Example items loading on the Positive symptom factor included Visual and Somatic Hallucinations and Grandiose Delusions. The Schneiderian symptom factor incorporated Thought Withdrawal and Broadcasting and Voices Discussing items, and the Negative symptom factor included items such as Alogia, Avolition, and Affective Flattening.

We genotyped a total of 12 single nucleotide polymorphisms (SNPs) in *DTNBP1* which is transcribed in opposite orientation to the human genome sequence. Markers were selected from those included in previous reports from our group and others, and were chosen to allow reconstruction of the specific haplotypes widely observed in this locus (Van den Oord et al, 2003a).

All markers were genotyped using fluorescence polarization detection of template-directed dye-terminator incorporation (FP-TDI). We used the AcycloPrime SNP detection kits appropriate for the specific polymorphism (PerkinElmer Life Sciences, Boston, MA) and manufacturer's instructions. Genotypes were called using an automated allele scoring platform (Van den Oord et al, 2003b). PCR and single-base extension primers were designed manually and are available on request. The average genotyping completion rate was 94.7% (range 89.3-98.7%).

We used a sequential addition approach well suited to data such as these in which a quantitative trait is measured in cases but not controls (Macgregor et al, 2006). Briefly, this method consists of an initial chi-square test between all cases and controls evaluating risk allele or haplotype proportions between groups. Next, the cases with the highest level of the quantitative trait are analyzed against all controls and the resulting chi-square value is compared to that for the full sample. Subjects with progressively lower levels of the quantitative trait are incorporated into the case group until the chi-square statistic meets or exceeds that of the full sample. For these analyses, we used the sum scores for the three symptom factors, Positive, Negative, and Schneiderian, as the quantitative traits and the previously determined risk haplotype, GCACTT, defined by the minor allele of rs2619538 and the major alleles of rs1474605, rs1018381, rs2619522, rs760761, and rs2005976 (Riley et al, submitted to *Schizophrenia Research*). All analyses were implemented using SAS (SAS Institute Inc, 2005) and UNPHASED (Dudbridge, 2003).

Linear regression analyses were also utilized to assess potential relationships between symptom factors and the high-risk dysbindin haplotype. Using only data from the affected subjects, scores for each factor were separately regressed against the high-risk haplotype. Additionally, t-tests of sum scores for each factor by risk haplotype carrier status were performed using SAS (SAS Institute Inc, 2005).

Results

We found no association of the high-risk dysbindin haplotype with any of the symptom factors using sequential addition. While the Negative symptom factor sequential addition analyses did ultimately yield a chi-square value exceeding the full sample chi-square value, 84% of the cases were incorporated into the analyses before this was accomplished. There is no established cutoff for the percentage of included case subjects necessary to demonstrate association; however, with such a high percentage of cases used, a strong association with negative symptoms no longer seems plausible. Additionally, no relationships between the risk haplotype and symptom factors were found using linear regression (Negative p=.53, Positive p=.51, Schneiderian p=.96) or t-tests (Negative p=.52, Positive p=.54, Schneiderian p=.78) (Table 3.6).

Table 3.6. Sum score statistics for all factors by risk haplotype carrier status.
SD = standard deviation

Factor	Carrier Status	Subjects	Mean	SD	t	p-value
Negative Symptoms	non-carriers	181	1.507	0.37	-0.65	0.5156
	carriers	435	1.528	0.35		
Positive Symptoms	non-carriers	163	1.516	0.34	0.61	0.5439
	carriers	375	1.498	0.33		
Schneiderian Symptoms	non-carriers	137	1.502	0.35	-0.27	0.7846
	carriers	330	1.512	0.34		

Discussion

None of the symptom factors analyzed demonstrated an association with the dysbindin risk haplotype in this cohort. This was unexpected considering two prior studies examining negative symptoms and dysbindin did find an association (Fanous et al, 2005; DeRosse et al, 2006), but several possible reasons may account for this discrepancy.

First, the symptom factor structure differed between the Irish family sample and the present Irish case-control sample. For the three symptom factors used here, negative symptoms were possibly more broadly defined than for the Irish family sample which had five symptom factors, and the items in each differed as a result of the instruments used. Three items were similar across samples: inappropriate affect, affective flattening, and thought disorder. Negative symptoms in this sample included four additional items and in the Irish family sample, eight more items. DeRosse et al (2006) used only three items, affective flattening, alogia, and avolition, all of which were incorporated into the negative symptom factor in the present study.

Additionally, the high-risk haplotypes defined in the Irish samples are highly cladistically divergent from each other. It is conceivable that one haplotype only confers risk to schizophrenia while the other predisposes to schizophrenia as well as a high degree of negative symptoms. Another possibility is that the dysbindin haplotype association with

negative symptoms is actually driven by linkage disequilibrium with an evolutionarily recent, causal polymorphism.

Manifestations of schizophrenia involving a high degree of negative symptoms are often considered the most severe. Negative symptoms are among the most persistent and refractory to treatment, making these symptoms imperative to examine (Stahl and Buckley, 2006). Since prior reports of modification by dysbindin have included negative symptoms (Fanous et al, 2005; DeRosse et al, 2006), cognitive function (Donohoe et al, 2007; Burdick et al, 2007), low hostility/excitability (Corvin et al, 2008) and poor clinical outcome (Tosato et al, 2007), this suggests two distinct possibilities. First, all of these interrelated disease dimensions may be tapping a common physiological process partially underlying each of them. Alternatively, of the various dysbindin haplotypes that have been associated with schizophrenia, they may have distinct symptom modifying properties or none at all. Dysbindin variation has also been associated with cognitive function in normal individuals (Luciano et al, 2009; Burdick et al, 2007).

Continuing efforts to explore the genetic underpinnings of clinical variation in schizophrenia are vital to understanding and treating this disorder. Advances in identifying susceptibility genes have been hindered by genetic and clinical heterogeneity, but understanding the nature and extent of the relationship between them may bring us closer to a useful comprehension of schizophrenia.

Chapter 4: Detection of Susceptibility Genes as Modifiers Due to Subgroup Differences in Complex Disease

Adapted from:

Bergen SE, Maher B, Fanous AH, Kendler KS. Detection of susceptibility genes as modifiers due to subgroup differences in complex disease. (in revision at *European Journal of Human Genetics*)

Abstract:

Complex diseases invariably involve multiple genes and often exhibit variable symptom profiles. The extent to which disease symptoms, course, and severity differ between affected individuals may result from underlying genetic heterogeneity. Genes with modifier effects may or may not also influence disease susceptibility. In this study, we have simulated data in which a subset of cases differ by some effect size on a quantitative trait and are also enriched for a risk allele. Power to detect this "pseudo-modifier" gene in case-only and case-control designs was explored blind to case substructure. Simulations involved 1000 iterations and calculations for 80% power at $p<.01$ while varying the risk allele frequency (RAF), sample size (SS), effect size (ES), odds ratio (OR), and proportions of the case subgroups. With realistic values for the RAF (.20), SS (3000) and ES (1), an OR of 1.7 is necessary to detect a pseudo-modifier gene. Unequal numbers of subjects in the case groups result in little decrement in power until the group enriched for the risk allele is less than 30% or greater than 70% of the total case population. In practice, greater numbers of subjects and selection of a quantitative trait with a large range will provide researchers with greater power to detect a pseudo-modifier gene. However, even under ideal conditions, studies involving alleles with low frequencies or low ORs are usually underpowered for detection of a modifier or susceptibility gene. This may explain some of the inconsistent association results for many candidate gene studies of complex diseases.

Introduction:

As the risk genes for complex diseases are rapidly being identified (Wellcome Trust Case Control Consortium, 2007; Barrett et al, 2008; Jakobsdottir et al, 2005), there has been increasing attention to the factors influencing variability within these disorders. For complex genetic disorders in which multiple genes act in concert to produce the disease, variability in phenotypic expression seems likely to result, at least in part, from underlying genetic heterogeneity. Genes impacting age of onset, severity, and differences in symptom clusters, but not susceptibility to illness have been termed modifier genes (Fanous & Kendler, 2005). Several such genes have now been identified for diseases such as sickle cell anemia (Steinberg, 2005), cystic fibrosis (Salvatore et al, 2002), nonsyndromic cleft lip (Zucchero et al, 2004), and spinocerebellar ataxia type II (Hayes et al, 2000, Pulst et al, 2005).

While susceptibility genes – those which influence disease liability - and modifier genes – those which impact on clinical variation within the illness - can be distinct, it is also possible for one gene to predispose individuals to a disease as well as specific symptom dimensions within the illness (Fanous & Kendler, 2005). In fact, for schizophrenia, a number of genes are reported to have both susceptibility and modifier influences including dysbindin (Straub et al, 2002; Fanous et al, 2005; DeRosse et al, 2006), COMT (de Chaldee et al, 1999; McClay et al, 2006; DeRosse et al, 2006), and DISC1 (Thomson et al, 2005; DeRosse et al., 2007). These have been termed susceptibility-modifier genes.

Difficulty in replicating findings for association studies of complex diseases has led to the stratification of subjects based on variation in disease characteristics as one plausible way to enhance the signal to noise ratio by reducing heterogeneity. Consequently, there have been attempts to identify biological features specific to particular forms of illness. However, classifying clinical subtypes of complex diseases has proven exceedingly difficult. Age of onset, severity, and familial or sporadic inheritance patterns have sometimes been used to subdivide case populations. Other strategies for grouping patients have focused on symptomatology, either through use of *a priori* criteria or by the more statistically rigorous methods of cluster or latent class analysis. Subjects can then be categorized as high or low scorers for a given factor. There are also methods which allow for the maximization of evidence for association by covariate-based subdividing without *a priori* cutpoints or data processing (Perdry et al, 2007; Macgregor et al, 2006). Each of these categorization approaches has some appeal, but the best approach for each complex disease has yet to be determined.

Since genes that confer susceptibility to a form of illness with a distinct symptom profile would manifest as susceptibility-modifier genes, the results of modifier gene association studies may yield information regarding variation in the genetic architecture of complex disease liability in addition to variability in symptom expression.

We suggest there are two particularly plausible mechanisms whereby a gene variant is associated with a symptom in a complex disease. First, the disorder is etiologically homogeneous and this gene "truly" impacts on that symptom – a true modifier. Second, the disorder is etiologically heterogeneous. This "pseudo-modifier" gene is really a risk gene but only for one subtype AND the subtypes differ on the levels of this particular symptom. We term this type of gene a "pseudo-modifier" because its effects on the symptoms in question actually arise from it conferring liability to a particular disease subtype. In this paper, we study this second mechanism to see under what circumstances it might be detected.

To do so, we simulated two case groups, for one of which the gene variant influencing symptom variability also confers disease susceptibility. The other case group arrives at the disease state through another, unspecified mechanism. A control group was simulated as well, but since power for case-control (susceptibility) analyses has been thoroughly investigated elsewhere, these results are included here for comparison purposes only. Case-only designs (for modifier effects) were considered, blind to case substructure, as risk allele frequency, sample size, odds ratio, effect size, and proportion of cases with the pseudo-modifier allele were varied.

Methods:

Two case groups and a control group were simulated according to a range of specified parameters, then tested for power to detect the pseudo-modifier gene of interest. Simulations were carried out using the software program SAS 9.1 or 9.1.3 (SAS Institute, 2005). All sets of simulations involved 1000 iterations and calculations of power given the risk allele frequency (RAF), odds ratio (OR), sample size (SS), and effect size (ES). We did not directly simulate an effect of the variant on the quantitative trait. Instead, we simulated a variant with population allele frequency in controls and type I cases and RAF*OR in type II cases. Importantly, this results only in an increased RAF among the type II cases. In case-control comparisons where the number of cases and controls is equal, the effective OR is then 1 + ((OR-1)/2). For example, a risk allele with a frequency of .1 at an OR of 1.4 would yield frequencies of .1, .1, and .14 in controls, type I cases and type II cases respectively in a sufficiently large sample. In case-control comparisons, grouping the heterogeneous case sets, the allele frequencies would be .1 in controls and .12 in cases.

For use in case only analyses, a quantitative phenotype was simulated sampling from a normal distribution with a mean of 0 and standard deviation of 1 in type I cases and a mean equal to the effect size (ES), the standardized mean difference of a trait between the two groups of cases, in type II cases, thus indirectly creating an association between the SNP and the quantitative trait. That is, the case group enriched for the risk allele also has a mean difference from the other case group.

For each set of parameter specifications, power to detect the influence of the impact of genotype on variation in the combined case groups (case-only) was calculated as well as power to detect the allele as conferring disease susceptibility (case-control). Although the case-control power calculations are not novel, they provide a useful comparison for the case-only investigations. We chose to use a one stage design since studies exploring modifier gene influences are not contingent on susceptibility gene association results.

Main analyses

We created two case subgroups differentiated on mean group differences for an unspecified, normally distributed, quantitative trait. The type II cases were enriched for the pseudo-modifier allele of interest, while the type I cases were not. Figure 4.1 illustrates the two case population distributions and their combined distribution when subgroup membership is unknown. All analyses were performed blind to case type. Unless otherwise specified, simulations included 3000 total subjects in which 750 were type I cases, 750 were type II cases and 1500 were controls. RAFs were varied from .10 to .50, and ORs of 1.1 to 2.0 by increments of .10 were modeled, initially holding the ES at 1. We defined sufficient power for detection as .8 or greater with a p-value $\leq .01$.

Additional analyses

The RAF was fixed at .20 and ES at 1 for analyses in which other parameters varied. To explore the effects of sample size, the total number of subjects was increased incrementally by 1000 from 2000 to 6000 while maintaining the same proportion of subjects in each group. Additionally, since it is implausible that two subpopulations of affected individuals would naturally divide the subject pool neatly in half, we also varied the percent of Type II subjects, possessing an enriched proportion of the risk-conferring allele. Total case and control numbers were held even. Furthermore, the effect sizes that might be observed could vary considerably and depend entirely on the phenotypic trait assessed. We consequently modeled a broad range of effect sizes from .5 to 3.0 with increments of .5 representing mean differences of half a standard deviation to three full standard deviations.

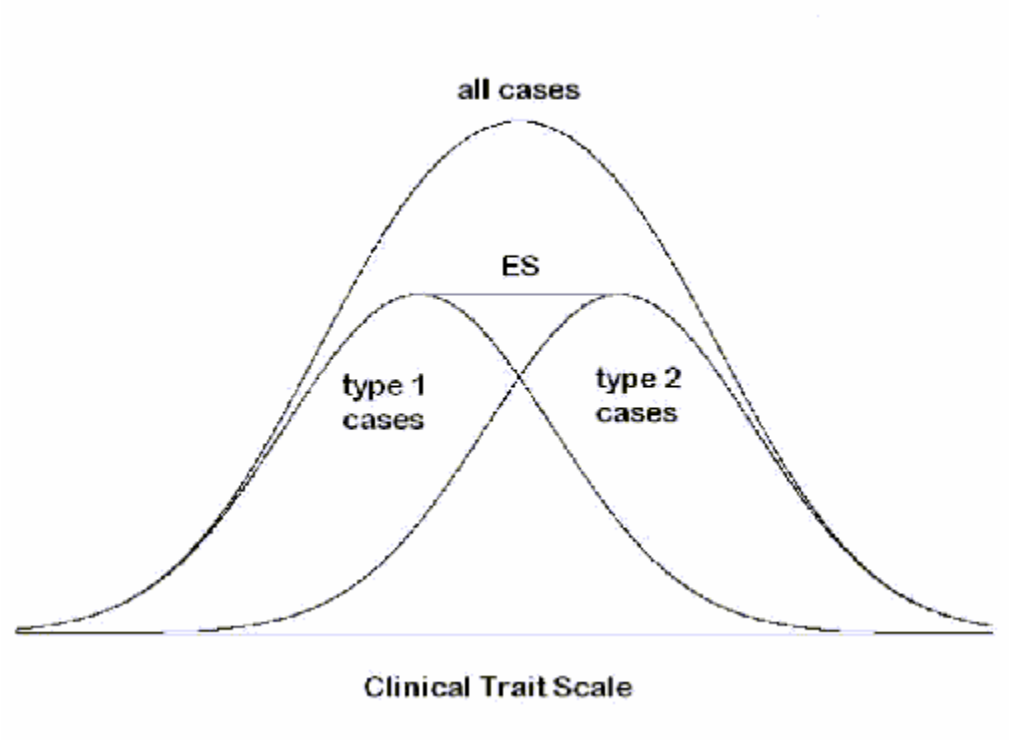


Figure 4.1. Case population distributions in relation to a clinical trait scale. Type I cases are depicted as scoring lower on the scale. Type II cases, enriched for the pseudo-modifier allele of interest, score higher by an effect size (ES) difference of one standard deviation in most simulations. The combined case population is also shown since investigators (and our analyses) are blind to case substructure.

Results:

To detect modifier associations that result from underlying genetic heterogeneity, in which the allele impacts on disease risk in only one subgroup, with our core set of parameter specifications (sample size of 3000, effect size of 1, and RAF of .20) we had

sufficient power to detect a pseudo-modifier association with an OR of 1.7 or greater (Figure 4.2). For comparison, a susceptibility allele with an OR of 1.2 is detectable under the same conditions.

Allele Frequency

RAFs of .10 or less are not sufficient to detect pseudo-modifier genes with a one standard deviation mean trait difference. However, susceptibility gene detection is possible with an OR of 1.3 or greater. Power curves grow progressively steeper as the RAF increases, culminating with detection of OR 1.15 and 1.3 for the susceptibility and pseudo-modifier gene analyses respectively.

Sample Size

With 2000 total subjects, only ORs of 1.8 and greater are sufficient for detection of pseudo-modifier effects. However, each additional 1000 subjects lowers the detectable OR by ~.10 until a sample size of 6000 is used. With this large sample size, power to detect an OR of 1.4 is just under .80 but falls sharply to ~.50 for OR detection of 1.3. Case-control simulations for susceptibility gene effects exhibit much steeper curves with a smaller range, allowing for detection of a RAF of .20 or greater and OR of 1.15-1.25 across all sample sizes examined.

Effect Size

When the mean phenotypic differences between subgroups is less than half a standard deviation, detecting pseudo-modifier effects is unlikely with odds ratios under two. Standard deviation differences of 1 and 1.5 are distinguishable at odds ratios of 1.7 and 1.5 respectively. From standard deviations of 2 to 3, however, the increased phenotypic disparity does not confer markedly enhanced detectability for pseudo-modifier genes.

Unbalanced Case Groups

Little decrement in power for the detection of pseudo-modifier effects was observed across a broad range of the percent of subjects with the subtype containing an enriched proportion of the risk-conferring allele. At any OR for which there is sufficient power for detection, 30-70% of the Type II cases are sufficient, and for high ORs, an even broader range may suffice (Figure 4.3).

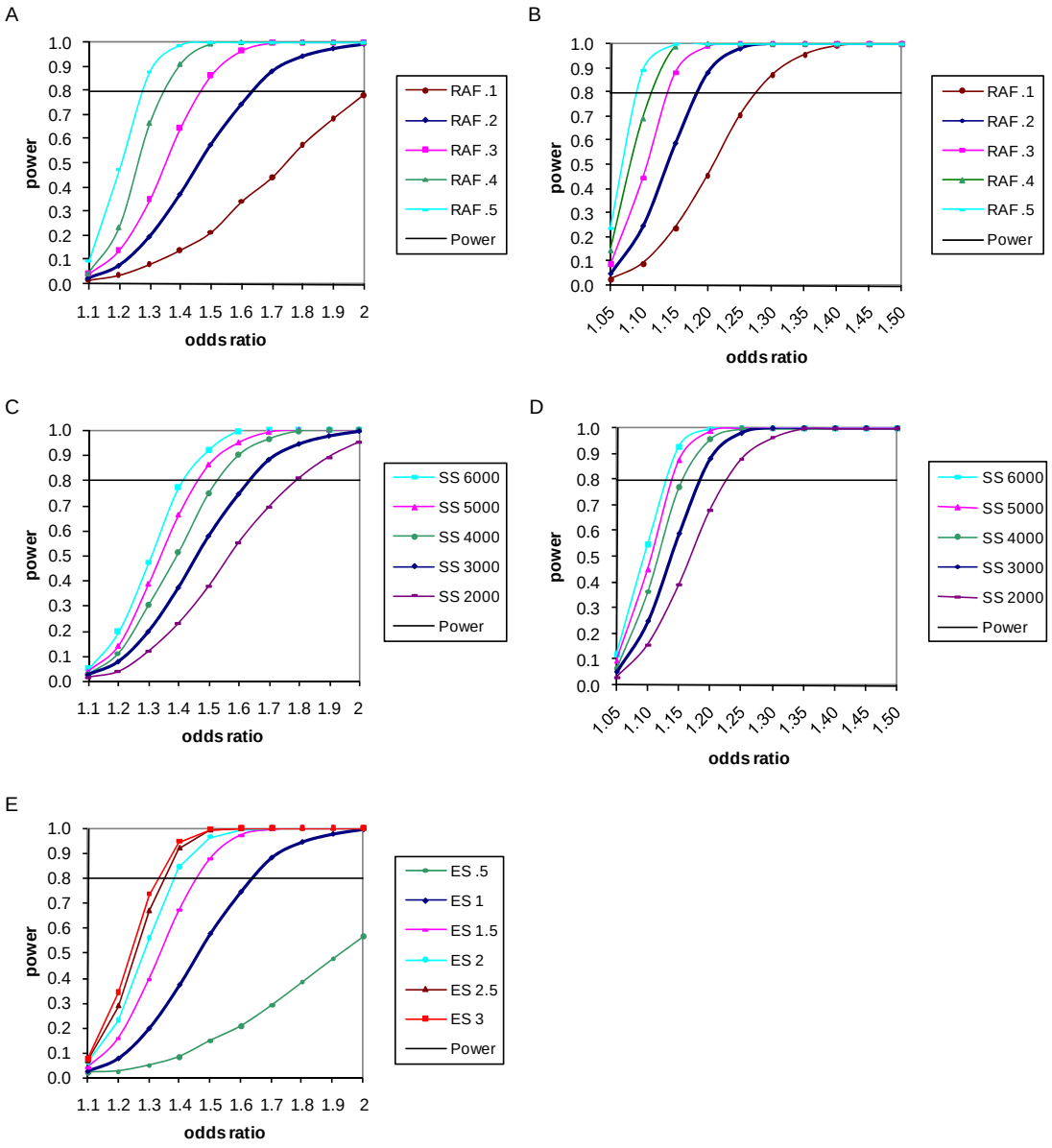


Figure 4.2. Power by odds ratio (OR) curves. When fixed, the risk allele frequency (RAF) = .2, effect size (ES) = 1 and sample size (SS) = 3000. Lines with these parameter specifications are represented on each plot and emphasized for frame of reference. A) Power to detect pseudo-modifier genes for RAF of .1-.5. B) Power to detect a susceptibility gene in case-control analyses varying the RAF from .1-.5 and OR from

1.05-1.50. C) Power for pseudo-modifier gene detection for SS = 2000-6000. D) Power for susceptibility gene detection with SS = 2000-6000. E) ES .5-3.0 impact on power to detect pseudo-modifier effects.

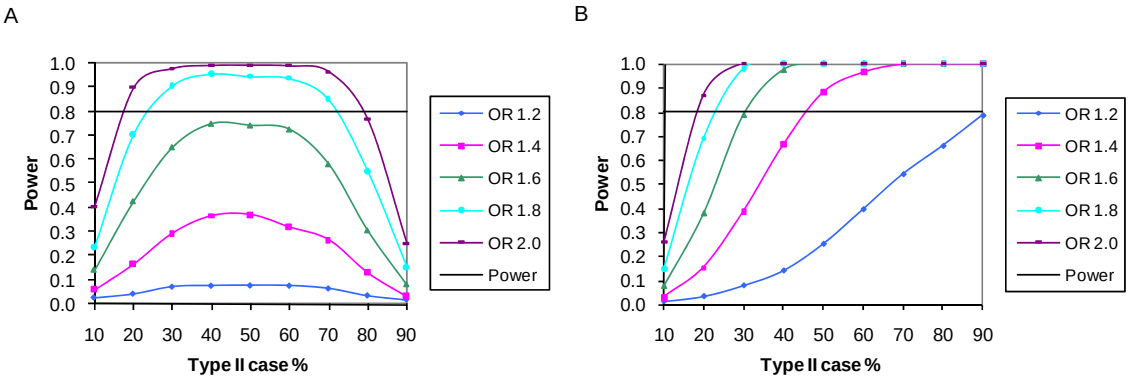


Figure 4.3. Effects of varying the proportion of Type II cases, enriched for the risk allele, on power across odds ratios (OR) 1.2-2.0. A) Case-only results for pseudo-modifier discrimination. B) Power for risk allele detection in case-control analyses.

Discussion:

From these results, it is clear that the discovery of modifier gene effects that arise from genetic heterogeneity in complex diseases is critically dependent on most of the parameter estimates examined here. However, it is interesting to note that the proportion of cases with the risk-conferring allele can vary between 30-70% with little observed deviation in power. Since subtypes are unlikely to evenly divide an affected population,

this is a reassuring finding. On the other hand, RAF is a vital factor in detecting pseudo-modifier genes. With 3000 subjects, a RAF of .10 (or less) is insufficient for detection of a pseudo-modifier gene for any OR under 2.0, and even under ideal circumstances with a RAF of .50, the OR must be 1.3 or greater.

Effect size is another important determinant of pseudo-modifier gene detection, although the phenotypic differences between case groups have no impact on susceptibility gene detection. This is an important consideration when selecting a trait on which to explore modifier effects since the groups should minimally differ by one standard deviation and preferably two or more on the trait of interest. As group membership is generally unknown, high trait variance is the best selection criterion usually available.

In practice, sample size is the most manipulable of the parameters explored here. It is intuitively obvious that greater numbers of subjects confer greater power, but beyond 5000, the additional effort of subject recruitment and assessment may not yield sufficiently enhanced power to be worthwhile. Even with very high numbers of subjects, detection of genes with low RAFs or low ORs is extremely difficult. For many complex diseases this may explain conflicting results from association studies.

For the parameters tested, attempts were made to examine realistic values. For example, the Affymetrix Mapping 500K Array and GenomeWide Human Mapping 5.0 Array both report average minor allele frequencies of .22 (www.affymetrix.com, 2008). and the

Illumina HumanHap550 and 650Y detect SNPs with average minor allele frequencies of .20-.23 depending on the population sampled (www.illumina.com, 2008). Consequently, simulations in which risk allele frequencies were held constant were given values of .20. Also, the odds ratio range we used is comparable to ORs commonly reported for complex genetic diseases (eg ~1.1-2.0; Wellcome Trust Case Control Consortium, 2007; Allen et al, 2008; Barrett et al, 2008; Zeggini et al, 2008).

The added power conferred by greater subject numbers has led to recent increases in multi-center collaborations generating cohorts consisting of thousands of individuals. Accordingly, the number of subjects used for most simulations in this study approximated that of some of these cooperative efforts. These include several of the Genetic Association Information Network (GAIN) studies such as that for ADHD (involving 2877 participants), major depression (3720), bipolar disorder (3316), schizophrenia (5189), and psoriasis (2902) (The GAIN Collaborative Research Group, 2007). The Wellcome Trust Consortium is another large collaborative effort which examined 2000 cases for each of 7 major complex diseases and a shared set of 3000 controls (Wellcome Trust Case Control Consortium, 2007).

Despite our attempts at modeling realistic values, some limitations were imposed out of necessity. The simulations presented here only assess the impact of a single gene on the predisposition to a certain form of a complex disease. In fact, a more likely scenario involves overlapping constellations of susceptibility genes as well as environmental

insults which are also not included in these models. In addition, we have presumed that the minor allele is the risk-conferring allele. However, common alleles with small main effects may act in concert with alleles in other genes to additively or epistatically influence liability to complex diseases, and we have not modeled these possibilities. Furthermore, the simulations presented here were restricted to two subtypes, when in reality, many more subtypes may exist for some diseases.

We have additionally presumed that the modifier effects of the allele in question are restricted to (or only assessed in) the case population. Detection of more subtle expression in unaffected individuals, when possible, might allow for greater power to detect the allele (Fanous and Kendler, 2005). Gene detection then hinges not only on disease expression but degrees of symptom expression as well, drawing on increased information to yield enhanced power.

The results presented here are applicable to nearly every complex genetic disease for which subtypes may exist. Several diseases may manifest with convergent symptom profiles but arise through different etiological mechanisms. The extent to which subpopulations differ in their symptoms can yield clues to underlying biological differences. For example, diabetes has two main subtypes (I and II) both of which present with high blood glucose levels and similar symptoms such as extreme thirst, fatigue, and blurred vision. However, in type I diabetes, the symptoms are due to the destruction of insulin-producing cells, while type II diabetes occurs when the tissues

become resistant to insulin or produce too little. Later age of onset and higher body weight are linked to, but not diagnostic of, type II diabetes (http://diabetes.niddk.nih.gov/dm/pubs/overview/index.htm, 7-12-08). These subtle phenotypic differences indicative of very distinct pathophysiological origins are precisely the type of clues sought to unlock the etiology of complex diseases.

Association studies examining modifier effects may actually uncover subtype-specific susceptibility genes. Whether variable symptom profiles for numerous diseases are due to modifier genes and environmental influences, differing underlying genetic architecture, or some combination of these possibilities will likely remain under investigation for many years to come. The simulation results presented here indicate there is reasonable power to detect pseudo-modifier genes under favorable conditions such as a high effect size, OR, and RAF, but they may well be missed under less ideal circumstances. These results can be used to inform researchers as to the relative power for studies of complex disease under a range of conditions when symptom variation is due to different genetic subtypes.

Chapter 5: Myelin and Synaptic Gene Networks in Schizophrenia

Abstract:

Evidence implicating myelin and synaptic alterations in schizophrenia has been accumulating over the past several years from imaging, neuroanatomical, gene expression and association studies. Network analyses of SNP data may add to this literature by offering a potentially powerful method of identifying risk genes in biological processes theorized to be involved in a disease. We examined genes pertaining to myelin and synapses using networks generated through literature searches and then included additional genes to link them together using Ingenuity Pathway Analysis (IPA). This yielded four networks – core and expanded networks for both myelin and synaptic genes. In a sample of 2093 total cases and 2332 control subjects we tested for association between each network and two populations, African-ancestry (AA, case N = 921, control N = 954) and European-ancestry (EA, case N = 1172, control N = 1378). Significance was assessed through permutation and calculation of empirical-p values. Only the core myelin network association with the AA group demonstrated significance (empirical p=.02), further substantiating the involvement of myelin-related genes previously implicated in schizophrenia. This tentative finding, uncorrected for the multiple networks tested, also adds provisional corroboration with the growing literature supporting distinct genetic risk factors between AA and EA populations for this disorder.

Introduction:

Convergent lines of evidence, to be reviewed below, have implicated alterations in myelin and synaptic dysfunction in schizophrenia. Onset of schizophrenia is often coincident with the developmental timeframe during which maturation of the prefrontal cortex occurs. This involves the refinement of synaptic connections known as synaptic pruning and enhancement of myelination to facilitate neuronal signaling. These processes may be aberrant in and of themselves, or they may unmask preexisting neurodevelopmental problems in this brain region (Woo and Crowell, 2005).

Functional disconnectivity occurs when there is a breakdown in signal transmission between brain regions. This has been found in many studies of the p50 and p300 electrophysiological responses (Bramon et al, 2004) and pre-pulse inhibition (Braff et al, 2001). These findings as well as fronto-temporal disconnectivity related to auditory hallucinations (Lawrie et al, 2002; Norman et al, 1997; Ford et al, 2002) could result from myelin deficits, synaptic dysfunction or a combination of both. Oligodendrocyte involvement with axons confers enhanced viability of the axon (Wilkins et al, 2003). Conversely, if fewer axons are available to myelinate, fewer oligodendrocytes are likely to be viable as well (Burne et al, 1996). These distinct but interrelated physiological processes have been increasingly examined in recent years and are frequently found to be altered in schizophrenia, but the reciprocal nature of this relationship makes it difficult to distinguish the primary pathological insult.

Myelin and schizophrenia

Myelin is the axon-insulating substance exclusively formed by oligodendrocytes in the central nervous system. Axons are mitogenic for oligodendrocytes, and myelination is initiated following contact of oligodendrocyte processes with axons of at least 1μm diameter. These processes extend around the axon wrapping inward multiple times. Next, compaction occurs in which the cytoplasm is extruded from all middle layers, excluding the inner- and outer-most (Compston et al, 1997). Oligodendrocytes myelinate about 20-30 sites on numerous adjacent axons (Butt & Ransom, 1989). The insulation provided by myelination facilitates much faster propagation of an action potential by allowing the signal to jump from node to node (termed saltatory conduction). Myelination increases in a region and age-dependent progression, culminating in maximal white matter volumes at approximately age 43 (Sowell et al, 2003).

Evidence from imaging studies

A relatively new imaging modality, diffusion tensor imaging (DTI), has been employed in the examination of white matter integrity in schizophrenia. This technique assesses the anisotropic (directional) diffusion of water molecules in the brain which corresponds to tissue properties such as the diameter and density of axon fibers and the thickness of

myelin sheaths (Kubicki et al, 2007). A review of 18 published studies using DTI in schizophrenia found 15 studies revealing anisotropic alterations throughout the brain (Kubicki et al, 2007). White matter tracts throughout the brain critically depend on intact myelin to convey signals (or water by proxy) from one region to another. Reduced anisotropy strongly suggests that inter-regional connections in the schizophrenic brain are structurally impaired, and this is due, at least in part, to myelin deficits.

Neuroanatomical findings

Many histopathological studies have focused on the prefrontal cortex and have demonstrated reduced numbers or density of glia in schizophrenia (Stark et al, 2004; Vostrikov et al, 2004, 2007; Hof et al, 2003; Uranova et al, 2004, 2007). Examination of glial cell density in Heschl's gyrus revealed no differences between schizophrenia and control groups (Cotter et al, 2004), but a reduced oligodendrocyte to neuron ratio was observed in schizophrenia in the anterior principle thalamic nucleus (Byne et al, 2006). These findings suggest a relative sparing of primary sensory cortical regions, but processing centers and association cortices may be preferentially affected in terms of glial deficits. Additional studies of other brain regions would be informative for this hypothesis.

Gene expression

Results from gene expression studies confirm on a cellular level the broad changes observed in functional, imaging, and anatomical studies. Hakak et al (2001) conducted a genome wide expression study of dorsolateral prefrontal cortex tissue that revealed reduced expression of six myelination genes in contrast to increased expression for most other changed genes. Tkachev et al (2003) focused on genes expressed by oligodendrocytes in the prefrontal cortex and also found reduced expression for several genes in the schizophrenia group using qPCR and microarray assays: OLIG1, OLIG2, SOX10, MBP, MAG, PLP1, MPZL1, CLDN11, MOG, ERBB3, and TF. Haroutunian et al (2007) carried out a thorough microarray study using brain tissue from many regions. Myelin-related genes were the most affected in regions with the greatest number of changed genes, the cingulate and hippocampus. Dracheva et al (2006) validated these findings in the cingulate gyrus using qPCR. This technique was also used to examine MAG, CNP, and MBP in individual thalamic nuclei, but no differences were found between the schizophrenia and control groups (Byne et al, 2007). Mitkus et al (2008) focused on expression of four genes in the dorsolateral prefrontal cortex. No decreases in expression levels were found by diagnosis, but carriers of risk alleles in CNP and OLIG2 did show reduced expression. Expression by genotype analyses were not performed for the other two genes, MOBP and MAG.

Prior association studies

Many myelin-related genes have been associated with schizophrenia hinting at a potential primary role in the pathology of schizophrenia. Three transcription factors expressed by oligodendrocytes are involved in differentiation and associated with schizophrenia: QKI (Aberg et al, 2006), OLIG2 (Georgieva et al, 2006; Huang et al, 2007), and SOX10 (Maeno et al, 2007). Several other genes encode proteins localized to myelin or involved in the process of myelination and have been associated with schizophrenia including: CNP (Peirce et al, 2006; Voineskos et al, 2008), MOG (Liu et al, 2005), PLP1 (Qin et al, 2005), and TF (Qu et al, 2007). Additionally, NRG1 is one of the most replicated genes associated with schizophrenia (Stefansson et al, 2002; Bakker et al, 2004; Corvin et al, 2004; Li et al, 2004, Tang et al, 2004; Kim et al, 2006; Lachman et al, 2006; Petryshen et al, 2005; Thomson et al, 2006; Benzel et al, 2007; Hanninen et al, 2007; Ikeda et al, 2008; So et al, 2009) along with one of its receptors found in oligodendrocytes, ERBB4 (Norton et al, 2006; Nicodemus et al, 2006; Silberberg et al, 2006; Benzel et al, 2007) but not the other, ERBB3 (Kanazawa et al, 2007; Benzel et al, 2007). Another receptor-ligand group has been associated with schizophrenia – the Nogo receptor (Novak et al, 2002; Tan et al, 2005; Budel et al, 2008) and ligand MAG (Yang et al, 2005; Wan et al, 2005), which function in neurite outgrowth inhibition. Although all of the aforementioned genes have been associated with schizophrenia by at least one study, nearly all also have negative reports of association as well.

Synaptic proteins and schizophrenia

Synaptic proteins common to the majority of neurotransmitter systems are predominantly involved in exocytosis and termed SNAREs. The soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins include synaptotagmin, synaptobrevin, syntaxin, and SNAP-25, but many different forms of these proteins exist making the total number of SNARE proteins much higher. Some SNARE proteins have also been localized to oligodendrocytes and are theorized to serve in membrane trafficking (Kramer et al, 2001; Madison et al, 1999). Since nearly every major neurotransmitter system has been implicated in schizophrenia, only synaptic proteins common to most synapses were included in these analyses.

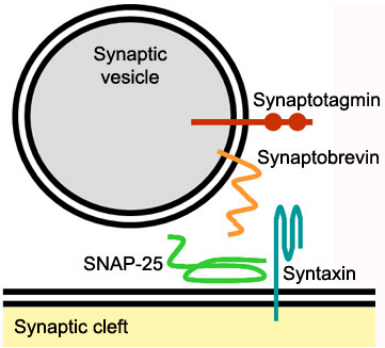


Figure 5.1. SNARE proteins. (Washington University, Neuromuscular website, 2007)

Gene expression and neuroanatomical findings

Synaptic loss has been demonstrated in schizophrenia repeatedly and in numerous brain regions, but neuronal loss does not seem to be a consistent feature of this illness (Pakkenberg, 1992; 1993). This has given rise to the reduced neuropil hypothesis of schizophrenia (Selemon & Goldman-Rakic, 1999). Reductions in cortical thickness of 3-13% (Brown et al, 1986; Pakkenberg, 1987, 1993; Zipursky et al, 1992; Andreasen et al,

1994; Ward et al 1996; Sullivan et al, 1996; Lim et al, 1996), increased density of neurons (Pakkenberg, 1993; Selemon et al, 1995, 1998), and reductions in synaptic terminal density (Sweet et al, 2007; Kung et al, 1998; Lewis et al, 2001; Pierri et al, 1999) all support this hypothesis. A review of 11 studies assessing mRNA and protein levels for GAP43, SNAP25, VAMP, and synaptophysin in the PFC in schizophrenia reported mixed results but more decreased levels than increased (Halim et al, 2003). One recent study of over 30,000 mRNA transcripts from the prefrontal cortex of 44 schizophrenics and 50 controls found changes in gene sets pertaining to neurotransmitter release, vesicle recycling, and cytoskeletal dynamics (Maycox et al, 2009).

The paucity of neuropil in schizophrenia may result from generation of fewer synapses in early development, or over-pruning of synapses in later development. Sub-lethal apoptosis has also been proposed as a mechanism by which this could occur (Glantz et al, 2006), but none of these hypotheses preclude the involvement of genetic alterations as the origin of this pathology.

Prior association studies

Despite numerous studies documenting alterations of synaptic proteins and mRNA expression in schizophrenia, few association studies have been conducted for these genes. The SNAP29 promoter region (Wonodi et al, 2005) and syntaxin 1A (Wong et al, 2004) did yield positive associations with schizophrenia, and two negative reports were linked

to SNAP25 polymorphisms (Tachikawa et al, 2001; Wong et al, 2003; Kawashima et al, 2008). Kawashima et al (2008) also reported no association of syntaxin 1A and VAMP2 in a Japanese population. Aside from these few examples, associations between genes encoding ubiquitous synaptic proteins and schizophrenia have remained largely uninvestigated compared with the synaptic proteins specifically related to particular neurotransmitter systems.

Heterogeneity in the genetic underpinnings of schizophrenia is a likely cause of inconsistent replication frequently observed in association studies of this disorder. It has been suggested that multiple rare variants with high penetrance could be responsible for schizophrenia (McClellan et al, 2007). Evidence supporting this hypothesis has arisen implicating the 22q11 deletion long held to be responsible for some cases of schizophrenia, as well as two more recently associated regions, 1q21.1 and 15q13 (International Schizophrenia Consortium, 2008; Stefansson et al, 2008; Kirov et al, 2008). Additionally, *de novo* copy number mutations have been observed at higher rates in sporadic versus familial schizophrenia cases (Xu et al, 2008).

Ample support also exists for schizophrenia arising through convergence of numerous allelic influences. The lack of consensus for the involvement of any single gene in schizophrenia liability is compelling evidence in and of itself. In addition, a recent paper from the International Schizophrenia Consortium presented data indicating several thousand loci may play a role in the development of schizophrenia (Purcell et al, 2009).

The approach suggested here is predicated on the common disease-common variant model in which multiple causal alleles contribute incrementally to schizophrenia liability. By grouping genes involved in neurological processes consistently shown to be altered in schizophrenia, locus heterogeneity can be examined within the context of gene clusters. Since mutations within a metabolic pathway can yield similar disease phenotypes (Held, 2006), it seems reasonable that similar effects may manifest in complex disorders.

Network analyses are association analyses with multiple interacting or similar genes considered together. They are based on the concept of locus heterogeneity which states that a disease or other phenotype may arise from genetic variants at different chromosomal loci. While a susceptibility allele at one locus might be too rare or have too low penetrance to be detectable, susceptibility alleles distributed throughout a network of related genes are more likely to be detected in aggregate.

The analyses presented here involved four networks: the core myelin network consisting of genes identified in the literature, the core synaptic network composed of SNARE proteins, and the extended myelin and synaptic networks generated by incorporating additional genes to link as many of the initial genes as possible through gene-gene interactions. We hypothesize that grouping genes into functional networks for association analyses will facilitate determination of our supposition that genes encoding myelin and synaptic proteins contribute to schizophrenia liability.

Methods:

Subjects and Genotyping

Subjects were drawn from the GAIN schizophrenia study described in detail in chapter 1. For these analyses 2093 total cases and 2332 control subjects were used (AA = 1875, EA = 2550, 2242 males, 2063 females). All subjects were genotyped using the Affymetrix GenomeWide Human SNP Array 6.0. The 906,600 SNPs assayed were subjected to quality control steps specifying that the minor allele frequency be greater than or equal to .01, the call rate be greater than or equal to 95%, and the Hardy Weinberg Equilibrium p-value be greater than .000001. This resulted in 845,817 SNPs remaining in the AA group and 729,454 SNPs in the EA group.

Network generation

For the myelin gene network, genes were initially drawn from previously published association and microarray expression studies. The synaptic genes were selected based on their presence at virtually all synapses since all major neurotransmitter systems have been implicated in schizophrenia. This resulted in a list comprised of synaptic docking protein genes which function in the docking of synaptic vesicles at the presynaptic membrane and subsequent release of neurotransmitters.

Using Ingenuity Pathway Analysis (Ingenuity Systems, Inc, 2009), initial lists of myelin and synaptic genes were connected (separately) according to published reports and interaction database information.

Statistical analyses

All genotyped SNPs within 10kb of the genes of interest were included. Association analyses were implemented in PLINK (Purcell et al, 2007) for each network and ancestry group. Significance was assessed using the R Q-value package involving generation of empirical p-values resulting from 1000 association tests using all SNPs from equal numbers of randomly selected genes for each network. The proportion of tests significant at $p<.05$ was calculated for each permutation and compared to network results.

Results:

The core synaptic network was not significantly associated with schizophrenia for the AA or EA groups ($p=.72$, .13 respectively). The extended synaptic network was similarly nonsignificant ($p=.88$, .27 respectively). However, the core myelin network did demonstrate significance for the AA group ($p=.02$), but not the EA group ($p=.88$). The addition of related myelin genes in the extended myelin network diluted the effect to the point of non-significance ($p=.18$) for the AA group and the EA group remained nonsignificant ($p=.56$).

Discussion:

Myelin-related genes previously implicated in schizophrenia do appear to be etiologically relevant, but this relationship was only observed in subjects of African ancestry. This finding adds to mounting evidence supporting distinct risk genes in African and European ancestry groups. Linkage and association studies examining both groups have detected different genetic regions impacting liability to schizophrenia (Takahashi et al, 2003; Faraone et al, 2005; Suarez et al, 2006; Holliday et al, 2008). A recent paper by the International Schizophrenia Consortium showed that there is greater risk gene commonality between schizophrenia and bipolar disorder for people of European ancestry than between schizophrenics of European and African ancestry (2009).

In terms of the biological processes examined here, the core myelin network, comprised solely of genes already linked to schizophrenia, had the greatest *a priori* likelihood of significant association in this study. In a similar study, Jungerius et al (2008) constructed a pathway of 138 myelin genes for association analyses in a Dutch cohort of 310 cases and 880 controls. The strongest result was for the PIK4CA gene, with five additional genes more weakly identified. Using a different approach to pathway analysis, Reitkerk et al (2009) examined a gene expression dataset and discovered that two myelin-relevant pathways, sphingolipid metabolism and the PI signaling pathway, were most strongly

overrepresented in schizophrenics. Taken together, these reports help strengthen the idea that myelin-related processes may be primary contributors to the schizophrenia phenotype.

Methods for conducting network analyses of SNP data are still under development, and our study contains some limitations we would like to address. For instance, in conducting the permutation tests to assess significance of the network results, the number of genes selected was fixed, but the gene sizes and numbers of SNPs per gene were not taken into account. If these measures differed between the specified networks and the average randomly generated gene set, it could influence the results. Also, no multiple testing correction was implemented for the multiple networks tested in both ancestry groups. Since the core networks were a subset of the expanded networks, they were non-independent, and it was therefore difficult to determine an appropriate correction method. However, some caution is warranted in interpreting our one positive result out of four networks tested.

There are numerous ways of generating gene networks, and alternate strategies to those tried here may prove more fruitful in defining a more precise cluster of genes conveying liability to schizophrenia through these biological processes. Furthermore, by using multiple concentric gene networks for each biological process of interest, the optimal size network could be determined for maximal association. This may differ across processes and/or across ancestry groups.

The results of this project prompt additional questions which remain to be answered. First, which genes are driving the AA-myelin association? It is unlikely that all genes in the core myelin network contribute equally to the significant association in the AA group. Deciphering which genes are more strongly associated would strengthen their position as candidate genes and could lead to possible intervention or therapeutic targets. Additionally, when taking admixture into account, do AA subjects with more European DNA demonstrate less myelin association? The African-American population is highly admixed with European-American populations (Parra et al, 1998), so if the myelin gene association is truly specific to the AA group, this association should grow weaker with increasing admixture. Use of ancestry-informative markers to gauge individual proportions of AA and EA DNA could be used to resolve this question. Furthermore, the differences observed across ancestry groups highlight the importance of broadening the populations included in these types of analyses to give us a better understanding of the mechanisms leading to schizophrenia (and other diseases) throughout the world.

The extensive genetic heterogeneity of schizophrenia has led to limited success with commonly used gene finding methods. Investigating related genes together may enhance the power to detect risk genes of very small effect. Ultimately, network analyses could become vital tools in the determination of aberrant biological processes and the risk genes impacting them in schizophrenia and other complex diseases.

Chapter 6: Power Differences for Family-Based Epistasis Detection Methods

Abstract:

The impact of genetic variation on common diseases is complex, involving many loci and their interactions. In population-based association samples, modeling gene-gene interaction, or epistasis, the conditional impact of one genetic polymorphism on another, is a relatively straightforward exercise. In these samples, genotypes at multiple loci, with their interaction term(s), can be included in a regression model as predictors. Detection of gene-gene interactions in family-based association samples is more complicated, but several methods have been proposed. In these approaches pseudocontrols are created based on the alleles or genotypes that were not transmitted to an affected offspring from genotyped parents. Two main approaches to generating pseudocontrols involve using nontransmitted two-locus genotypes (Cordell approach) and nontransmitted alleles (two-locus haplotype relative risk approach; HRR). In the former, 15 pseudocontrols would be generated, while the latter yields only one. These cases and pseudocontrols are then analyzed using logistic regression conditional on the parental genotypes. The core distinction between these approaches is the inclusion of transmitted alleles in the creation of pseudocontrol genotypes in the Cordell approach. We have explored the power for both of these methods under a variety of models of epistasis in 1000 simulated samples of 500 parent-child trios. Under "classic" epistasis models and particularly for dominance models, the HRR approach yields greater power, but under most other models (additive, heterogeneity, and crossover) the Cordell approach demonstrates increased power for detecting epistasis. These results suggest different approaches to family-based epistasis testing are indicated depending on the expected model of epistasis in effect.

Introduction:

Genes never act in isolation. By only investigating independent main effects of genetic variants, the ways in which alleles influence each other goes undetected, and information contributing to disease predisposition is lost. Finding the best ways to explore gene interactions and capture this variation is highly relevant to studies of complex disease.

Epistasis is the general term for interactions between genes, but it has been defined somewhat differently between studies. Departures from additivity or multiplicativity on the penetrance scale have been termed epistasis. Biological epistasis, the physical interaction of proteins or other molecules leading to a different phenotype than when expressed alone, is currently difficult to study given our limited knowledge of protein-protein interactions at this time. Statistical epistasis, in which the penetrance of a two-locus genotype differs from additivity on a multiplicative scale, combined with a plausible biological foundation is probably a more reasonable goal, and this is the definition to be used here.

There are numerous ways in which the genotype at one locus can influence the effect of the genotype at another locus. In a typical conception of epistasis, the risk conferred by one locus is variable depending on the alleles present at another locus. A scenario in which one locus changes the direction of the effect at another locus is called a crossover effect. In a heterogeneity model, a two-locus recessive disease model is used in which

two recessive alleles at either (or both) loci confer disease risk. The penetrance need not be the same for both loci (Cordell, 2002). For an additive model, the allelic loading at each locus influences penetrance in an additive fashion. The dominance model involves one locus masking the effects of an allele at another locus.

Epistatic interactions are one possible reason for the inconsistent replication in schizophrenia association studies. If a particular configuration of allelic variants predisposes individuals to this illness to a greater extent than these variants in isolation, individual alleles may be difficult to detect. In studies of schizophrenia genetics, epistasis is of increasing interest with several recent articles giving evidence for the involvement of epistatic processes in this disorder (Ott et al, 2005; Corvin et al, 2007; Nicodemus et al, 2007; Morris et al, 2007; Edwards et al, 2008; Burdick et al, 2008; Prata et al, 2009).

Epistasis detection

Although epistasis detection is a more straightforward process in case-control datasets, it can also be accomplished using family data with the added benefit of being relatively robust to population stratification. Two main family-based approaches have been proposed. In one, the multifactor dimensionality reduction-pedigree disequilibrium test (MDR-PDT), discordant sib pairs or trios can be used in the detection of joint effects of multiple loci in the absence of detectable main effects with significance assessed through

permutation (Martin et al, 2006). In the other, pseudocontrols are created based on the alleles or genotypes that were not transmitted to an affected offspring from genotyped parents. There are two published approaches to generating pseudocontrols which involve using nontransmitted two-locus genotypes (Cordell approach) to create 15 pseudocontrols (Cordell et al, 2004) or using nontransmitted alleles (two-locus haplotype relative risk approach; HRR) which yields only three (Falk and Rubenstein, 1987) (Figure 6.1). Through the simulated scenarios explored here, we sought to determine which approach yielded greater power to detect epistatic interactions.

Figure 6.1. Depiction of Cordell and HRR approaches to pseudocontrol generation. A = affected, P = pseudocontrol. The hypothetical cross was between double-heterozygote parents and the genotype selected to be affected was arbitrary.

Cordell

	AA	Aa	aA	aa
BB	P	P	P	P
Bb	P	P	P	P
bB	P	P	P	P
bb	P	P	P	A

Haplotype Relative Risk

	AA	Aa	aA	aa
BB	P			
Bb				
bB				
bb				A

Methods:

All simulations and analyses were conducted using SAS (SAS Institute, 2005)
Each test was comprised of 1000 simulated samples of 500 parent-child trios. Pseudocontrols were generated in accordance with the two family-based epistasis detection methods under examination. For the Cordell approach, 15 pseudocontrols were generated from the nontransmitted two-locus genotypes. The HRR approach yields one pseudocontrol arising from the nontransmitted alleles.

For the simulation, the prevalence, K, was fixed at .005 and modeled using the following equation in which π = the baseline risk, GRR = genotype relative risk, p and q are the major and minor allele frequencies for locus 1, respectively, and r and s are the respective major and minor allele frequencies for locus 2:

$$K = p^2r^2\pi + 2pqr^2\pi \times GRR2 + r^2q^2\pi \times GRR3 + p^2rs\pi \times GRR4 + pqrs\pi \times GRR5 + q^2rs\pi \times GRR6 + p^2r^2\pi \times GRR7 + pqs^2\pi \times GRR8 + q^2s^2\pi \times GRR9$$

The minor (risk) allele frequencies were fixed at .1 for both loci. Logistic regression was used to test for epistatic effects. Models of additive, heterogeneity, crossover, and dominance epistatic effects were examined with different magnitudes of effects. (See Appendix B: Penetrance matrices used in epistasis simulations) Several models were adapted from Howson et al (2005).

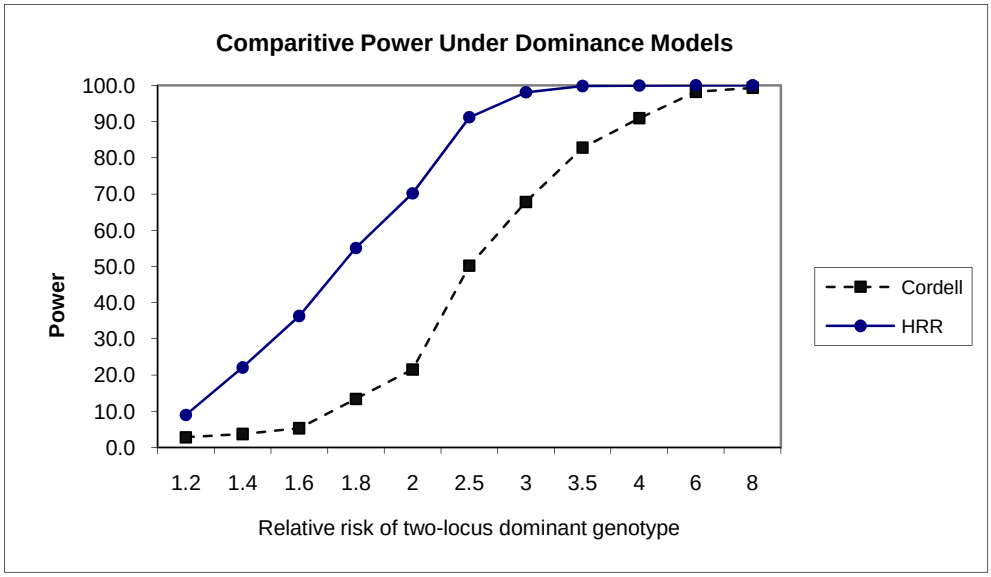
Results:

The power differences between approaches varied substantially according to the models used and the magnitude of the epistasis effects established by the penetrance matrices (Appendix C). Under dominance models, the HRR approach has greater power for epistasis detection (Figure 6.2). Over a broad range of dominance models, those with very low and high marginal effects exhibited floor and ceiling effects attenuating the differences between approaches. Although differences were most dramatic for intermediate penetrance values, the HRR approach showed superior power for detection for all dominance models tested. However, the Cordell approach yields greater power for most other epistasis models. Models termed "epistatic effects" yielded quite similar results between detection approaches, while additive and heterogeneity models were far more easily detected by the Cordell approach. Crossover models exhibited intermediate results.

Table 6.1 Comparative power for Cordell and HRR approaches under different models of epistasis. Numbers in parentheses denote the models adapted from Howson et al (2008).

	Power	
model type	Cordell	HRR
epistatic (4)	31.5	32.7
epistatic (5)	19.6	23.1
additive (7)	99.9	47.6
additive	82.2	5.3
heterogeneity (11)	49.6	4.4
heterogeneity (12)	11.1	12.0
crossover (14)	87.4	62.6
crossover (15)	100	76.7
dominance	2.8	9.0
dominance	3.7	22.1
dominance	5.3	36.3
dominance	13.4	55.1
dominance	21.5	70.2
dominance	50.2	91.2
dominance	67.8	98.1
dominance	82.8	99.8
dominance	90.9	99.9
dominance	98.2	100
dominance	99.3	100

Figure 6.2. Comparative power for Cordell and HRR approaches under dominance models.



Discussion:

From these results, we conclude that different approaches to family-based epistasis testing are indicated depending on the expected model of epistasis in effect. Under conditions of dominance, it is advantageous to generate pseudocontrols without the susceptibility alleles to enhance contrast with the affected individual. For this reason, the HRR approach with only one pseudocontrol, lacking the alleles present in the affected individual, would be advisable. Other epistatic interactions are more easily detected through inclusion of more pseudocontrols even if they do harbor the risk alleles. In real data, it is unlikely that the model of epistasis would be known *a priori*, but the Cordell approach may be a wise choice given its higher power under most models.

Once evidence for a statistical epistatic interaction has been revealed, determining the biological meaning it confers is challenging (Moore and Williams, 2005). It is possible that the statistical result is a false positive, and consequently, corrections for multiple testing should be implemented to reduce this possibility. Also, it could be the case that the alleles demonstrating epistasis are in linkage disequilibrium with the true causative alleles. Additional genotyping or in vitro experiments may be useful in resolving that question. Biological investigations of the purported interactions will certainly lend credence to any report of statistical epistasis. For example, protein-protein interactions can be assayed by yeast two hybrid experiments, and knockout mice have also been used to assess downstream effects of removing one protein.

Only two-locus interactions have been investigated here, but higher-order interactions are entirely plausible. The computational feasibility of detecting these interactions, however, is low given the number of possible permutations. Power for detection of two-locus interactions diminishes rapidly with lower allele frequencies and is even further compromised by addition of a third locus.

Most investigations of epistasis in schizophrenia have been limited to genes that are known or theorized to biologically interact. Thus far, little evidence for epistasis in GWAS has been presented (Wellcome Trust Case Control Consortium, 2007), however, the development of new ways of analyzing large datasets will hopefully allow us to tap

the multitudes of interactions waiting to be discovered. Determining the most powerful methods of epistasis detection in conjunction with appropriate multiple testing corrections will provide an important tool to extend our understanding of complex disease etiology.

Chapter 7: General Discussion

The studies presented here have either directly or indirectly been aimed at developing a better understanding of schizophrenia and its various manifestations. Since it is increasingly apparent that multiple genetic pathways to schizophrenia are possible and likely, it follows logically that some genetic influences may vary by population. Support for this has been given through linkage studies in groups of African and European ancestry (Takahashi et al, 2003; Faraone et al, 2005; Suarez et al, 2006; Holliday et al, 2008) as well as a recent paper showing greater risk gene concordance between schizophrenia and bipolar disorder in European-Americans than between schizophrenics of European and African descent (Purcell et al, 2009).

Two of the studies detailed here tentatively corroborate differences in symptom profiles and genetic foundations of schizophrenia between subjects of African and European ancestry. In phenotypic assessments, these two groups demonstrated distinct factor structures. The greatest differences were observed in affective symptoms with individuals of European ancestry having higher levels of manic and depressive symptoms. Explorations of myelin and synaptic gene networks in these samples yielded a significant association for one myelin network and only in the African-ancestry group. Potential population differences should be kept in mind when comparing association or other studies conducted in different ancestry groups.

In a series of studies exploring the relationships between seven genes and symptoms of schizophrenia, we found a significant association of PAH with delusions, GABRB3 with hallucinations, and SNAP25 with both of these symptom factors. AKT1 alleles conferred greater levels of Schneiderian symptoms, but dysbindin, MAOB, and SLC6A4 were not related to any symptom dimensions. Understanding the genetic influences on the symptoms of schizophrenia could contribute to the generation of more targeted treatments for them. It may also lead to the identification of reliable subtypes and eventually aid in uncovering the predisposing biological mechanisms. Similar studies investigating modifier gene effects but encompassing a greater number of genes would be useful in these regards.

The question of whether schizophrenia is one disease or several has lingered since its identification and remains one of the most pertinent questions in this field. Distinct genetic risk factors giving rise to similar symptom profiles presents challenges to the detection of susceptibility genes. The simulation study addressing detection of a pseudo-modifier gene in one subgroup with a slightly different set of symptoms explores a variety of parameters impacting association results under these conditions.

It is possible that some of the inconsistent associations reported for schizophrenia candidate genes are due to epistatic interactions. If the interaction of two genes confers greater disease risk than the genes individually, the genes may not be detected in examinations of main effects. The final study presented here explored approaches for

detecting epistatic effects in family data under different models of epistasis. The haplotype relative risk (HRR) approach, involving generation of one pseudocontrol, yields greater power for detection under conditions of dominance, but the Cordell approach with fifteen pseudocontrols is more powerful under most other models.

As national and international collaborations grow to produce ever larger cohorts of subjects, better detection of genetic loci influencing schizophrenia should follow. But if many thousands of loci, most with very small effect sizes, are contributing to the schizophrenia phenotype, it may not be possible to identify all of them. The gene-finding strategies implemented for monogenic disorders and complex disorders with limited genetic heterogeneity are not well suited for complex diseases with hundreds or thousands of susceptibility loci. Novel approaches must be developed and applied to data from many populations if we are to fully understand the genetic underpinnings of schizophrenia and other highly complex diseases.

Environmental risk factors between or within populations could also influence the diagnosis and manifestation of schizophrenia. Although with a heritability of ~81% (Sullivan et al, 2003) it is appropriate to focus most efforts on the genetic risk factors for schizophrenia, environmental influences on the liability to schizophrenia and the ways they may interact with susceptibility genes also deserve mention here. However, ~20% of the variance in liability to schizophrenia is environmental in origin, and this certainly merits investigation in its own right. The prenatal viral exposure theory of schizophrenia

etiology has been championed through season of birth studies (Davies et al, 2003), animal model generation through prenatal maternal immune system activation (Ozawa et al, 2006), and recent GWAS studies pointing to immune system genes (Purcell et al, 2009; Shi et al, 2009; Stefansson et al, 2009). Other environmental factors have been posited such as immigrating to another country (Cantor-Graae and Selten, 2005). The disorienting nature of adapting to another culture may lead to psychosis in some individuals, or discrimination could foster persecutory beliefs that spiral into psychotic features. Cannabis use may confer additional risk in people predisposed to developing schizophrenia (Moore et al, 2007). Additional environmental theories abound, and their study should not be marginalized since environmental manipulation is more tractable and palatable than genetic alterations for prevention and intervention strategies.

Nevertheless, the majority of people who experience these risk factors do not go on to develop schizophrenia, underscoring the importance of genetic liability to these influences. Genetic variation remains the primary cause of schizophrenia and the best hope for development of more efficacious treatments. It is obvious that with copious genetic heterogeneity and a variety of potential environmental risk factors, many different causal mechanisms are likely acting to produce the phenotype termed schizophrenia.

Decades of research have highlighted the complexity of the genetic foundations giving rise to schizophrenia, but conclusive findings have remained elusive. The advent of high-throughput genotyping and new computational strategies has made more complex

analyses feasible. Nascent methods such as network or pathway analysis may capture variants disrupting biological process known to have gone awry or even uncover previously unknown mechanisms leading to the disease. Investigations of epistatic interactions could also lead to a better understanding of the constellation of genetic alterations resulting in schizophrenia, and explorations of symptoms and their genetic bases will also likely shed light on this enigmatic disease.

Such diverse research tactics are appropriate for this symptomatically and genetically heterogeneous disease. The rapid progress from a broad range of research domains engenders hope that we will eventually unlock the etiological mechanisms of schizophrenia, but it is clear that many more years of research in this field lie ahead. The ultimate goals of preventing schizophrenia when possible and treating it when not will be best served by understanding the mechanisms giving rise to this disease.

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Appendix A: Exploratory Factor Analysis factor loadings for the combined GAIN sample and individual groups.

Combined Sample

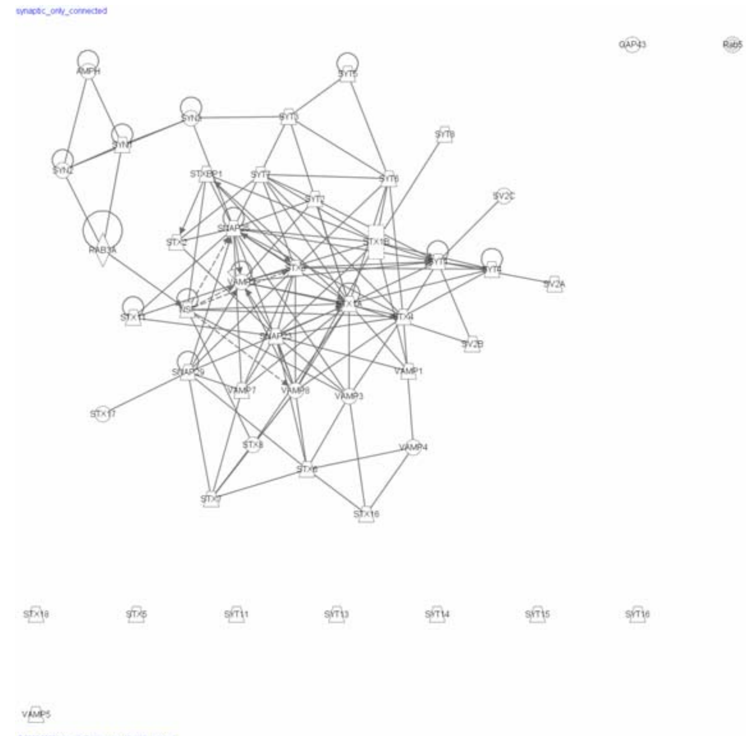
	1	2	3
delusions	0.836	0.233	0.288
paranoia	0.773	0.188	0.243
hallucinations	0.779	-0.108	0.088
control delusions	0.546	0.148	0.156
conversing/commenting/cont hall	0.746	-0.066	0.012
abnormal perception of thought	0.504	0.118	0.100
blunted affect	0.145	-0.157	0.668
poverty of speech	0.076	-0.162	0.707
formal thought disorder	0.175	0.084	0.597
bizarre behavior	0.188	0.114	0.565
depression	0.185	0.450	-0.283
mania	-0.033	0.897	0.063
depression with psychotic features	0.263	0.465	-0.172
mania with psychotic features	0.015	0.934	0.140

	African Ancestry			European Ancestry		
	1	2	3	1	2	3
delusions	0.897	0.175	0.100	0.677	0.579	0.242
paranoia	0.813	0.109	0.079	0.634	0.503	0.205
hallucinations	0.781	-0.165	0.075	0.810	0.107	-0.071
control delusions	0.558	0.125	0.134	0.535	0.176	0.183
conversing/commenting/cont hall	0.715	-0.164	-0.008	0.786	0.018	-0.008
abnormal perception of thought	0.523	0.115	0.069	0.502	0.105	0.118
blunted affect	0.188	-0.193	0.678	0.175	0.575	-0.194
poverty of speech	0.136	-0.261	0.729	0.099	0.596	-0.149
formal thought disorder	0.278	0.206	0.580	0.058	0.617	0.071
bizarre behavior	0.271	0.236	0.515	0.113	0.585	0.093
depression	0.221	0.070	-0.476	0.255	-0.270	0.486
mania	0.043	0.908	-0.125	-0.015	0.063	0.867
depression with psychotic features	0.351	0.037	-0.369	0.324	-0.200	0.490
mania with psychotic features	0.077	0.971	-0.050	0.023	0.171	0.922

Appendix B: Myelin and synaptic gene networks

myelin-core (16)	myelin-extended (54)	synaptic-core (48)	synaptic-extended (69)
QKI	APP	SNAP23	AMPH
OLIG1	CANX	SNAP25	CAMK2B
OLIG2	CAV1	SNAP29	CFTR
SOX10	CD44	VAMP1	GAP43
TF	CD82	VAMP2	GOSR1
CNP	CDKN1B	VAMP3	GOSR2
MOG	CLDN11	VAMP4	HNF4A
PLP1	CNP	VAMP5	NAPA
MOBP	CSDA	VAMP7	NRXN1
CLDN11	DAB1	VAMP8	NSF
MAL	EGFR	STX1A	NSFL1C
NGFR	ERBB2	STX2	PLCG1
RTN4R	ERBB3	STX3	PRKCA
MAG	FN1	STX4	PRKCB1
ERBB3	FYN	STX5	PRKCD
NRG1	HBEGF	STX6	PRKCE
	HLA-A	STX7	PRKCG
	HNF4A	STX8	PSEN1
	IL2	STX11	RAB3A
	IL4	STX16	Rab5
	IL6	STX17	RABEP1
	INS	STX18	SNAP23
	ITGB1	SYN1	SNAP25
	LCK	SYN2	SNAP29
	MAG	SYN3	SPTAN1
	MAL	SYT1	SRC
	MAP1B	SYT2	STX11
	MBP	SYT3	STX16
	MOBP	SYT4	STX17
	MOG	SYT5	STX18
	NEUROG3	SYT6	STX1A
	NGFR	SYT7	STX1B
	NOTCH1	SYT8	STX2
	NRG1	SYT11	STX3
	OLIG1	SYT13	STX4
	OLIG2	SYT14	STX5
	PIK3R1	SYT15	STX6
	PLG	SYT16	STX7
	PLP1	SV2A	STX8

PRKCE	SV2B	STXBP1
PRNP	SV2C	SV2A
PTK2	NSF	SV2B
PTPN11	STXBP1	SV2C
QKI	AMPH	SYN1
RANBP9	GAP43	SYN2
RHOA	RAB3GAP2	SYN3
SHC1	RAB3A	SYNCRIP
SOX10	RAB5A	SYT1
SRC		SYT11
SYK		SYT13
TF		SYT14
TNF		SYT16
TSPAN3		SYT2
TSPAN4		SYT3
		SYT4
		SYT5
		SYT6
		SYT7
		SYT8
		USO1
		VAMP1
		VAMP2
		VAMP3
		VAMP4
		VAMP5
		VAMP7
		VAMP8
		VCL
		ZDHHC7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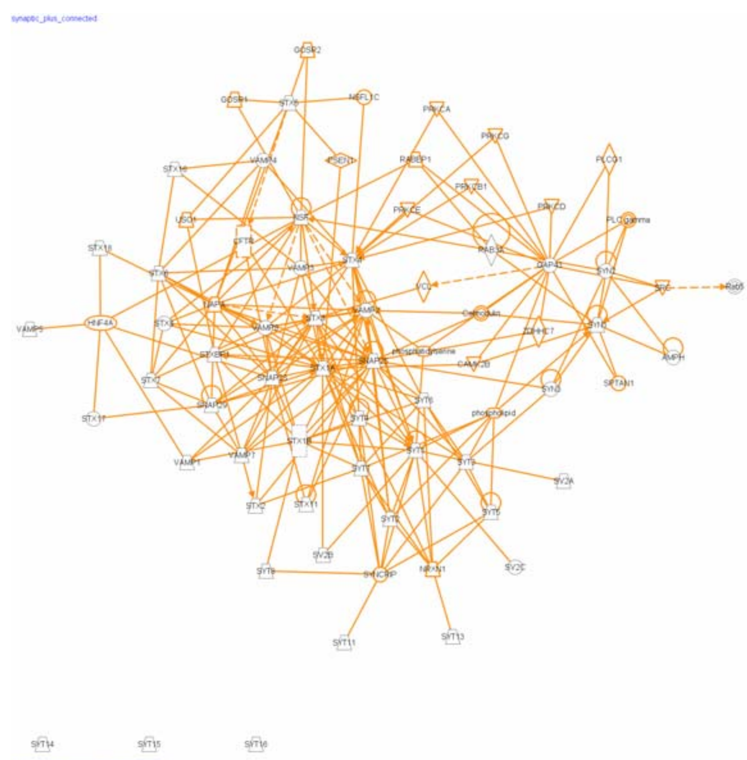
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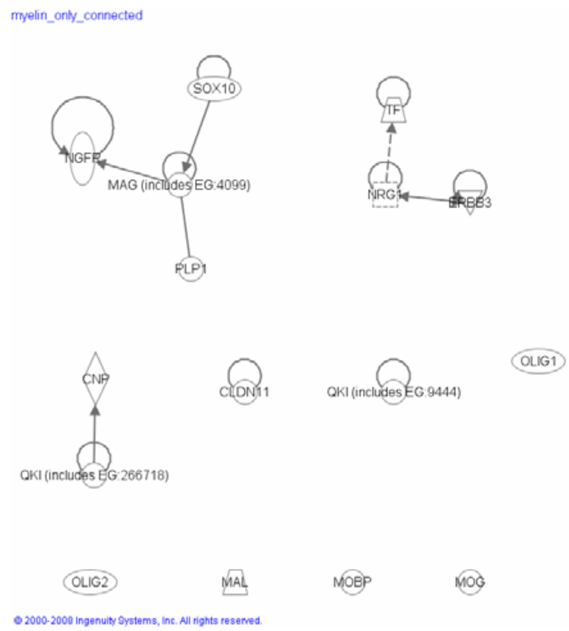
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TF
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CNP
CLDN11
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OLIG1
QKI (includes EG:266718)
OLIG2
MAL
MOBP
MOG
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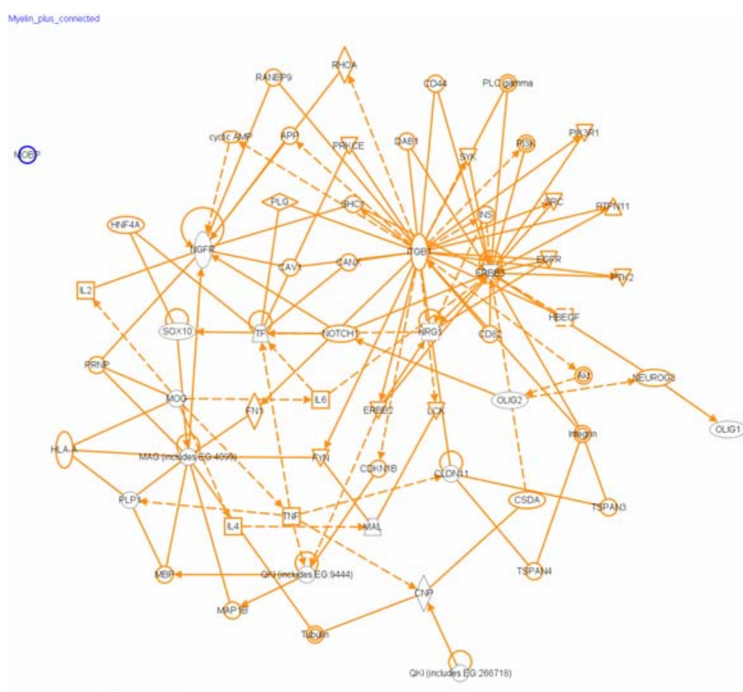
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PLG
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INS
SRC
PTPN11
HNF4A
NGFR
CAV1
CANX
ITGB1
EGFR
ERBB3
PTK2
IL2
HBEGF
SOX10
TF
NOTCH1
NRG1
CD82
PRNP
Akt
NEUROG3
MOG
IL6
OLIG2
FN1
ERBB2
LCK
Integrin
OLIG1
HLA-A
MAG (includes EG:4099)
FYN
CDKN1B
CLDN11
PLP1
CSDA
TSPAN3
TNF
IL4
MAL
MBP
QKI (includes EG:9444)
TSPAN4
CNP
MAP1B
Tubulin
QKI (includes EG:266718)
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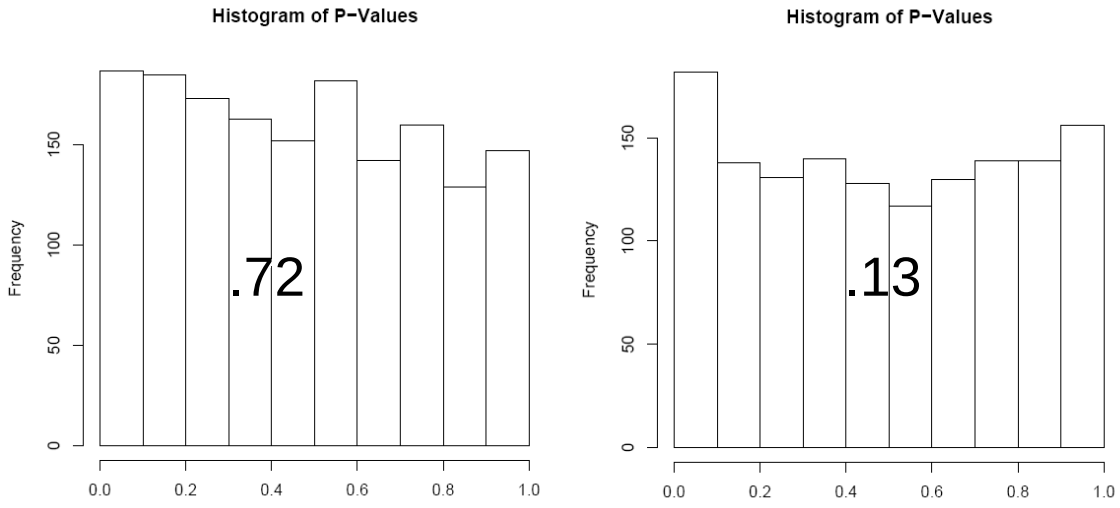
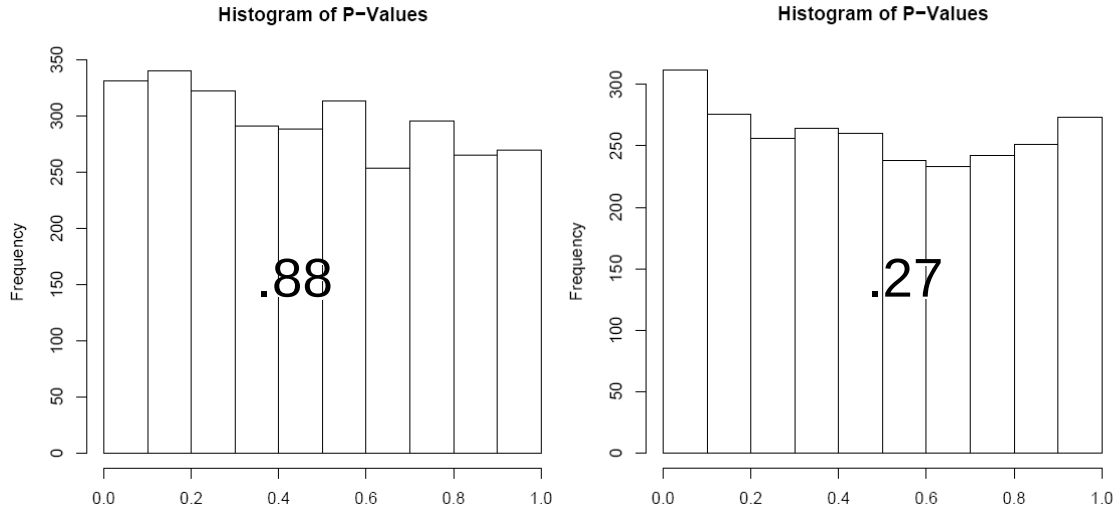
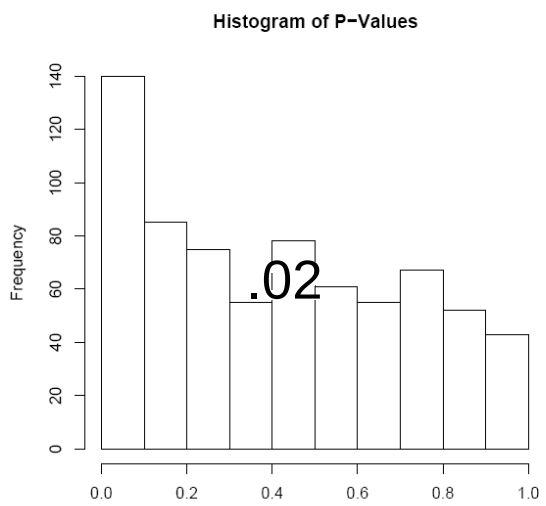
Core Synaptic Network with superimposed empirical p-values

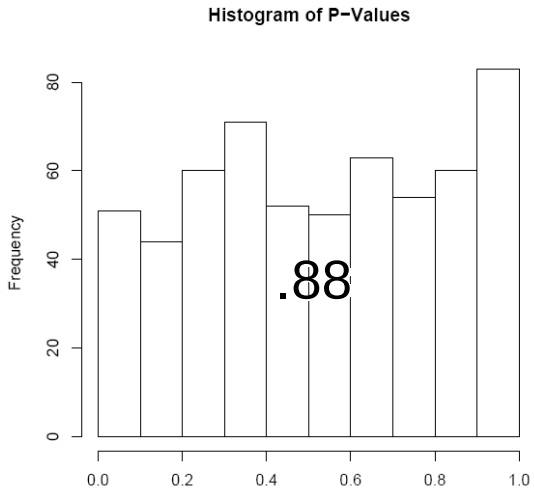


Extended Synaptic Network with superimposed empirical p-values

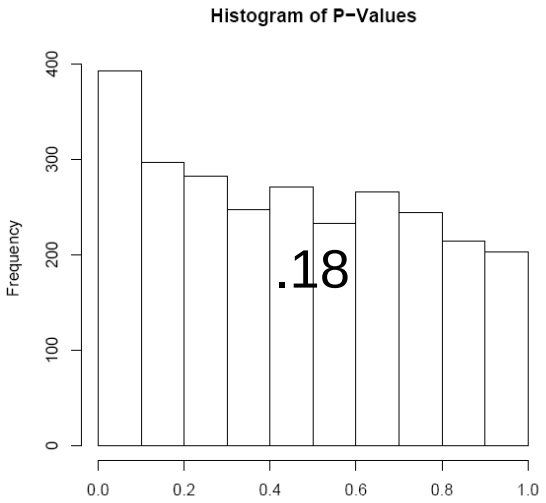


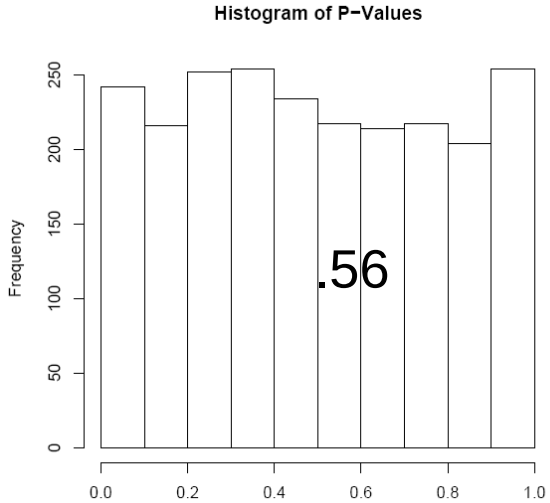
Core Myelin Network results with superimposed empirical p-values





Extended Myelin Network results with superimposed empirical p-values





Appendix C: Penetrance matrices used in epistasis simulations
Numbers in parentheses denote the models adapted from Howson et al (2008).

epistatic (4)

	0	1	2
0	1	1	20
1	1	1	40
2	1	1	80

additive (7)

	0	1	2
0	1	10	100
1	2	11	101
2	4	13	103

epistatic (5)

	0	1	2
0	1	1	1
1	1	1	1
2	1	1	50

additive

	0	1	2
0	1	2	4
1	2	4	6
2	4	6	8

heterogeneity (11)

	0	1	2
0	1	1	20
1	1	1	20
2	4	4	20

crossover (14)

	0	1	2
0	3	3	15
1	2	2	30
2	1	1	60

heterogeneity (12)

	0	1	2
0	1	1	2
1	1	1	2
2	2	2	2

crossover (15)

	0	1	2
0	1	1	150
1	2	2	100
2	4	4	50

dominance

	0	1	2
0	1	1	1
1	1	x	x
2	1	x	x

x = 1.2, 1.4, 1.6, 1.8, 2, 2.5, 3, 3.5, 4, 6, 8

Vita

Sarah Elizabeth Bergen was born on September 30, 1977 in Ames, Iowa. Following her high school graduation in Plano, TX, she attended Macalester College in St. Paul, MN and earned a BA in Neuroscience and Psychology in 2000. After working at UCSF for several months, she entered a Neuroscience graduate program at the University of Pittsburgh. She graduated in 2004 with an MS degree and moved back to California to work at the Neurogenetics Institute of USC in Los Angeles. She moved to Richmond, VA in 2005 to begin doctoral work in Genetics at VCU.