Schizophrenia Bulletin vol. 35 no. 1 pp. 96–108, 2009
doi:10.1093/schbul/sbn155
Advance Access publication on November 20, 2008

A Genome-Wide Association Study of Schizophrenia Using Brain Activation as a Quantitative Phenotype

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Background: **Genome-wide association studies (GWASs) are increasingly used to identify risk genes for complex illnesses including schizophrenia. These studies may require thousands of subjects to obtain sufficient power. We present an alternative strategy with increased statistical power over a case-control study that uses brain imaging as a quantitative trait (QT) in the context of a GWAS in schizophrenia.** ***Methods*****: Sixty-four subjects with chronic schizophrenia and 74 matched controls were recruited from the Functional Biomedical Informatics Research Network (FBIRN) consortium. Subjects were genotyped using the Illumina HumanHap300 BeadArray and were scanned while performing a Sternberg Item Recognition Paradigm in which they learned and then recognized target sets of digits in an functional magnetic resonance imaging protocol. The QT was the mean blood oxygen level–dependent signal in the dorsolateral prefrontal cortex during the probe condition for a memory load of 3 items.** ***Results:*** **Three genes or chromosomal regions were identified by having 2 single-nucleotide polymorphisms (SNPs) each significant at $P < 10^{-6}$ for the interaction between the imaging QT and the diagnosis (*ROBO1-ROBO2*, *TNIK*, and *CTXN3-SLC12A2*). Three other genes had a significant SNP at $<10^{-6}$ (*POU3F2*, *TRAF*, and *GPC1*). Together, these 6 genes/regions identified pathways involved in neurodevelopment and response to stress.** ***Conclusion:*** **Combining imaging and genetic data from a GWAS identified genes related to forebrain development and stress response, already implicated in schizophrenic dysfunction, as affecting prefrontal efficiency. Although the identified genes require confirmation in an independent sample, our approach is a screening method over the whole genome to identify novel SNPs related to risk for schizophrenia.**

Key words: genome-wide scan/schizophrenia/working memory/genes/DLPFC/fMRI

Introduction

Genome-wide scans offer the opportunity to interrogate the entire genome to identify risk genes for complex illnesses. Recent published studies have successfully identified risk genes in a variety of illnesses including diabetes (type 2),[1] macular degeneration,[2] Crohn disease,[3] bipolar disorder, Alzheimer disease, and Parkinson disease, to name a few. Several investigators have argued that very large samples of many thousands of subjects per group are needed to have sufficient power to conduct such studies.[1,4] It is difficult to obtain such samples, and combining the needed data from multiple sites and studies encounters considerable challenges in diagnostic and methodological standardization, as well as increasing the genetic and population heterogeneity (Salvi E., Guffanti G., Orro A., Lupoli S., Torri F., Potkin S., Turner J., Barlassina C., Cusi D., Milanesi L., Macciardi F. Ancestry correction in genome-wide association studies: Comparison of different methods to control for population stratification. 2008, Manuscript Submitted).[5–7] Interpretation of such studies is further complicated by the difficulty in obtaining an independent large sample for replication.

Some of these power limitations and related sample size requirements can be mitigated by using a quantitative trait (QT) strategy. The use of a QT brings considerably more power, up to 4–8 times, than typical case-control approaches in which a group of patients (cases) is compared with a group of controls.[8] Case-control approaches, when applied to multifaceted disorders such as schizophrenia, are largely dependent on subjective and nonquantitative information to identify and separate cases from controls, and differences in disease severity and other more subtle characteristics are lost. In comparison, a

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QT is objectively measureable, provides more variation than a simple dichotomous classification, and may be more proximal to the genetic etiology than clinical symptoms.

In this study, we use differential brain imaging activation patterns as the starting point in our analyses, based on the assumption that brain imaging will reveal important pathophysiological differences in subjects with and without schizophrenia. We then determine the impact of genetic variation on these brain activation phenotypic patterns to identify genetic influences potentially key to understanding the pathophysiology. In this way, we use brain imaging activation as the QT as a reflection of schizophrenia dysfunction. We chose activation in the dorsolateral prefrontal cortex (DLPFC) as the QT in this study based on statistically significant brain activation differences that we observed between schizophrenia patients and healthy controls during a working memory task. This choice was bolstered by the extensive literature implicating the DLPFC in schizophrenia.[9–18]

The role of the DLPFC in schizophrenic dysfunction goes beyond merely the question of hypo- or hyperfrontality. It is an area implicated in schizophrenia both structurally and functionally, both in the left and right hemispheres, from differences in local gene expression,[19] to differences in cell morphometry,[20] to structural circuitry differences,[21,22] and both local and distributed differences in functional activation.[23,24] One of the most replicated results is the difference in blood oxygen level–dependent (BOLD) signal in the DLPFC in schizophrenics during a working memory task. In the N-back task, schizophrenics fail to activate the DLPFC as much as healthy volunteers do, even when performance is accounted for. In the Sternberg Item Recognition Paradigm,[9,15,17,25] schizophrenics show more activation in the DLPFC than do controls.[15,26] This is true whether a region of interest analysis or a multivariate analysis is applied.[27] This complex relationship between the precise cognitive demands and the neural dysfunction, in conjunction with the known structural differences, makes the BOLD signal changes in the DLPFC a good choice for a cognitive biomarker in schizophrenia.

Previous twin studies of schizophrenia have identified both the behavioral and functional magnetic resonance imaging (fMRI) measures obtained with the Sternberg working memory task as heritable traits.[12] Using the same task, working memory performance decreased with increasing genetic load among schizophrenic twins discordant for schizophrenia and control twins.[14] The Sternberg task-related abnormalities are found in the relatives of persons with schizophrenia as well as in the patients both on medication, and with no medication, which is also consistent with a heritable trait.[9,15]

Our large Functional Biomedical Informatics Research Network (FBIRN) sample, collected across 10 different universities, allowed matching for performance accuracy with the identical level of memory load, addressing possible confounds in previous efforts. In the FBIRN sample, the major difference in brain activation between schizophrenia subjects and controls was during the retrieval condition of a working memory task (SIRP).[26] The lack of differences observed in the encoding condition suggested that schizophrenia subjects were able to store the memoranda similarly to controls (over a range of 1 to 5 items) but required greater activation of the DLPFC to achieve the same level of performance accuracy as controls during item retrieval from memory. The major DLPFC activation difference observed in the retrieval conditions was present at memory load of 3 items. Schizophrenia subjects activate the DLPFC to a greater degree than healthy controls to achieve the same level of performance at that level of demand. This is consistent with the inefficiency hypothesis put forward by Callicott[28] and Manoach and colleagues.[9,15] We used activation in the DLPFC at the memory load of 3 items as the QT for this study.

The use of QTs with a comprehensive genome-wide scan has not been commonly applied to neuropsychiatric disorders, perhaps because of difficulty in determining the QT. A notable exception was the discovery of KIBRA using memory performance as the QT, based on quartile ranking in verbal episodic memory, in a genomics scan of pooled DNA.[29] Recently, Almasy et al. used a measure of cognitive function as a QT in conjunction with 386 microsatellite markers in a family study of schizophrenia.[30] Neuroimaging, however, has been used to reveal the function of candidate genes, eg, *COMT*,[31,32] using studies designed to begin with a specific gene and explore its effects on various phenotypes. Brain imaging has been used to study the function of a number of other genes such as *SLC6A4* transporter, *DRD4*, *DRD1*, *HTR3A*, *TPH2*, and *MAOA*[33–37] and genes associated with schizophrenia including *NRG1*, *RGS4*, *COMT*, *GRM3*, *G72*, *DISC1*, and *BDNF*.[38–41]

In functional neuroimaging studies of neuropsychiatric patients and healthy controls, differential activation in regions of interest or putative circuits can be identified. In this study of schizophrenia, we limit our imaging phenotypes to the left and right DLPFC (BA 46), known to be an area of schizophrenic dysfunction—and then examine the role of individual genetic variation on these phenotypes at an individual level, ie, how each single-nucleotide polymorphisms (SNPs) predicts activation in the DLPFC. Our approach reverses the candidate gene strategy: Rather than beginning with a specific candidate gene as a grouping factor and searching for differences in neuroimaging results within groups, we begin with brain imaging as a phenotype and determine the SNPs that influence that phenotype.

Methods

The participating institutions in this study were University of California Irvine (UCI), University of California

Table 1. Clinical and Demographic Summaries

Demographic Characteristics		Patients	Controls	Statistical Significance	Percent reporting
Number of subjects		64	74	-	100
Race (% Caucasian)		76.6	81.1	ns	99
Gender (% male)		71.9	60.8	ns	100
Handedness (% right)		87.5	91.9	ns	100
Mean age (SD)	range: 19-65	37.3 (11.2)	37.8 (11.7)	ns	100
Subject's mean years of education (SD)	range: 10-20	13.4 (1.8)	15.9 (1.9)	< 0.001	91
Mother's mean years of education (SD)	range: 0-20	13.0 (3.4)	13.6 (3.5)	ns	83
Father's mean years of education (SD)	range: 4-22	14.6 (3.3)	14.7 (3.6)	ns	81
Mean premorbid FSIQ[a] estimate (SD)	range: 85-126	105.2 (9.2)	113.3 (7.7)	< 0.001	95
		Range	Mean	SD	% Reporting
Calgary Depression Scale (total score)		0–20	5.24	5.1	98
Scale for Assessment of Positive Symptoms (global measures sum)		0–19	8.93	4.4	95
Scale for Assessment of Negative Symptoms (global measures sum)		0–13	6.45	3	94
InterSePT Suicidality Scale (sum of 11 items)		0–10	1.56	2.7	78
Deficit Syndrome Scale—global categorization (% classified as "3"):		72.7			86

[a]Full Scale Intelligence Quotient, derived from the North American Adult Reading Test (Blair and Spreen, 1989).

Los Angeles (UCLA), University of New Mexico/MIND Research Network, University of Iowa, University of Minnesota, Duke University/University of North Carolina, Brigham and Women's Hospital (BWH), Massachusetts General Hospital (MGH), and Yale University.

Subjects

The sample consisted of 64 subjects with chronic schizophrenia and 74 controls overall matched for gender and age, diagnosed according to *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition*, criteria with a Structured Clinical Interview for Diagnosis. This was part of a larger cohort collected by the FBIRN. The demographics and summary of the clinical measures for these subjects can be found in table 1. Subjects with schizophrenia (SZ) and healthy volunteers did not differ significantly in age, race, gender, handedness, or parental education levels. SZ were significantly lower on the FSIQ measure than controls (105.9 vs 113.3, $P < .001$) and had significantly fewer years of schooling (13.4 vs 15.9, $P < .001$).

Neuroimaging Methods

The neuroimaging methods have been reported in detail elsewhere.[26,27,42] In summary, all subjects were scanned while performing a Sternberg Item Recognition Paradigm (SIRP),[15] in which they learned and then recognized target sets of digits. The target set could include 1, 3, or 5 items; during the retrieval period, subjects indicated by pressing a button to indicate whether or not a single probe digit was a member of the immediately preceding target set. fMRI data were collected while subjects performed this task, at a variety of 1.5T and 3T scanners around the United States. Generally, the imaging protocol was a linear or spiral echo-planar imaging sequence, using 22–27 anterior-posterior commisures (AC-PC) aligned slices, 4 mm thick with a 1 mm gap, Repetition Time (TR) = 2 s, Time to Echo (TE) = 30 ms (40 ms for the 1.5T scanners), flip angle (FA) = 90°, 64 × 64 in-plane resolution, Field of View (FOV) = 220 mm. Each run of the SIRP task lasted 6 minutes, and subjects performed 3 of them in a single scanning session, performing 2 repetitions of each memory load in each run.

All subjects' data were visually checked, preprocessed, and analyzed as described in Potkin et al, this issue.[26] Analysis was performed using the FBIRN Image Processing Scripts, which use FMRIB Software Library[43] to analyze large datasets efficiently. Individual datasets were motion corrected, slice timing corrected, where possible the B0 distortion de-warping was applied; and all datasets were smoothed to a common level of 8 mm full width half maximum[44]. Each subject's data were analyzing within FSL using a general linear model, based on convolving the canonical hemodynamic response function with the onset and duration of the encoding and retrieval conditions of the different memory loads. The mean BOLD signal change for the various conditions (encoding and recalling 1, 3, and 5 items relative to fixation) from each subject's data was extracted from both the right and left DLPFC, as defined using the WFU Pickatlas.[45,46] As described elsewhere,[26] mean BOLD signal in either hemisphere was significantly greater in schizophrenic subjects than in the controls in the condition of retrieving the 3 item target set from memory. The implications of the diagnostic group differences are discussed in the companion article that same phenotype was used here for the initial genome-wide association study (GWAS).

Genotyping Methods

Genotyping was performed with the Illumina Infinium HumanHap300 BeadArrays. The HumanHap300 BeadArray assayed 317 503 SNPs, derived from the Phase I HapMap and selected to tag haplotype blocks, with a mean call rate of 99.7%. Approximately 750 ng of genomic DNA was used to genotype each subject of the discovery sample according to the Illumina Infinium 2 assay manual. Each sample was whole genome amplified, fragmented, precipitated, and hybridized overnight for a minimum of 16 hours at 48 °C to allele-specific (Human1) or locus-specific (Hap300) probes on the BeadArray. Nonspecifically hybridized fragments were removed by washing while remaining specifically hybridized DNA were processed for the single base extension reaction, stained, and imaged on an Illumina Bead Array Reader. Normalized bead intensity data obtained for each sample were loaded into the Illumina Beadstudio 2.0 software which generated SNP genotypes from fluorescent intensities using the manufacturer's default cluster settings.

Five samples with less than 90% of markers successfully genotyped across all SNPs were excluded from the analysis. We removed 5342 SNPs with more than 10% missing genotypes across subjects and 232 SNPs with minor allele frequency (MAF) less than 1% (some overlapped with the previous category). After removal of SNPs that did not pass the quality control measures we had 302 783 (autosomal) markers to analyze, with a mean call rate of 98.9% indicating a very high rate of successful genotyping.

Statistical Methods

To correct for possible population stratification in our sample(s), we used the program EIGENSTRAT[47] that controls for the risk of stratification by performing a principal component analysis with the highest possible number of available SNPs. We thus used the entire set of SNPs from HumanHap300, as suggested by the method, and additionally performed a parallel analysis using a subset of SNPs (18 036) that (a) were not in linkage disequilibrium (LD) to each other, (b) showed a MAF > 0.30, and (c) were not in chromosomal regions previously known to be related to schizophrenia. With both approaches, our samples did not show evidence of stratification.

All autosomal SNPs that passed quality control checks were tested for QT association interaction using the "G × E" tool implemented in PLINK (http://pngu.mgh.harvard.edu/purcell/plink/).[48] The statistical model is based on comparing the differential effects of SNP association by diagnosis, thus G × D rather than G × E in our case, on the brain imaging QT.

Out of the possible 4 models (ie, additive, codominant, dominant, and recessive) G × D implements the additive model that generally reflects the additive contribution to risks for complex diseases.[49] Additive models also can detect strong non-additive effects. When appropriate, SNPs were subsequently analyzed with additional genetic models, eg, we used the dominant model when the hypothesized risk allele (B) was rare, with few risk allele homozygotes (BB) observations in cases and controls, pooling risk allele homozygotes (BB) and heterozygotes (AB) genotypes together in the analysis. Such a model tests the hypothesis that carrying even one copy of that particular allele increased risk of disease.[50,51]

There are no definitive methods for determining a statistical threshold for a QT interaction, like G × D, in a context of a GWAS. Given 302°783 SNPs any results at 10^{-6} or smaller should provide enough evidence for an association of a given SNP with a QT. This threshold is in keeping with WTCCC recommendations.[52] While interaction terms generally have fewer subjects with the combination of events due to the interaction than main effects, however defining a definite and appropriate statistical threshold is complex (Potkin, S.G.; Guffanti, G.; Lakatos, A.; Turner, J.A.; Kruggel, F.; Fallon, J.H.; Saykin, A.; Orro, A.; Lupoli, S.; Salvi, E.; Weiner, M.; and Macciardi, F. Brain Imaging as a Quantitative Trait to Identify Novel Susceptibility Genes for Alzheimer's Disease in a Genome-wide Association Study: Initial Analysis and Data Release. 2008, Under Review.).[53] For the purposes of presenting the initial analyses of our data, considering the number of variables analyzed and the complexity of the model, we chose the threshold of 10^{-6} for our interaction term and also added an even more conservative rule requiring observing at least 2 SNPs $\leq 10^{-6}$ in either the left or right hemisphere. Of these, we discuss the biological plausibility and their potential in schizophrenia.

The genetic annotation was performed with WGAViewer software, Version 1·25N, 2008 (http://www.genome.duke.edu/centers/pg2/downloads/wgaviewer.php).[54]

Results

Table 1 represents the demographic and clinical characteristics of the sample. The average age was 38 (range 18–61) and 36.2 (range 18–65) years of age for the subjects with schizophrenia and the controls, respectively. The mean duration of illness was 14.3 years (range 2–43 years). All were treated with stable doses of antipsychotic drugs. This sample is typical of chronic schizophrenic patients in treatment with a moderate degree of stable symptoms (see table 1).

Table 2a shows the significant results obtained for the interaction term (SNP × diagnosis) using the criteria of the QT analysis of $\leq 10^{-6}$ for at least 2 SNPs in either the left or right DLPFC. Only results using the right DLPFC phenotype passed the significance threshold and are presented. We identified 3 genes or chromosomal regions associated with our phenotype. The 3 genes or regions are (1) *ROBO2-ROBO1* region on chromosome 3, (2) *TNIK* and surrounding area on chromosome 3, and (3)

Table 2a. Genes / Chromosomal Regions Identified in the Quantitative Trait Analysis With At least 2 Independent SNPs at a 10^{-6} Significance Level

Chromosome	Gene	SNP	Location	Type	MAF_CTRL	MAF_SZ	*P* Value
3	ROBO2-ROBO1	Rs7610746	78138637	Intergenic	0.31	0.41	7.56E-06
3	ROBO2-ROBO1	rs9836484	78127379	Intergenic	0.32	0.41	4.23E-06
3	TNIK	rs2088885	172453985	Intronic	0.47	0.45	6.24E-06
3	TNIK	rs7627954	172462669	Intronic	0.47	0.45	6.24E-06
5	CTXN3-SLC12A2	rs245178	127231091	Intergenic	0.32	0.30	1.22E-06
5	CTXN3-SLC12A2	rs245201	127197111	Intergenic	0.32	0.30	9.31E-08

Note: Quantitative trait analysis for the interaction between right dorsolateral prefrontal cortex and single-nucleotide polymorphisms (SNPs) from the genome-wide association study. All listed genes have at least 2 SNPs in the right DLPFC at $\leq 10^{-6}$. The chromosome number (in order), gene name (build 36.3), SNP basepair position, physical location, and region are presented. The minor allele frequency (MAF) is presented for healthy controls and schizophrenic patients.

CTXN3-SLC12A2 region on chromosome 5. For all 3 findings, there are also additional clusters of independent, nominally significant ($10^{-5} < P < .05$) SNPs within a well-defined subregion, providing further support that the association is not by chance. The *ROBO2-ROBO1* region on chromosome 3 spans a total of 3.2 Mbp and includes 247 SNPs. Within that region, there is a small area of about 900 kbp including 70 SNPs, 11 of which are significant besides the original 2. The *TNIK* gene and surrounding area, from 172169267 to 172755182, includes 79 SNPs, 12 of which are nominally significant besides the original 2. The *CTXN3-SLC12A2* region spans about 518 kbp and includes 46 SNPs, 17 of which are significant besides the original 2. In figure 1, we present the relationship between the QT results for the right DLPFC and the significant genes or chromosomal regions. The *P* values for the QT are depicted with the physical location of the SNPs and the LD map. The details of the associations for these 3 genes or chromosomal regions are included in Supplemental tables 1, 2, and 3 (available online).

Additional genes are presented in table 2b that are in putative functional pathways related to the genes in table 2a and have at least 1 SNP at the 10^{-6} level. This list contains GPC1, belonging to a pathway involving *ROBO1–ROBO2*.[55] *POU3F2* and *TRAF3* appear to be related to TNIK. Supplemental tables 4, 5, and 6 (available online) details the most significant SNPs from the genes represented in table 2b.

A low MAF can affect the results of an additive model, with few observation of the minor allele homozygotes genotypes; therefore, we recalculated the statistics for using a dominant model for *POU3F2*.[50] A greater level of significance was observed for *POU3F2* with 3 SNPs at 10^{-6} (rs9321063, rs9491640, and rs9491646). The increased significance indicates that MAF bias did not account for the findings and suggests that even a single copy of the risk allele affects the quantitative phenotype that distinguishes schizophrenia patients from normal controls.

Discussion

We present an initial QT analysis that combines imaging and genetic data obtained in a GWAS from the FBIRN cohort to identify potential genes related to the susceptibility of schizophrenia. Using a brain imaging, quantitative phenotype is inherently different than a case-control categorical study. This approach could identify the same risk genes found in a typical case-control study or entirely different set of genes because of the specificity of the phenotype, a quantitative measure of DLPFC activation, rather than a diagnostic categorization. Thus, each method has advantages in identifying risk genes. Previous GWAS in schizophrenia using a case-control approach have produced several interesting candidate genes, although there has been little consistency in the findings, perhaps with the exception of *ZNF804A* found through a meta-analysis.[56] Our results are based on a QT analysis.

There is no agreed upon methods for adequately controlling for false positive while protecting against false negatives in an analysis of the size required by GWAS. We chose a threshold of $<10^{-6}$ for at least 2 independent SNPs within a gene/region defined according to the current status of genome annotation. We also examined the biological plausibility of the genes that had been identified.

It needs to be emphasized that the SNPs on the Illumina Infinium HumanHap300 BeadArrays are tagging SNPs (htSNPs), ie, surrogates for a given small region of DNA, in essence microloci on the chromosome. It is possible that any given SNP identified in this analysis is a surrogate for an adjacent causal SNP that may not have been present within the chip. Regardless of the *P* value for a SNP, it is a statistical representation of an area of the gene that can harbor the causal SNP and not necessarily itself be the causal SNP. The advantage of this approach is that each htSNP identifies a mean of 5 kb in which a causal DNA variation may be found, pointing to an area of focus for gene sequencing and subsequent studies of molecular mechanism. However, some of the SNP's annotation in the current genome

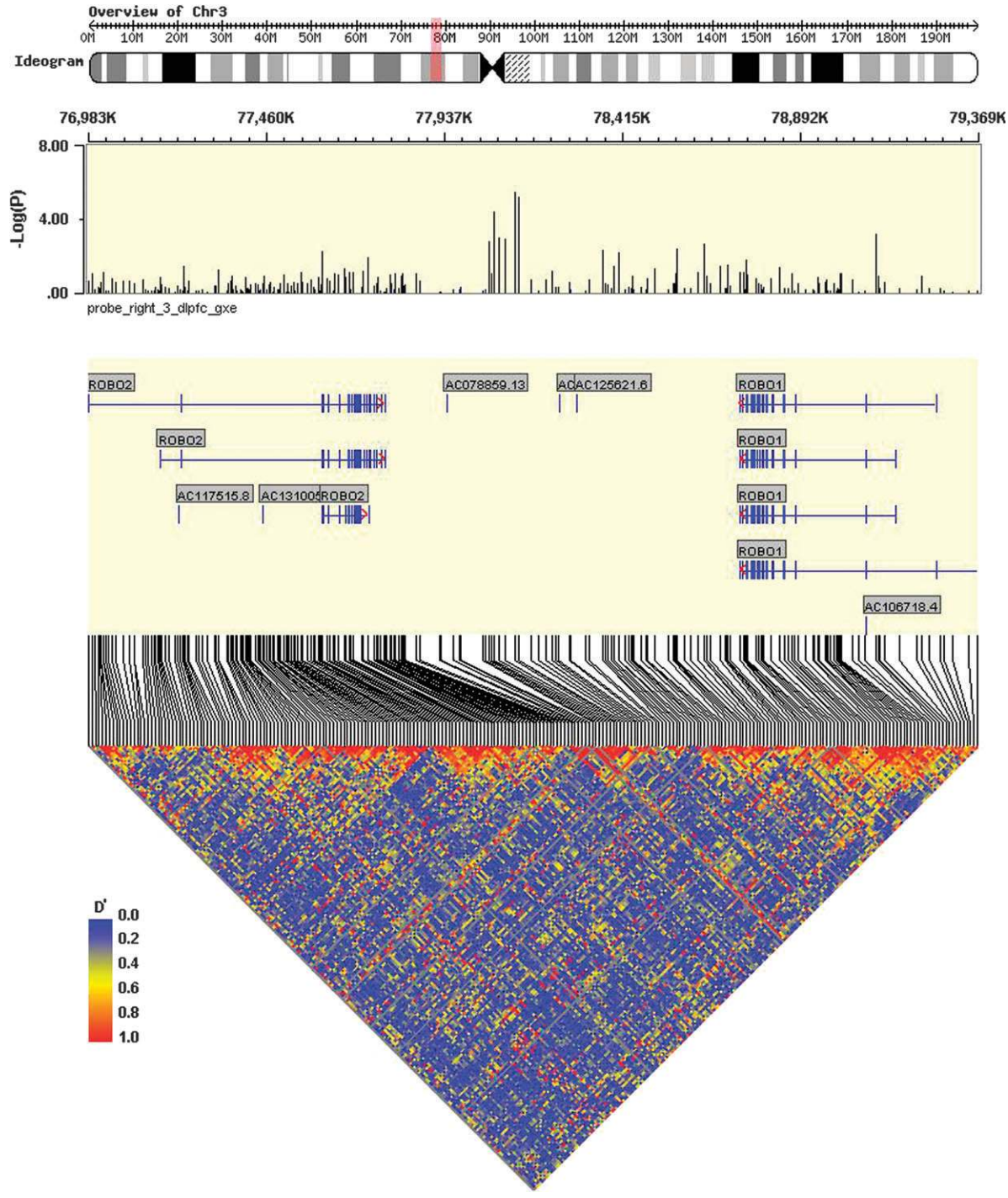

Fig. 1. Regions of the Genome Showing Evidence of Association in the Quantitative Trait (QT) analysis. Physical map of the single-nucleotide polymorphisms (SNPs) associated with (a) ROBO2-ROBO1 region, (b) TNIK gene, and (c) CTXN3-SLC12A2 region produced by WGAViewer.[54] The topmost sector is the ideogram of chromosomes 3 or 5; the vertical red line shows the relative of location of gene region. Below this is the graph showing the –log *P* significance values of the individual SNPs on the imaging phenotype (dorsolateral prefrontal cortex) for the right hemisphere. The small blue lines below this indicate the location of the exons in the transcripts annotated (translated region of the DNA). The vertical lines above the accompanying triangular matrix indicate the SNP locations and demonstrate the LD pattern between SNPs (D') calculated using Haploview 4·1.[89] The warmer colors on the flame scale indicate greater LD, while the blue indicates absence of LD.

build is not definitive, nor in some cases even known, and the possibility of finding additional genes especially in the intergenic areas is likely as the structure of the genome is more fully understood.[57–59] Finally, we must emphasize that the identified genes are candidates that require confirmation in an independent sample of schizophrenic subjects as well as in other illnesses such as bipolar disorder and autism to determine their specificity.

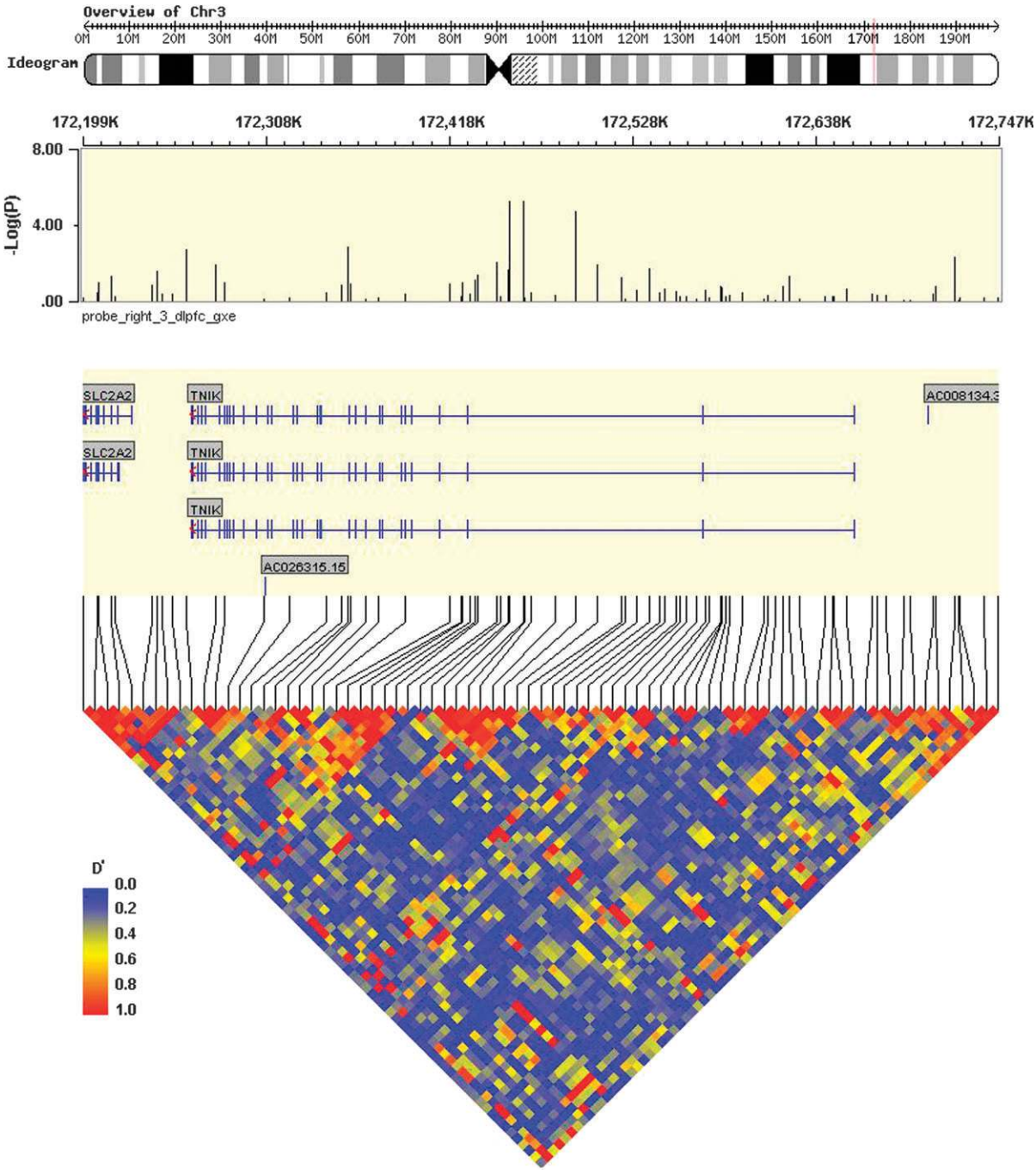

Fig. 1. Continued.

The 6 genes listed in table 2 form a multifaceted picture of forebrain development and stress response implicated in schizophrenia. A cluster of the most significant genes are involved in the development of cortex, particularly the forebrain and midline/callosal connections. While several of these genes have not previously been implicated in schizophrenia, they do support schizophrenia as a neurodevelopmental disorder, at least in part.

GPC1 (glypican, slit receptor) and ROBO2-ROBO1 are involved in dorsal forebrain development including neural precursor migration and axonal connectivity in the ipsilateral and contralateral hemisphere, eg, midline crossing and guidance of axons related to prefrontal cortices including DLPFC.[55,60] CTXN3 (cortexin) is a brain-specific integral membrane protein highly enriched in cortex. It is expressed in fetal brain and increases in density perinatally. *SLC12A2* found in the same region as *CTXN3* is another possible candidate gene. *SLC12A2* is involved in the regulation of GABA neurotransmission and has been shown to be differentially expressed in the

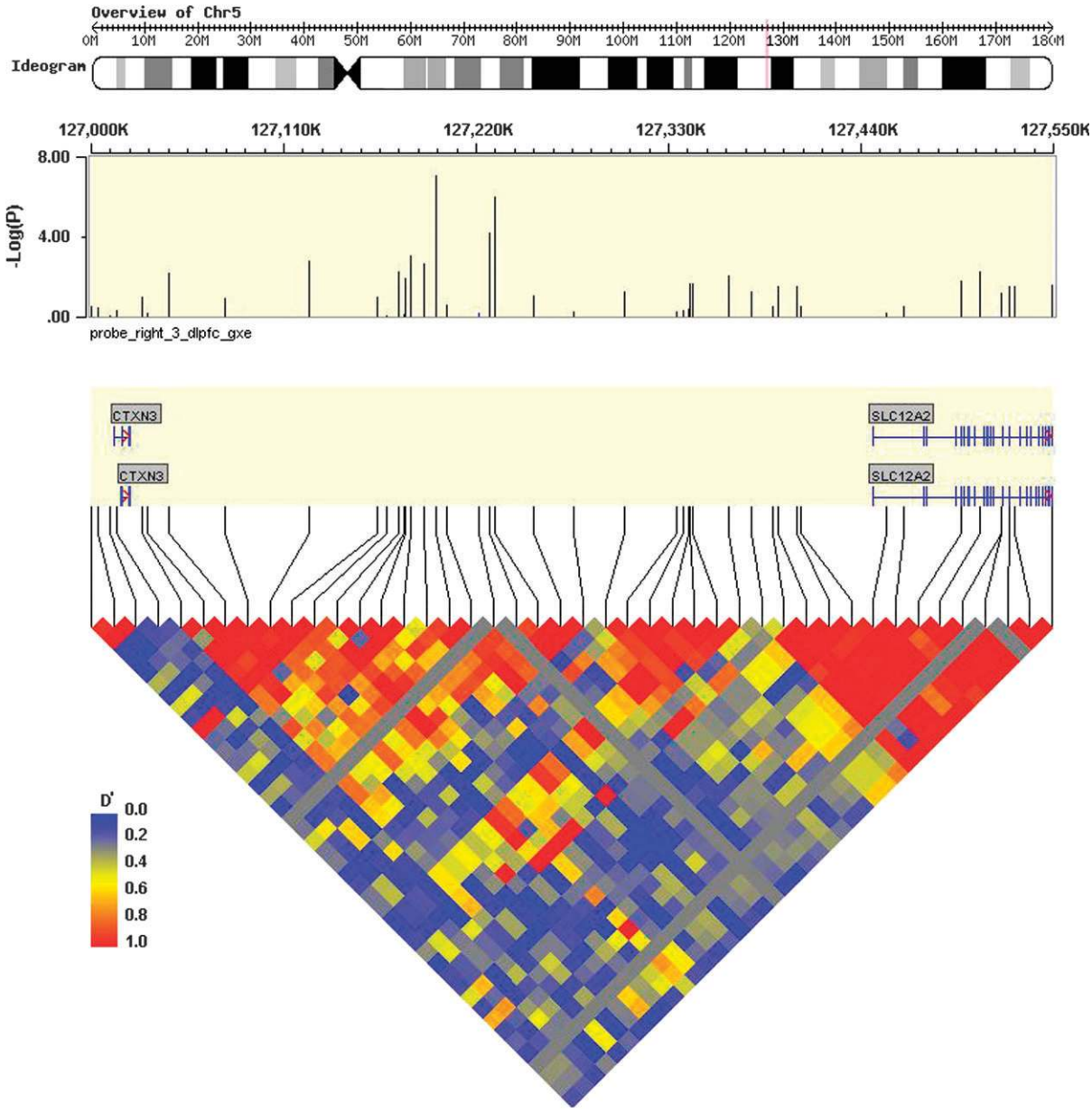

Fig. 1. Continued.

DLPFC of schizophrenic patients compared with controls.[61] None of these genes, however, has been identified as a risk gene for schizophrenia.[62]

While these genes have not been previously implicated in schizophrenia by other association studies, callosal morphometry, however, is known to be abnormal in schizophrenia[63] and ipsilateral and contralateral cortico-cortical and cortico-subcortical connectivity in general have been implicated[64–66] and could be affected by these genes. These findings are consistent with a wealth of literature implicating abnormal development and connectivity of the DLPFC in schizophrenia.[18,67–69] Importantly, the *CTXN3-SLC12A2* region found in our analysis is in the second most important region linked to schizophrenia in the meta-analysis by Lewis et al,[70] and it is on the boundary of the chromosome 5 region implicated in multiple cognitive measures in schizophrenia by Almasy et al.[30]

The significant SNPs in table 2 serve as tags or markers for possible causative risk genes for schizophrenia. Some of the SNPs such as those for the *ROBO2-ROBO1* region are actually in intergenic areas and may be reassigned to other genes. This may be the case for rs9836484 that could be more strongly associated with snRNA AC078859.13 (small nuclear RNA) or to the scRNA AC117462.5 (small cytoplasmic RNA) than to *ROBO1* or *ROBO2*. Even though the annotation of these 2 small noncoding RNA genes is not complete, it is possible that they serve as regulators for the transcription and subsequent expression of *ROBO1* and/or *ROBO2*.[71]

Table 2b. Additional Genes Idenitified by Having At least 1 Single-Nucleotide Polymorphism (SNP) at a 10^{-6} Significance Level and Putatively Belonging to a Pathway From a Gene in Table 2a

Chromosome	Gene	SNP	Location	Type	MAF_CTRL	MAF_SZ	*P* Value
3	ROBO2-ROBO1	rs7610746	78138637	Intergenic	0.31	0.41	7.56E-06
3	ROBO2-ROBO1	rs9836484	78127379	Intergenic	0.32	0.41	4.23E-06
3	TNIK	rs2088885	172453985	Intronic	0.47	0.45	6.24E-06
3	TNIK	rs7627954	172462669	Intronic	0.47	0.45	6.24E-06
5	CTXN3-SLC12A2	rs245178	127231091	Intergenic	0.32	0.30	1.22E-06
5	CTXN3-SLC12A2	rs245201	127197111	Intergenic	0.32	0.30	9.31E-08
2	GPC1	rs1574192	240957178	Intergenic	0.38	0.30	3.92E-06
6	POU3F2	rs9491640	99025985	Intergenic	0.06	0.02	9.23E-06
14	TRAF3	rs10133111	102447074	Downstream	0.20	0.21	4.77E-06

Three of these genes have functions related to the hypothalamus-pituitary-adrenal (HPA) stress axis. The HPA axis influences widespread responses to stress including modulating immune and inflammatory responses. DLPFC function is strongly influenced by prenatal (second trimester) and postnatal/adult stress,[72,73] and HPA axis is overactivated in schizophrenia, particularly in paranoid schizophrenia.[74] The 3 genes related to HPA function were *TRAF3*, *TNIK*, and *POU3F2*. TNIK is involved in responses to environmental stress, primarily through immediate early gene activation (JUN); in the adult, it enables changes in the responses of neurons due to stress and affect long-term potentiation in concert with *RAPT2*.[75] TRAF3 is a signal transducer in the TNF alpha, JNK, and NF-kappa-B cascades in T lymphocyte immune responses. POU3F2 is transcription factor that regulates genes associated with CRH and CRH promoters and affects cell survival and brain development via BRN-2 for differentiation of neuronal cells and transcription factors GLIS1. The significant SNP associated with *POU3F2* is approximately 200 Kb from the gene, and the minor allele was underrepresented in our sample. To address the allele distribution, we performed an analysis using the dominant model and found three *POU3F2* SNPs to be significant at 10^{-6}.

Exposure to stress exacerbates schizophrenic symptoms and causes marked DLPFC cortical dysfunction. Hains and Arnstein[76] suggest that patients with serious mental illness have weaker endogenous regulation of stress pathways possibly related to DISC1 and RGS4. DISC1 (disrupted in schizophrenia) normally regulates cAMP, and RGS4 (regulator of G protein signaling) inhibits phosphatidyl inositol protein kinase C intracellular signal. Cortisol released during stress binds to receptors in the cortex as well as in the HPA. Cortisol inhibits COMT (an enzyme which degrades dopamine) in the cortex,[77,78] leading to increased extracellular dopamine that can disrupt prefrontal functioning. Schizophrenic subjects with the met-met form of COMT may be especially vulnerable to such disruption.[31,79–83]. This example demonstrates how genetic vulnerability and stress could converge to disrupt DLPFC functioning, creating impaired working memory and other psychiatric symptoms.

A limitation of our study is the sample size and lack of availability of an independent sample for replication. Given the costs and logistic issues, it is not surprising that such replication samples are not yet available, although one is underway in Norway (TOP Project, Institute of Psychiatry, University of Oslo, Oslo, Norway).

There are 6 published GWASs in schizophrenia.[6,30,84–87] Each of these GWAS has identified 1 or 2 genes (SNPs) that have passed genome wide significance. These studies except for the Kirov et al study did not use the SNP panels that we studied, therefore severely limiting comparisons. In the published reports and their supplemental tables, there is no information regarding our top SNPs, and these studies did not provide raw data or *P* values in their tables or supplemental tables for other SNPs representing our top genes. The Kirov study also did not provide raw data or *P* values below their genome-wide threshold of significance. Such values are necessary to confirm our findings.

Our sample only contained chronic schizophrenia patients and therefore may not fully representative of the disease. Also, the FBIRN sample was not an epidemiological sample, further limiting its generalizability. The small sample size was somewhat mitigated by the increase in statistical power of a QT design over a categorical case-control analysis.[88] Nevertheless, the problem of false positives remains. There is no established method for determining appropriate statistical threshold values for an interaction term (ie, SNP × diagnosis) in a QT analysis in the context of a genome-wide scan. Our solution was to focus on loci identified by at least 2 independent SNPs with a $P < 10^{-6}$ because the conjunction of these results is less likely than a single result alone. This is in keeping with the WTCCC and O'Donovan et al,[56] considering genome-wide thresholds of $P < 10^{-5}$ and 10^{-7} as "moderately strong" and "strong" evidence for an association.

Other loci identified by a single SNP at $P < 10^{-6}$ (of which there were 6 originally) were included if their biological plausibility was supported through a putative relationship to any of the 3 most significant loci. In the supplemental tables, we report the full set of SNPs and significance values within each significant loci. This allows the reader to consider our preliminary results in terms of their statistical and biological consistency given the multiple testing issues. This is similar to the strategy used by Almasy et al[30] in addressing the issue of corrections for multiple tests.

Another issue to be addressed is the phenotype. DLPFC activation during a working memory task was chosen as our quantitative phenotype because it is a well-documented characteristic of schizophrenia. In this sample, abnormal activation in the DLPFC as measured by BOLD was observed between schizophrenia and controls. This was most prominent when testing the 3 item condition of the SIRP working memory task. This difference in activation was bilateral and not a consequence of performance. Identifying genes that contribute to this difference in activation has the potential to identify novel genes and pathways involved in working memory dysfunction in schizophrenia. It is possible that other brain imaging phenotypes would identify other candidate genes.

The approach described is a screening method that makes GWAS data usable and exploratory in preparation for future studies, eg, molecular studies, expression, and transgenic studies, and all other functional genomic approaches. It allows for completely novel SNPs to be identified as playing a role in the disease phenotype.

Conclusion

In summary, we report data from a whole-genome association study using a well-studied cohort of chronic schizophrenia patients and matched controls. In contrast to a case-control analysis, we used a QT design, in which DLPFC activation during a working memory task was the QT. The QT analysis has considerably greater power than a case-control design. The goal of this preliminary analysis was to identify unanticipated risk genes for the development of schizophrenia. The genes identified require confirmation in an independent sample. This method of gene discovery complements other established strategies such as case-control designs with large sample sizes, family-based design, and targeted candidate gene approaches.

Supplementary Material

Supplementary tables 1–6 are available at http://schizophreniabulletin.oxfordjournals.org.

Funding

This research was supported by grants to The Transdisciplinary Imaging Genetics Center (TIGC-P20 RR020837-01) and to the FBIRN (1 U24 RR021992) from the National Center for Research Resources at the National Institutes of Health.

Acknowledgments

We acknowledge the help and support of Mita Mancini and Yann Legros from Illumina, as well as Cristina Barlassina, Chiara Dal Fiume, Alessandro Orro, and Federica Torri (University of Milan) for performing the HumanHap 300 Bead Array procedures. We also acknowledge the editorial support of Liv McMillan and Divya Rajpoot.

Authorship contributions: The inclusion of the FBIRN as an author represents the efforts of many otherwise unlisted researchers over the years who also had explicit input into the conception, design, and implementation of the work. The following authors conceived, designed, and/or implemented the SIRP and experiment and/or facilitated data acquisition or data sharing for that experiment and manuscript review: Ford, Lauriello, Mathalon, Turner, and Potkin.

The following authors contributed to genetic analysis and interpretation of the imaging genetics and writing the manuscript: Potkin, Turner, Lakatos, Fallon, Guffanti, and Macciardi.

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