Schizophrenia Bulletin vol. 41 no. 4 pp. 909–918, 2015
doi:10.1093/schbul/sbu171
Advance Access publication December 22, 2014

Glutamate Networks Implicate Cognitive Impairments in Schizophrenia: Genome-Wide Association Studies of 52 Cognitive Phenotypes

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Cognitive impairments are a core feature in patients with schizophrenia. These deficits could serve as effective tools for understanding the genetic architecture of schizophrenia. This study investigated whether genetic variants associated with cognitive impairments aggregate in functional gene networks related to the pathogenesis of schizophrenia. Here, genome-wide association studies (GWAS) of a range of cognitive phenotypes relevant to schizophrenia were performed in 411 healthy subjects. We attempted to replicate the GWAS data using 257 patients with schizophrenia and performed a meta-analysis of the GWAS findings and the replicated results. Because gene networks, rather than a single gene or genetic variant, may be strongly associated with the susceptibility to schizophrenia and cognitive impairments, gene-network analysis for genes in close proximity to the replicated variants was performed. We observed nominal associations between 3054 variants and cognitive phenotypes at a threshold of $P < 1.0 \times 10^{-4}$. Of the 3054 variants, the associations of 191 variants were replicated in the replication samples ($P < .05$). However, no variants achieved genome-wide significance in a meta-analysis ($P > 5.0 \times 10^{-8}$). Additionally, 115 of 191 replicated single nucleotide polymorphisms (SNPs) have genes located within 10 kb of the SNPs (60.2%). These variants were moderately associated with cognitive phenotypes that ranged from $P = 2.50 \times 10^{-5}$ to $P = 9.40 \times 10^{-8}$. The genes located within 10 kb from the replicated SNPs were significantly grouped in terms of glutamate receptor activity (false discovery rate (*FDR*) $q = 4.49 \times 10^{-17}$) and the immune system related to major histocompatibility complex class I (*FDR* $q = 8.76 \times 10^{-11}$) networks. Our findings demonstrate that genetic variants related to cognitive trait impairment in schizophrenia are involved in the *N*-methyl-D-aspartate glutamate network.

Key words: schizophrenia/genome-wide association study/cognitive phenotypes/glutamate receptor activity/immune function/functional gene network

Introduction

Schizophrenia is a common and complex psychiatric disorder characterized by clinical and genetic heterogeneity; the lifetime risk of schizophrenia is approximately 1%. This disorder has a strong genetic component, and the estimated heritability is 81%.[1] Genome-wide association studies (GWAS) that examine hundreds of thousands of single nucleotide polymorphisms (SNPs) could be powerful tools to identify common susceptibility variants for complex traits, including schizophrenia and cognitive phenotypes. Several large-scale GWAS on schizophrenia have successfully identified several genome-wide significant risk variants located in the *ZNF804A*, *NRGN*, and *TCF4* genes, *MIR137*, and a major histocompatibility complex (MHC) region.[2–4] In addition, the latest GWAS in Schizophrenia Working Group of the Psychiatric Genomics Consortium have identified several SNPs in 108 independent loci including above regions.[5] These loci harbor over 100 candidate genes. However, each risk

genetic variant for schizophrenia only has small effects, with odds ratios that range from 1.06 to 1.32. Polygenic schizophrenia risk scores with the additive effects of many SNPs that have a small effect from GWAS data explained only ~20% of the variance in the disease status.[5,6] Although it is commonly accepted that the risk for developing schizophrenia is mediated by many genes or genetic variants, previous findings of GWAS on schizophrenia only explain a small aspect of the genetic architecture of this disorder. To solve this problem and to minimize genetic heterogeneity, intermediate phenotypes, such as cognitive traits, rather than the diagnosis of schizophrenia are emphasized.

Cognitive impairments are a core feature and reasonable treatment target of this illness, and they contribute to social dysfunction and life outcomes.[7] Substantial evidence suggests that most cognitive functions have a genetic basis and are heritable (h^2 = 0.33–0.85).[8–11] The cognitive impairments include problems in processing speed, attention/vigilance, working memory, verbal learning, visual learning, reasoning and problem solving, social cognition, and general intelligence; these impairments are typically stronger in patients with schizophrenia,[12] and dysfunctions have also been observed in unaffected relatives or unaffected twin siblings of schizophrenia patients.[13] A substantial portion of the phenotypic correlation between schizophrenia and cognitive traits is caused by identical genetic effects,[13] although not all genes that affect susceptibility to schizophrenia affect all cognitive phenotypes. Therefore, cognitive traits have been proposed as useful intermediate phenotypes to understand the genetic mechanisms implicated in the pathophysiology of schizophrenia. To date, several GWAS that investigated cognitive phenotypes impaired in schizophrenia, such as general cognitive ability, executive function, processing speed, and verbal fluency, have been conducted in other cohorts that did not include patients with schizophrenia.[14–18] These studies reported that no single variant was associated with any of the cognitive phenotypes at the level of genome-wide significance despite the large number of subjects. Several studies have indicated that a substantial proportion of the variances in cognitive ability are because of several common genetic variants with small effects.[14,18] Polygenic cognitive scores have been associated with a risk of schizophrenia, whereas polygenic schizophrenia risk scores have been associated with lower cognitive ability.[17] However, these studies that used cognitive phenotypes did not use schizophrenia samples because the cognitive functions of the patients had potential confounding factors related to the illness process, state, and medications. These studies focused on a limited number of cognitive phenotypes. Recently, to explain the genetic architecture in cognitive functions, a hypothesis-driven gene-network analysis was performed, which examined whether genetic variants in a hypothesis-driven network showed a greater association with cognitive phenotypes compared with variants outside the hypothesis-driven network.[19] The study determined that genetic variants in the genes that encode the *N*-methyl-D-aspartate (NMDA)/membrane-associated guanylate kinase receptor complex were enriched by the association with intelligence. According to previous findings, we hypothesized that it would be difficult to detect a genome-wide significant SNP related to each cognitive phenotype using relative small samples, but genetic variants detected through a "hypothesis-free" GWAS approach that tested a broad range of cognitive phenotypes at a suggestive level of significance might be aggregated in gene networks related to the pathophysiology of schizophrenia, eg, glutamate or immune functions. The study consisted of 4 stages. First, we performed discovery GWAS of a large battery of cognitive phenotypes impaired in schizophrenia using healthy subjects. Second, we attempted to replicate the associations between the prominent SNPs in the discovery GWAS and the cognitive phenotypes in schizophrenia patients. Third, a meta-analysis of the associations between the discovery GWAS and the replication results was performed. Finally, to obtain better understanding of schizophrenia and its cognitive impairments, we searched the functional gene network of a set of genes located within 10 kb from the replicated SNPs that were connected to the cognitive phenotypes.

Materials and Methods

Subjects

The subjects for the discovery GWAS consisted of 411 healthy subjects (49.9% males, 205 males and 206 females, mean age ± SD, 36.3 ± 12.8 years). The subjects for the replication analysis consisted of 257 patients with schizophrenia (53.3% males, 137 males and 120 females, mean age ± SD, 36.5 ± 12.3 years). Demographic information is shown in supplementary table 1. The subjects were biologically unrelated within the second degree of relationship and were of Japanese descent.[20,21] The subjects were assessed and diagnosed as previously described.[22] The participants provided written informed consent after the study procedures had been fully explained. This study was performed in accordance with the World Medical Association's Declaration of Helsinki and was approved by the Research Ethical Committee of Osaka University.

Cognitive Phenotypes

To assess general intellectual abilities, memory function, executive function, speed of processing, verbal learning and memory, attentional function, and social cognition, which are impaired in schizophrenia, 52 cognitive phenotypes were obtained by the Wechsler Adult Intelligence Scale-third edition (WAIS-III), the Wechsler Memory Scale-Revised (WMS-R), the Wisconsin Card Sorting Test (WCST), the Verbal Fluency Test (VFT), the Rey Auditory Verbal Learning Test (AVLT), the Continuous

Performance Test-Identical Pairs version (CPT-IP), and the Facial Emotion Labeling Test (FELT). The list and details of each cognitive test are provided in the supplementary table 2 and supplementary methods. Although we attempted to examine all phenotypes from all subjects, complete findings from all phenotypes were not obtained for all subjects. The reason why the whole cognitive battery was not completed by all individuals is because these data were obtained from subjects in each daily medical treatment and some cognitive tests were started in the middle of the overall study. Each test was administered as follows: WAIS-III, 393 controls and 182 patients; WMS-R, 410 controls and 231 patients; AVLT, 410 controls and 191 patients; VFT, 408 controls and 213 patients; CPT, 411 controls and 202 patients; WCST, 303 controls and 171 patients; and FELT, 342 controls and 191 patients.

Genotyping

The genotyping was performed using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix) according to the manufacturer's protocol. The genotypes were obtained from the CEL files using Birdseed v2 for the 6.0 chip implemented in Genotyping Console software (Affymetrix). Quality control (QC) was performed as previously described.[23,24] The details of the QC are provided in the supplementary methods. After applying the QC, 517 946 SNPs remained for the GWAS.

Statistical Analyses

The statistical analyses of the demographic variables were performed using PASW Statistics 18.0 software (SPSS Japan, Inc.). Based on the assumption that most demographic variables, such as age and years of education, and cognitive phenotypes were not fitted to a normality distribution with the Kolmogorov-Smirnov test ($P < .05$), the differences in the continuous variables, such as age and years of education, were analyzed using the nonparametric Mann-Whitney U test; the differences in the categorical variables, such as the gender and ratios of the SNPs, were analyzed using the Pearson's chi-square or Fisher's exact tests. The correlations among the cognitive phenotypes were assessed using nonparametric Spearman's correlation coefficient. We performed multiple linear regression analyses with each cognitive phenotype as a dependent variable and the additive dosage of each SNP (the number of major alleles: 0, 1, or 2) as the independent variables in the discovery GWAS and in the replication samples using PLINK 1.07 software. Gender, the years of education, and age (if the phenotype scores were not adjusted for age) were included as the covariates. We set an arbitrary a priori P value of $<1.0 \times 10^{-4}$ to avoid type II errors and to obtain as many SNPs as possible for the discovery GWAS. We extracted the SNPs that attained a P value of $<1.0 \times 10^{-4}$ in the discovery GWAS and performed a replication analysis of the extracted SNPs in the replication samples. For the replication analysis, we applied 1-tailed $P < .05$ based on a hypothesis that the directionally associated SNPs were identical to the SNPs detected in the discovery GWAS. A meta-analysis of the regression estimates (β) and the SEs between the discovery GWAS and the replication samples was undertaken for each SNP that reached a significance threshold of $P < .05$ in the replication analysis, based on the inverse-variance weighted-effect-size approach as implemented in the METASOFT program.[25] Cochran's chi-square-based Q statistical test was performed to assess the possible heterogeneity between the sample sets. The fixed-effect model was applied in the absence of heterogeneity ($P > .05$). The SNP annotations and identification of the genes within 10 kb or the closest gene to the detected SNP were confirmed by the SNP info web server[26] and the dbSNP (http://www.ncbi.nlm.nih.gov/snp). A functional gene-network analysis was performed using GeneMANIA software (Multiple Association Network Integration Algorithm; http://www.genemania.org/), which was used to discover the following: to identify whether there are known interactions between the query genes and, if so, the mechanism for their interaction; to add extra genes strongly connected to the query genes; to search for genetic, physical pathway and co-expression networks; and to identify functional gene networks.[27] To create the final composite network, this software uses 6 main sources: IRefIndex and BioGRID for physical interactions and genetic interactions; Gene Expression Omnibus for co-expression networks; InterPro via Ensemble for protein domains; I2D for networks of interologs of physical interactions; and their own manual curation efforts.[27] We used query-dependent weighting method (a default setting) to weight networks. The functional gene-networks analysis tests the query plus the related genes for enrichment of a selection of Gene Ontology (GO) term annotations against the background of all genes in human with any GO annotation. For the calculations of the gene networks, the genes within 10 kb from the detected SNPs were used as the query genes. Based on evidence for 108 susceptible loci for schizophrenia harbor over 100 genes[5] and our hypothesis that schizophrenia is complex disorder and multiple genes with interactions would contribute to pathogenesis of this disorder, additional 100 genes related to the query genes were used. These 100 related genes were identified using available genomics and proteomics data as described above. When testing GO enrichment in the gene-network analysis, we applied Benjamini-Hochberg false discovery rate (FDR) $q < 0.05$ to control multiple testing.

Results

Stage 1: The GWAS on 52 Cognitive Phenotypes in Healthy Subjects

A flow chart of this study is presented in figure 1. We used 52 cognitive phenotypes that focused on general intellectual ability, memory, executive function, processing

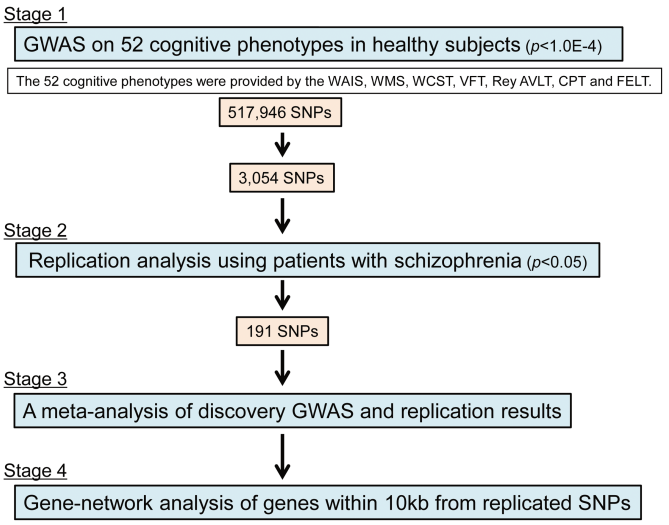

Fig. 1. A flow chart of this study.

speed, verbal learning and memory, and attentional and social cognitive functions. As expected, all of the cognitive phenotypes used in the current study were impaired in patients with schizophrenia and these phenotypes were significantly correlated with each other, as shown in supplementary table 2 and supplementary figure 1, respectively. We first performed GWAS on the comprehensive cognitive phenotypes in the discovery samples. The Quantile-Quantile and Manhattan plots for each phenotype in the samples are shown in supplementary figure 2. We observed associations between 3054 variants and the cognitive phenotypes at a suggestive threshold level of $P < 1.0 \times 10^{-4}$. The top 20 SNPs are shown in table 1. Of these SNPs, the strongest association was observed on the Visual Paired Associates II (ViPA2) of WMS-R at rs10757641, an intronic SNP in the TEK tyrosine kinase, endothelial (*TEK*) gene on 9p21.2 ($P = 3.62 \times 10^{-10}$). We applied Bonferroni correction ($\alpha = 5.0 \times 10^{-8}/52$) to control multiple testing although this correction might be conservative because these phenotypes were not independent as shown in supplementary figure 1. The result still showed suggestive association after applying the correction ($P = 1.88 \times 10^{-8}$). Although it is known that the encoded protein mediates the signaling pathway that functions in embryonic vascular development, the effect of the gene on cognitive function is unknown. Fourteen closest genes (*ZNF804A*, *MAD1L1*, *CNTN4*, *VRK2*, *MRPS21*, *PAK6*, *DISP2*, *ZNF536*, *CSMD1*, *GRIN2A*, *PRICKLE2*, *TMTC1*, *FAM5B*, and *KCNB1*) of the 3054 SNPs have been associated with schizophrenia in 108 loci of the latest GWAS.[5] The numbers of the suggestive SNPs at the threshold level of $P < 1.0 \times 10^{-4}$ did not concentrate solely on a particular cognitive phenotype ($P > .05$, supplementary figure 3); they were scattered in each phenotype and ranged from 17 SNPs for Comprehension in the WAIS-III to 107 SNPs for ViPA2 in the WMS-R, indicating that certain cognitive phenotypes did not have significantly more or less SNP hits than others.

Stage 2: Replication Analysis Using Patients With Schizophrenia

We attempted to replicate the associations between the 3054 genetic variants detected in the discovery GWAS and the cognitive phenotypes in 257 schizophrenia patients. Of the 3054 SNPs, we replicated the associations between 191 SNPs and the cognitive phenotypes in the second-stage samples ($P < .05$). The replicated SNPs accounted for 6.3% of the discovery SNPs (191/3054). However, the top 20 SNPs did not cluster the higher end of the discovery SNPs, as shown in supplementary table 3. None of the top 20 SNPs at the first stage, as listed in table 1, was replicated ($P > .05$). As expected, some of the replicated SNPs were associated with several phenotypes (supplementary figure 4), eg, rs10741845 located on chromosome 11 was associated with several phenotypes, such as general memory, verbal memory, and logical memory I and II on the WMS-R. Twenty-one SNPs were associated with multiple phenotypes. The highest association was observed for Symbol Search on the WAIS-III at rs4315839, located 1.9 kb upstream of the cyclin-dependent kinase inhibitor 2A interacting protein (*CDKN2AIP*) gene on 4q35.1 ($P = 3.33 \times 10^{-4}$). This gene is a vital dual regulator of cellular senescence and apoptosis.[28] Because the SNP is located on the 5′ side of the *CDKN2AIP* gene, we searched for potential transcription factor binding sites in sequences that included rs4315839 with the pattern search program (P-Match 1.0) using TRANSFAC 6.0 public sites (http://www.gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi). This SNP may be related to the regulation of gene transcription (core score = 0.963, matrix score = 0.955). The c-Rel binding site was altered by a single nucleotide change; the sequence GTACTCCA**C**C (C allele is a risk allele for lower cognitive function) is a c-Rel binding site, whereas the sequence GTACTCCA**T**C is not a c-Rel binding site. This alteration could lead to dysregulation of the transcriptional activity of the *CDKN2AIP* gene and cause apoptosis.

Stage 3: A Meta-analysis That Combined the Discovery GWAS and Replication Results

We performed a meta-analysis that combined the discovery GWAS and the replication associations of the replicated 191 SNPs with the cognitive phenotypes. Of these SNPs, the SNPs that attained $P < 1.0 \times 10^{-6}$ and all SNPs are shown in table 2 and supplementary table 4, respectively. The strongest SNP in the meta-analysis was identical to the highest replicated SNP, rs4315839, located upstream of the *CDKN2AIP* gene, for Symbol Search on the WAIS-III ($P = 9.40 \times 10^{-8}$). Next, we investigated

Table 1. Top 20 SNPs Detected in the Discovery GWAS

					Healthy Subjects				Gene	
Phenotypes	Chr	SNP	Bp	m	*N*	β	SE	*P*	±10 kb	Closest
ViPA2	9p21.2	rs10757641	27212360	C	407	−0.34	0.05	**3.62E-10**	*TEK*	*TEK*
ViPA2	9p21.2	rs633903	27196119	G	410	−0.30	0.05	**8.28E-09**	*TEK*	*TEK*
D′2	10p15.3	rs2813400	1642998	C	410	−0.21	0.04	**1.50E-08**	*ADARB2*	*ADARB2*
PEM	5q32	rs17108927	148286317	C	300	2.46	0.43	**2.20E-08**	—	*ADRB2*
Info	3p14.3	rs1526590	54916350	C	401	−0.41	0.07	**2.39E-08**	*CACNA2D3*	*CACNA2D3*
Picture Arrangement	2q12.1	rs6731116	104908503	C	391	−1.30	0.23	6.09E-08	—	*POU3F3*
D′2	10p15.3	rs7898120	1643368	A	411	−0.19	0.03	6.90E-08	*ADARB2*	*ADARB2*
FIQ	12q23.1	rs11108839	97534882	G	392	−7.42	1.35	7.03E-08	—	*NEDD1*
FIQ	12q23.1	rs17026471	97519429	A	384	−7.39	1.35	7.26E-08	—	*NEDD1*
VIQ	12q23.1	rs11108839	97534882	G	392	−7.78	1.42	8.51E-08	—	*NEDD1*
CA	2p16.1	rs12612495	57941597	T	303	−0.69	0.13	1.04E-07	—	*VRK2*
Picture Arrangement	2q12.1	rs7582242	104917154	G	385	−1.27	0.24	1.10E-07	—	*POU3F3*
VIQ	12q23.1	rs17026471	97519429	A	384	−7.69	1.42	1.13E-07	—	*NEDD1*
ViR1	6q16.1	rs9345419	94720479	A	396	1.10	0.21	1.42E-07	—	*EPHA7*
ViPA2	9p21.2	rs581724	27187422	T	407	−0.28	0.05	1.44E-07	*TEK*	*TEK*
Info	3p14.3	rs10510775	54928174	A	410	−0.37	0.07	1.49E-07	*CACNA2D3*	*CACNA2D3*
FIQ	12q23.1	rs2193371	97523634	T	391	−7.27	1.36	1.50E-07	—	*NEDD1*
PEM	12q24.23	rs474932	119950367	T	301	1.82	0.34	1.66E-07	*CCDC60*	*CCDC60*
ViPA2	12q24.31	rs7959363	125108927	G	408	−0.40	0.08	1.80E-07	—	*NCOR2*
VerPA1	1q42.12	rs6426075	225637606	A	410	−1.05	0.20	1.93E-07	—	*LBR*

Note: Chr, chromosome; Bp, nucleotide location; m, minor allele; SE, standard error of beta. See the supplementary table 2 for abbreviations of each phenotype. *P* values of <5.0E-08 are shown in boldface and underlined.

the locational characteristics of the 191 replicated SNPs identified at stage 2 compared with the 3054 discovery GWAS SNPs detected at stage 1 (supplementary table 5). The ratio of the SNPs that had a gene within 10 kb from the detected SNP was higher in the replicated SNPs (115/191: 60.2%) compared with the discovery GWAS SNPs (1462/3054: 47.9%) ($\chi^2 = 10.95$, $P = 9.34 \times 10^{-4}$). There were no differences in the ratios of the intragenic SNP, the SNP located on the TBFS, the nonsynonymous SNP, the synonymous SNP, or the SNP located on 3′UTR between the replicated and discovery SNPs ($P > .05$).

Stage 4: Gene-Network Analysis

Based on the evidence for SNPs located within 10 kb of genes show abundance of associations with phenotypes compared with intergenic SNPs[29] and our finding that SNPs having genes within 10 kb from the SNP were assembled in the replicated SNPs (115/191), we hypothesized that the genes located within 10 kb on either side of the replicated SNPs would be aggregated in functional networks related to the pathophysiology of schizophrenia. The replicated 115 SNPs had 59 genes located within 10 kb from the SNP sites. By analyzing these genes as query genes using the GeneMANIA web server (figure 2), 2 prominent gene networks, the "glutamate receptor activity" (red circles; Coverage [numbers of genes in the network with a given function/all genes in the genome with the function]: 12/20, $FDR\ q = 4.49 \times 10^{-17}$) and the "antigen processing and presentation of exogenous peptide antigen via MHC class I (MHCI), transporter associated with antigen processing (TAP) dependent" (blue circles; Coverage: 13/75, $FDR\ q = 8.76 \times 10^{-11}$), as well as 2 marginal gene networks, "phospholipase activity" (orange circles; Coverage: 6/55, $FDR\ q = 1.30 \times 10^{-3}$) and "RNA polymeraseII regulatory region sequence-specific DNA binding" (purple circles; Coverage: 5/60, $FDR\ q = 2.80 \times 10^{-2}$), were observed. Glutamate receptor activity and the immune system related to MHCI have repeatedly been linked to the pathophysiology of schizophrenia, as well as to cognitive functions.[19,30]

Discussion

To our knowledge, GWAS in a broad range of cognitive phenotypes in healthy subjects remain to be examined. There is no replication analysis of the GWAS findings in schizophrenia patients. This study is the first analysis to detect functional gene networks accumulated by genetic variants related to cognitive impairments in schizophrenia through comprehensive GWAS on a range of cognitive phenotypes. The genes in close proximity to the replicated SNPs relevant to cognitive impairments in schizophrenia showed a strong association with the glutamate network.

We did not identify a genome-wide significant variant related to cognitive phenotypes in the meta-analysis.

Table 2. The SNP List of the Meta-analysis That Combined Discovery GWAS and the Replication Results

					Healthy Subjects			Schizophrenia			Meta-analysis				Gene	
Phenotypes	Chr	SNP	Bp	m	*N*	β	*P*	*N*	β	*P*	*N*	P_{hetero}	β	*P*	±10 kb	Closest
Symbol Search	4q35.1	rs4315839	184363856	C	389	−0.86	4.44E-05	182	−1.15	3.30E-04	571	0.46	−0.94	9.20E-08	*CDKN2AIP*	*CDKN2AIP*
LM1	11p15.1	rs10741845	20577844	A	408	−2.36	6.18E-06	231	−2.33	3.60E-03	639	0.97	−2.35	1.03E-07	—	*SLC6A5*
PO	1q41	rs2577138	220205980	A	385	−7.33	1.22E-05	179	−11.34	1.10E-03	564	0.32	−8.01	1.04E-07	*EPRS*	*EPRS*
ViR2	6p22.3	rs1555108	22694829	G	410	1.42	2.33E-06	231	2.45	7.30E-03	641	0.32	1.51	1.20E-07	—	*HDGFL1*
ViR2	6p22.3	rs9466427	22660332	T	396	1.49	2.58E-06	230	2.51	7.70E-03	626	0.34	1.57	1.32E-07	—	*HDGFL1*
VerM	11p15.1	rs10741845	20577844	A	408	−4.68	1.28E-05	231	−4.96	3.10E-03	639	0.89	−4.75	1.90E-07	—	*SLC6A5*
FM	3p11.1	rs12492805	88298205	G	403	0.55	6.16E-06	228	0.52	8.60E-03	631	0.90	0.54	2.30E-07	—	*C3orf38*
Block Design	13q13.3	rs1556060	36709477	T	392	1.36	1.02E-05	181	1.46	5.70E-03	573	0.88	1.38	2.63E-07	*DCLK1*	*DCLK1*
VFT-Category	3p26.3	rs10510211	1589530	C	406	1.63	2.35E-06	213	1.07	2.50E-02	619	0.38	1.47	3.37E-07	—	*CNTN6*
FM	13q21.2	rs4886229	61003863	C	400	−0.46	1.91E-05	225	−0.50	3.70E-03	625	0.83	−0.47	3.45E-07	*TDRD3*	*TDRD3*
PO	1q41	rs7545314	220181584	T	392	−7.60	7.88E-06	180	−8.11	1.00E-02	572	0.90	−7.70	3.55E-07	*EPRS*	*EPRS*
Picture Completion	7q22.3	rs10953515	106293682	A	392	−1.06	2.80E-06	181	−0.81	3.10E-02	573	0.60	−1.01	3.66E-07	*CCDC71L*	*CCDC71L*
PO	1q41	rs2789809	220185048	C	391	−7.51	7.44E-06	182	−7.90	1.20E-02	573	0.92	−7.58	3.78E-07	*EPRS*	*EPRS*
FIQ	16p12.1	rs974474	26803575	G	387	−3.73	7.86E-06	172	−4.36	1.10E-02	559	0.76	−3.83	3.85E-07	—	*C16orf82*
ViR2	6p22.3	rs9348530	22656971	G	408	1.36	8.40E-06	231	2.55	6.80E-03	639	0.27	1.46	4.87E-07	—	*HDGFL1*
PO	1q41	rs2789812	220190788	G	393	−7.32	1.11E-05	181	−8.07	1.00E-02	574	0.84	−7.45	5.10E-07	*EPRS*	*EPRS*
PO	1q41	rs7550249	220188195	T	391	−7.39	8.99E-06	181	−7.75	1.30E-02	572	0.93	−7.46	5.12E-07	*EPRS*	*EPRS*
ViPA2	15q21.3	rs12591795	53565627	G	404	−0.29	3.36E-06	230	−0.32	4.20E-02	634	0.87	−0.29	5.13E-07	—	*WDR72*
ViR2	6p22.3	rs6905924	22655599	T	410	1.37	8.40E-06	231	2.50	7.70E-03	641	0.29	1.46	5.16E-07	—	*HDGFL1*
PO	1q41	rs10779396	220188436	A	393	−7.32	1.11E-05	182	−7.90	1.20E-02	575	0.88	−7.42	5.76E-07	*EPRS*	*EPRS*
PO	1q41	rs2577136	220208049	C	393	−7.32	1.11E-05	182	−7.90	1.20E-02	575	0.88	−7.42	5.76E-07	*EPRS*	*EPRS*
PO	1q41	rs2647422	220177352	C	393	−7.32	1.11E-05	182	−7.90	1.20E-02	575	0.88	−7.42	5.76E-07	*EPRS*	*EPRS*
PO	1q41	rs2647447	220175777	T	393	−7.32	1.11E-05	182	−7.90	1.20E-02	575	0.88	−7.42	5.76E-07	*EPRS*	*EPRS*
PO	1q41	rs2647449	220176963	A	393	−7.32	1.11E-05	182	−7.90	1.20E-02	575	0.88	−7.42	5.76E-07	*EPRS*	*EPRS*
ViR2	6p22.3	rs9358578	22702647	A	395	1.35	1.44E-05	225	2.64	4.00E-03	620	0.21	1.47	6.01E-07	—	*HDGFL1*
PO	1q41	rs2647420	220179322	A	391	−7.34	1.21E-05	179	−7.90	1.20E-02	570	0.88	−7.44	6.26E-07	*EPRS*	*EPRS*
PIQ	1q41	rs2577138	220205980	A	385	−6.55	3.89E-05	179	−10.31	2.10E-03	564	0.33	−7.17	6.29E-07	*EPRS*	*EPRS*
VerPA2	1p21.2	rs11576210	99992372	T	408	−0.37	5.81E-05	231	−0.63	1.30E-03	639	0.25	−0.42	7.26E-07	—	*PALMD*
D′2	10p12.31	rs10828015	20608914	T	407	−0.18	4.55E-05	200	−0.39	8.30E-04	607	0.10	−0.20	7.40E-07	—	*PLXDC2*
TE	1q21.3	rs744536	151328794	T	303	2.02	2.59E-05	172	2.29	7.80E-03	475	0.80	2.08	8.83E-07	*SELENBP1*	*SELENBP1*
Delayed Recall	2p13.2	rs17348624	73312069	A	410	−0.76	5.18E-05	192	−1.34	1.70E-03	602	0.24	−0.85	8.93E-07	*RAB11FIP5*	*RAB11FIP5*
PO	1q41	rs2647430	220171786	G	392	−7.07	2.03E-05	181	−8.04	1.10E-02	573	0.80	−7.25	9.88E-07	*EPRS*	*EPRS*
Digit Symbol	1p22.3	rs12061951	86081453	C	393	−0.89	7.99E-05	182	−0.92	2.50E-03	575	0.94	−0.90	9.93E-07	—	*CYR61*

Note: Abbreviations are explained in the first footnote to table 1. P_{hetero}, $P_{heterogeneity}$. See the supplementary table 2 for abbreviations of each phenotype.

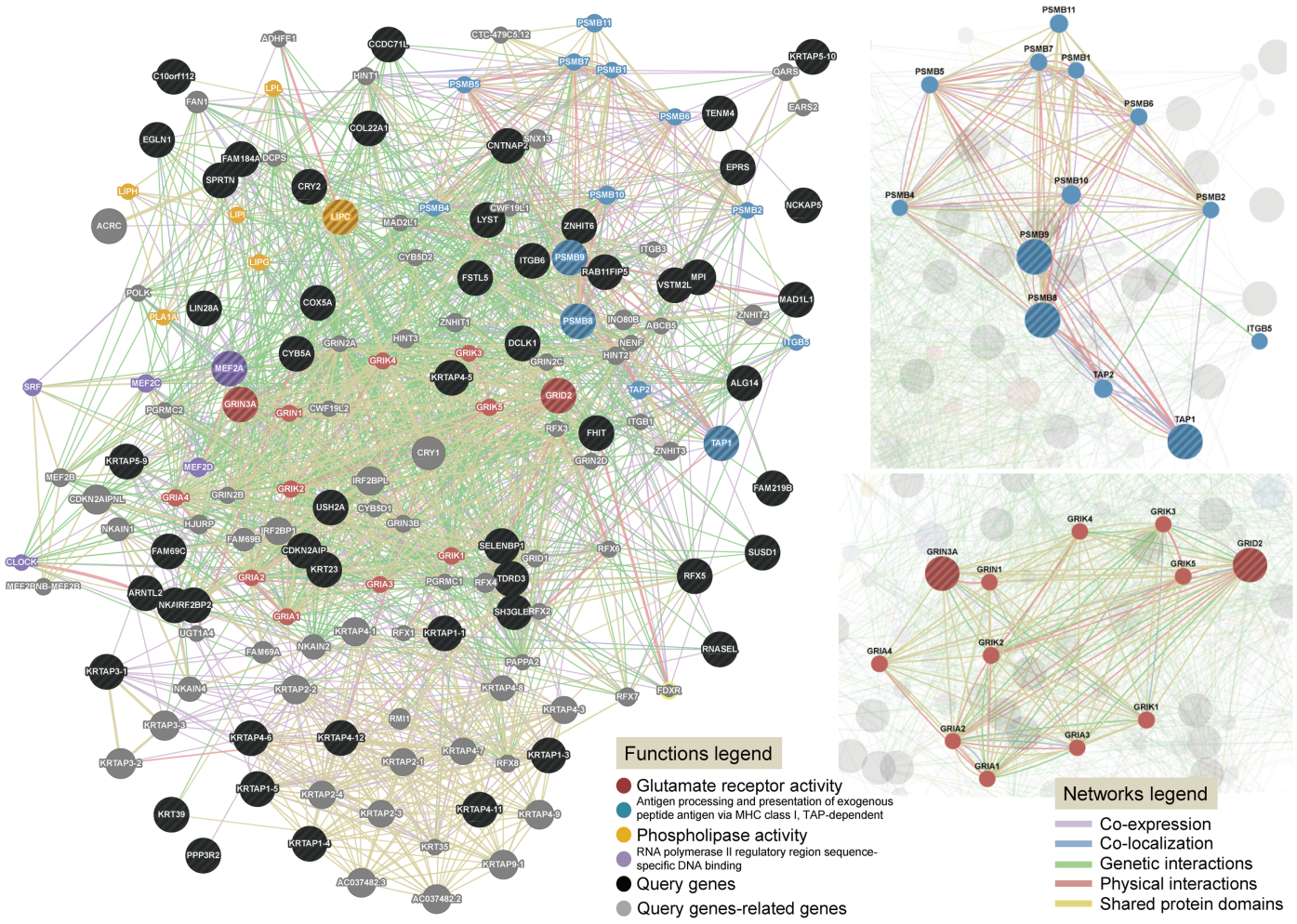

Fig. 2. Functional gene networks connected to cognitive phenotypes in schizophrenia. The functional networks, including co-expression, co-localization, genetic interaction, physical interaction and shared protein domains, are indicated by connecting genes with colored lines. All query genes are given the maximum node size and the size of the nodes for related genes is inversely proportional to the rank of the gene in a list sorted by the gene score assessed by the software. The "glutamate receptor activity" network was shown as the most predominant network composed of red-colored node genes, such as *GRIN3A* and *GRID2* (enlarged view of lower right). Other significant networks are as follows: "antigen processing and presentation of exogenous peptide antigen via major histocompatibility complex class I. TAP-dependent" (blue nodes) (enlarged view of upper right), "phospholipase activity" (orange nodes), and "RNA polymeraseII regulatory region sequence-specific DNA binding" (purple nodes).

Each genetic variant had weak to moderate associations with cognitive phenotypes that ranged from $P = 3.01 \times 10^{-5}$ to $P = 9.40 \times 10^{-8}$. Because integrated effects of several genetic variants with small effects, rather than each effect of an intensely associated variant, may be strongly associated with cognitive impairments in schizophrenia, we tested our hypothesis that genetic variants detected through GWAS on a large range of cognitive phenotypes at a loose threshold may be aggregated in functional gene networks related to the pathophysiology of schizophrenia. We determined that gene networks, such as glutamate and immune functions, rather than each genetic variant, were strongly associated with cognitive impairments in schizophrenia. We suggest that this approach would be an effective method to converge detected genetic variants and to detect functional gene networks.

Glutamate is the major excitatory neurotransmitter of the central nervous system (CNS) and is implicated in basic neuronal functions and CNS processes, including learning, memory, and synaptic plasticity. NMDA receptors are voltage-dependent ionotropic glutamate receptors. Hypofunction of glutamate transmission through NMDA receptors has been implicated in the pathophysiology of schizophrenia. The NMDA hypofunction theory is supported by various lines of evidence from pharmacological, genetic, postmortem, and brain imaging studies.[31] NMDA receptor antagonists, such as phencyclidine and ketamine, could induce a schizophrenia-like psychosis and cognitive deficits in individuals without schizophrenia and exacerbate symptoms in patients with schizophrenia.[32,33] Aberrances of the density and subunit composition of NMDA receptors have been reported in

postmortem brains of patients with schizophrenia.[34,35] The NMDA receptor consists of a heteromeric tetramer protein that contains 2 obligate NR1 subunits and 2 subunits from 2 NR2 (A, B, C, or D) and/or NR3 (A or B) families. One of the genes that comprise the glutamate receptor activity network detected in this study is the *GRIN3A* gene, which encodes the NR3A subunit of the NMDA receptor. NR3A acts to suppress NMDA receptor activity and is involved in the development of dendritic spines by modulating NMDA receptor activity.[36] An NR3A deficit increases spine density and initiates synapse maturation and memory consolidation during early postnatal neural development.[37] *GRIN3A* expression levels in the dorsolateral prefrontal cortex were elevated by approximately 30% in schizophrenia patients relative to controls.[38] Genetic associations between schizophrenia and genetic variants, such as rare SNPs, as well as copy number variants in the *GRIN3A* gene have been reported.[39,40] These findings suggest that aberrant enhanced *GRIN3A* function could be involved in the pathophysiology of schizophrenia and its cognitive impairments. The glutamate receptor, ionotropic delta 2 (*GRID2*), which comprises the glutamate network, encodes a protein that is a member of the ionotropic glutamate receptor family. Although it is known that D-Serine, which is reduced in the plasma and cerebrospinal fluid of schizophrenia patients,[41] binds to the δ2 glutamate receptor encoded by the *GRID2* gene,[42] whether the gene is involved in the risk of schizophrenia is unknown. Further research regarding this gene is needed to increase the understanding of the glutamate network.

We determined that the immune function network via MHCI was associated with cognitive functions. Recently, it has become increasingly clear that immune dysregulation plays an important role in the etiology of schizophrenia.[30] GWAS repeatedly indicate associations of schizophrenia with immune genes in the MHC region on chromosome 6.[2,4,5] MHCI molecules are expressed on neurons in the CNS throughout development and in adulthood, and these molecules regulate brain development, including neurite outgrowth, synapse formation and function, and long-term plasticity of excitatory synaptic transmission.[30,43] The MHCI molecules modulate glutamate receptor function.[44] The MHCI molecule–mediated glutamatergic receptor function may be related to cognitive impairments in schizophrenia.

To our knowledge, our study was the first to identify 5 suggestive variants at 3p14.3, 5q32, 9p21.2, and 10p15.3 associated with cognitive phenotypes relevant to schizophrenia, only in the discovery GWAS cohort, that could serve as a widely used benchmark for a genome-wide significance threshold of $P < 5.0 \times 10^{-8}$. Of 5 variants, rs10757641 at 9p21.2 was still suggestive after correcting multiple comparisons; however, we could not replicate any associations in the replication samples. No genetic variant in the GWAS of cognitive phenotypes related to schizophrenia has reached genome-wide significance despite the large number of subjects.[14–18] Given a small sample size used in the current study, there is a possibility of false-positive results because of the small number of samples. These findings suggest that much larger samples, such as the latest GWAS of schizophrenia,[5] would be required to detect genome-wide significance variants related to cognition.

To alleviate concern about multiple comparisons, it might be fruitful to reduce cognitive phenotypes into a general cognitive factor *g* and cognitive domains. To address the question, we applied our 4 stages strategy after reducing cognitive phenotypes into *g* and 6 cognitive domains, as shown in supplementary analysis. This approach did not detect any genome-wide significant variant (supplementary tables 6–8 and supplementary figure 5) or significant gene network (supplementary figure 6). At first stage, the 14 closest genes of the 3054 SNPs were listed in 108 susceptible loci for schizophrenia. At second stage, most SNPs were associated with 1 phenotype only, while 21 SNPs were influencing multiple phenotypes. These genes and SNPs may be associated with a particular gene network. Therefore, we performed additional gene-network analyses of the 14 closest genes (supplementary figure 7), the 6 genes located within 10 kb from 21 SNPs multiply related to cognitive phenotypes (supplementary figure 8), and the 53 genes within 10 kb from the remaining SNPs related to 1 phenotype only (supplementary figure 9) and their related 100 genes, respectively. There was no significant gene network of the 14 closest genes. On the other hand, there were significant gene networks of the 6 genes (glutamate receptor activity) and 53 genes (immune system related to MHCI). As these genes constituted a part of 59 genes used at fourth stage, it is no wonder that 2 prominent networks were divided into each gene network. These findings support our hypothesis that genetic variants detected through GWAS of a broad range of cognitive phenotypes at a suggestive level would be aggregated in gene networks related to the pathophysiology of schizophrenia.

Regardless of the examined phenotypes, the trait/disease-associated SNPs from GWAS have been significantly overrepresented in regions of regulatory genetic elements compared with the SNPs randomly selected from the genotyping arrays.[45] In addition, the SNPs in and in close proximity to the genes from all GWAS SNPs have been shown to explain more variation of the examined phenotypes and to replicate at higher rates compared with the intergenic SNPs.[29,46] We determined that the SNPs with genes within 10 kb from the detected SNPs were significantly larger in the replicated SNPs compared with the discovery GWAS SNPs. Our findings support that the regulatory genetic elements may be particularly enriched for the phenotype-associated SNPs. These associated SNPs may contribute to the regulation of gene expression, and they may be related to the pathophysiology of

cognitive impairment in schizophrenia. The relationships between the SNPs and the gene expressions are speculative, and the detected SNPs are not necessarily related to genes in close proximity to the SNPs.

There are some limitations to interpret our findings. Only 191 of 3054 associations were replicated at $P < .05$. This rate of replication (6.3%) was low and might be what was expected by chance. Based on our hypothesis that multiple genes with interactions would be related to pathogenesis of schizophrenia, we performed gene-network analysis using query and the related genes. However, genes that are strongly connected to the query genes but not necessarily showing effects on cognition might lead to a biased result.

In this study, we attempted to integrate the results of each GWAS of a large number of cognitive phenotypes by investigating functional gene networks. Our identified gene networks support important implications regarding cognitive impairments in the pathophysiology of schizophrenia. We suggest that a convergence of results from GWAS regarding multiple cognitive phenotypes that result from focusing on functional gene networks may be an informative strategy to detect prominent networks relevant to schizophrenia.

Supplementary Material

Supplementary material is available at http://schizophreniabulletin.oxfordjournals.org.

Funding

Japanese Ministry of Health, Labor and Welfare (H22-seishin-ippan-001); Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) (KAKENHI, 22390225-Grant-in-Aid for Scientific Research (B), 23659565-Grant-in-Aid for Challenging Exploratory Research, Grant-in-Aid for Scientific Research on Innovative Areas [Comprehensive Brain Science Network]); Japan Foundation for Neuroscience and Mental Health.

Acknowledgments

We would like to thank all individuals who participated in this study. The authors have declared that there are no conflicts of interest in relation to the subject of this study.

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