GENETIC MECHANISMS OF BIOMARKERS IN SCHIZOPHRENIA, BIPOLAR DISORDER AND DEPRESSION

EDITED BY: Hongsheng Gui, Shaohua Hu, Qiang Wang, Xiancang Ma and Sarah Tarbox-Berry

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GENETIC MECHANISMS OF BIOMARKERS IN SCHIZOPHRENIA, BIPOLAR DISORDER AND DEPRESSION

Topic Editors:
Hongsheng Gui, Henry Ford Health System, United States
Shaohua Hu, Zhejiang University, China
Qiang Wang, Sichuan University, China
Xiancang Ma, First Affiliated Hospital of Xi'an Jiaotong University, China
Sarah Tarbox-Berry, Wayne State University, United States

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Table of Contents

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Editorial: Genetic Mechanisms of Biomarkers in Schizophrenia, Bipolar Disorder and Depression

Yiguo Tang[1,2], *Hongsheng Gui*[3], *Sarah Tarbox-Berry*[4,5], *Shaohua Hu*[6], *Xiancang Ma*[7,8,9] *and Qiang Wang*[1,2*]

[1] *Mental Health Center and Psychiatric Laboratory, State Key Laboratory of Biotherapy, West China Hospital of Sichuan University, Chengdu, China,* [2] *West China Brain Research Center, West China Hospital of Sichuan University, Chengdu, China,* [3] *Behavioral Health and Psychiatry Research, Henry Ford Health System, Detroit, MI, United States,* [4] *Department of Neurology, School of Medicine, Wayne State University, Detroit, MI, United States,* [5] *Department of Psychiatry, School of Medicine, Yale University, New Haven, CT, United States,* [6] *Department of Psychiatry, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China,* [7] *Department of Psychiatry, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China,* [8] *Center for Brain Science, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China,* [9] *Clinical Research Center for Psychiatric Medicine of Shaanxi, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China*

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Editorial on the Research Topic

Genetic Mechanisms of Biomarkers in Schizophrenia, Bipolar Disorder and Depression

INTRODUCTION

Mental illnesses, one of seriously intractable chronic diseases, are exerting pernicious effect upon people's daily life. Most of the patients with psychiatric disorders are often unable to fulfill their social and occupational functions. Currently, symptom-based criteria of Diagnostic and Statistical Manual of Mental Disorders (DSM) or International Classification of Diseases (ICD) are indispensable to the diagnosis of mental disorders, in which there is no biological index clearly delineating the boundary between abnormality and normality of mental health. Moreover, considering clinical heterogeneity, genetic background underlying the same clinical symptoms can be very different. Thereby, delving into genetic mechanisms beneath is of vital importance for figuring out the pathophysiological changes and monitoring the disease progression and treatment response.

Although the exact etiology of psychiatric diseases still remains unclear, a large proportion of susceptibility to mental illnesses can be explained by genetic factors (1). Each genetic variation slightly increases the susceptibility, but the cumulative effect of the variations, particularly in the presence of interaction with environmental risk factors, may serve as a strong trigger for mental dysfunction. In the arena of genetic analyses, genome-wide association study (GWAS) is widely applied to detect the genetic polymorphism of individuals across the whole genome, hence can obtain millions of genotypes to be analyzed along with phenotypes at the population level (2). By virtue of sample statistics and their statistical significance (i.e., *p*-value), the genetic variations most likely to affect traits are picked out and the inferred susceptibility loci related to trait variations are then identified. Based on GWAS, the polygenic risk score (PRS) was later developed to quantify the cumulative effect of multiple genes or loci by switching genomic information into a comprehensive score measuring susceptibility to diseases (3). And over the past years, PRSs have been demonstrated to be a promising biomarker by a large amount of evidence. Moreover, neurocognitive functions and neuroimaging as quantitative characters (in other words, endophenotype) have also found their ways into GWAS analyses (4).

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Ingrid Melle,
University of Oslo, Norway

***Correspondence:**
Qiang Wang
wangqiang130@scu.edu.cn

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Studies within this Research Topic adopted an array of methods to assess biomarkers and molecular genetics in schizophrenia, bipolar disorder, and major depression disorder (MDD), mainly touching upon the following scopes:

(1) detection of genome variations;
(2) integrated analysis of genetics and biomarkers;
(3) exploration of the biological subtypes;
(4) study of biological markers for cross-diseases diagnosis;
(5) identification of the structural and functional brain abnormalities.

DETECTION OF GENOME VARIATIONS

Numerous studies have provided consistent evidence that genetic factors are involved in the pathogenesis of mental disorders. The heritability of schizophrenia, bipolar disorder, and MDD are 0.81, 0.75, 0.37, respectively (5); and this indicates that there is a significant genetic component to their etiology, especially for the former two. However, since there are no completely consistent results regarding to the exact genetic factors, identifying susceptibility loci from numerous candidate genes is still a huge ongoing challenge.

Levchenko et al. unveiled associations of alleles of NC_000008.11:g.32614509_32614510del, rs61731109, and rs10508649 (*NRG1* and *PIP4K2A*) with antidepressant treatment response, of alleles of rs35641374 (*NRG1*) and rs10508649 (*PIP4K2A*) with time to recurrence of depressive and manic or mixed episodes among patients with bipolar disorder, and of allele A of rs2248440 (*HTR2C*) with depression severity. In the systematic review by Rovný et al., two polymorphisms related to gating function (*HTR2A* rs6311 and *TCF4* rs9960767) were reported to be associated with schizophrenia at a meta-analytic or genome-wide level, providing further insight into the etiopathogenetic links between genetic variation, gating efficiency, and schizophrenia. According to the primary etiological hypotheses of schizophrenia, glutamate is believed to be a contributor to the onset of schizophrenia. However, Li W. et al. identified that genetic variations of *SLC1A1*, which were generally considered as a critical role in regulating the glutamatergic system, were not the susceptibility biomarkers for schizophrenia, but instead for psychopathology symptoms.

INTEGRATED ANALYSIS OF GENETICS AND BIOMARKERS

By integrating the information of multiple genetic susceptibility loci with other potential biomarkers, the capacity for predicting, screening, and intervening high-risk populations prior to the onset of mental diseases, which is the centerpiece of precise prevention of complex diseases, can be greatly enhanced.

Tao et al. first reported associations between cognitive impairment, insulin resistance (IR), and oxidative stress in the first-episode untreated schizophrenics, suggesting that IR may also be a peripheral biological marker of cognitive dysfunction in schizophrenics. A systematic review of potential biomarkers of MDD by Shao and Zhu elaborated upon the associations between monoamine signaling deficits, detrimental personality traits and MDD. As mentioned by Levchenko et al., protein interactions were also involved in the pathogenesis of mood disorders, hence proteomic biomarkers have attracted much attention for its involvement in the occurrence and development of various psychiatric disorders. Wu et al. first established the relationship between the *AQP4* polymorphisms and the risk of schizophrenia in the Southern Chinese Han population. Fu et al. reported a positive association between brain-derived neurotrophic factor (BDNF) polymorphism rs6265 and schizophrenia. In their cis-mQTL (Methylation Quantitative Trait Loci) analysis, an association of rs6265 with various methylation loci surrounding BDNF was detected, further supporting the promising role of BDNF-related methylation in the pathophysiology of schizophrenia. To hammer away at the proteomic biomarkers that can differentiate patients with schizophrenia, bipolar disorder, and MDD and predict the transition of the high-risk group to mental illness, Lee et al. have initiated the Seoul Pluripotent Risk for Mental Illness (SPRIM) study. Moreover, environmental factors, such as intestinal microorganisms, also exert a prominent influence on the etiology of mental illnesses. Cai et al. discovered that mGluR5$^{-/-}$ mice were susceptible to despair-like behavior and the systemic knockout of mGluR5 did not affect the gut microbiota or inflammatory.

EXPLORATION OF BIOLOGICAL SUBTYPES

Exploration of biological subtypes creates a fertile climate for the research domain criteria. Wang et al. reported that the occurrence and development of depressive symptoms in schizophrenia might be influenced by SNP rs3758391 through the dysregulation of *SIRT1* mRNA expression. In the study of genetic variations of *SLC1A1* mentioned above, Li W. et al. also found five SNPs (rs7032326, rs7860087, rs2039291, rs4742007, and rs301430) related to subtype symptoms. In addition, neuroimaging techniques and neuroelectrophysiology also provide further insight into biological subtypes of psychiatric illnesses.

STUDY OF CROSS-DISEASE BIOLOGICAL MARKERS

Comorbidities of different psychiatric disorders are prevalent, while some of these clinical sharing can be explained by an overlapping genetic basis. Recent post-GWAS analyses, as examples indicated from FUMA, Sherlock, and SMR can be further conducted to predicate candidate genes and identify the pleiotropic genes which contribute to various traits simultaneously. By adopting those *in silico* approaches above, Liu et al. revealed 21 potential pleiotropic genes and three biological pathways highly likely to be shared between schizophrenia and cardiometabolic disease. Ullah et al. established for the first time the association of highly recurrent copy number variations with schizophrenia and premenstrual dysphoric disorder. Cao et al.

not only revealed three novel microRNAs (hsa-miR-208b-3p, hsa-miR-494-5p, and hsa-miR-208a-3p) potentially contributing to schizophrenia, but also suggested that higher cardiovascular mortality and lower odds of glioma in schizophrenic patients could be explained by the sharing of regulatory networks between schizophrenia and other pathologies.

IDENTIFICATION OF THE STRUCTURAL AND FUNCTIONAL BRAIN ABNORMALITIES

Structural and functional brain abnormalities identified by neuroimaging techniques and neuroelectrophysiology, such as fMRI, DTI, and EEG, further enhancing the understanding of neural mechanisms of psychiatric diseases.

Li Z. et al. evaluated changes in brain gray matter structure by measuring cerebral cortex thickness and subcortical gray matter volume, and reported that *MTHFR* C677T polymorphism may be involved in the dysfunction of limbic–cortical–striatal–pallidal–thalamic (LCSPT) circuits mediating emotion processing. As mentioned above, neuroimaging techniques and neuroelectrophysiology also contribute to the exploration of the potential biomarkers of biological subtypes. Auditory verbal hallucinations (AVH), a core feature of schizophrenia, refer to hearing voices without external stimulation in the awake state (6, 7). Such symptoms not only occur in 60–90% schizophrenics (8, 9), but also feature in a range of other psychiatric disorders. Sun, Fang, Shi et al. pinpointed that inhibitory control was impaired in schizophrenia patients, and worse inhibitory top-down control might contribute causally to the onset of AVH, echoing the most influential hypotheses of AVH (10). And by taking advantage of EEG, they also discovered that AVH in schizophrenia may be related to neuropathological abnormalities in frontal-central brain regions. Additionally in another study lead by the same group, Sun, Fang, Peng et al. found that AVH patients showed higher activity level in the resting-state and may have impaired higher-order auditory expectations in the task-related state, speculating that the occurrence of AVH may occupy certain brain resources and compete for brain resources with external auditory stimuli. Furthermore, through resting-state fMRI scans, Gao et al. found abnormal connections related to auditory, speech, and memory circuits, including the STG, Wernicke's area, Broca's area, and hippocampus, in schizophrenia patients with AVH.

CONCLUSION

In a nutshell, through delving into the genetic mechanisms of biomarkers in schizophrenia, bipolar disorder, and MDD, a better understanding of the etiology of these mental disorders as well as comorbidity comes into being. With more development in this area of reseach, psychiatrists will be able to make more appropriate decisions in diagnosis, treatment selection and prediction of disease course.

AUTHOR CONTRIBUTIONS

All authors conceived and developed the presented ideas and contributed to the final manuscript equally.

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ORIGINAL RESEARCH
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miRNA-Coordinated Schizophrenia Risk Network Cross-Talk With Cardiovascular Repair and Opposed Gliomagenesis

Hongbao Cao[1,2,3†], Ancha Baranova[3,4†], Weihua Yue[5†], Hao Yu[6], Zufu Zhu[7], Fuquan Zhang[8] and Dongbai Liu[7*]*

[1] Department of Psychiatry, First Hospital/First Clinical Medical College of Shanxi Medical University, Taiyuan, China, [2] Department of Genomics Research, R&D Solutions, Elsevier Inc., Rockville, MD, United States, [3] School of Systems Biology, George Mason University (GMU), Fairfax, VA, United States, [4] Research Center for Medical Genetics, Moscow, Russia, [5] Department of Psychiatry Institute of Mental Health, Peking University, Bejing, China, [6] Department of Psychiatry, Jining Medical University, Jining, China, [7] Department of Neurology, Jiangyin People's Hospital Affiliated to Southeast University, Jiangyin, China, [8] Department of Psychiatry, The Affiliated Brain Hospital of Nanjing Medical University, Nanjing, China

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Edited by:
Shaohua Hu, Zhejiang University, China

Reviewed by:
Owen Murray Rennert, Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), United States
Radka Kaneva, Medical University-Sofia, Bulgaria

***Correspondence:**
Fuquan Zhang
zhangfq@njmu.edu.cn
Dongbai Liu
dliu02@gousinfo.com

[†]These authors have contributed equally to this work

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Background: Schizophrenia risk genes are widely investigated, but a systemic analysis of miRNAs contributing to schizophrenia is lacking.

Methods: Schizophrenia-associated genetic loci profiles were derived from a genome-wide association study (GWAS) from the Schizophrenia Working Group of the Psychiatric Genomics Consortium (PGC) dataset. Experimentally confirmed relationships between miRNAs and their target genes were retrieved from a miRTarBase. A competitive gene set association analysis for miRNA-target regulations was conducted by the Multi-marker Analysis of GenoMic Annotation (MAGMA) and further validated by literature-based functional pathway analysis using Pathway Studio. The association between the targets of three miRNAs and schizophrenia was further validated using a GWAS of antipsychotic treatment responses.

Results: Three novel schizophrenia-risk miRNAs, namely, miR-208b-3p, miR-208a-3p, and miR-494-5p, and their targetomes converged on calcium voltage-gated channel subunit alpha1 C (CACNA1C) and B-cell lymphoma 2 (BCL2), and these are well-known contributors to schizophrenia. Both miR-208a-3p and miR-208b-3p reduced the expression of the RNA-binding protein Quaking (*QKI*), whose suppression commonly contributes to demyelination of the neurons and to ischemia/reperfusion injury. On the other hand, both QKI and hsa-miR-494-5p were involved in gliomagenesis.

Conclusion: Presented results point at an orchestrating role of miRNAs in the pathophysiology of schizophrenia. The sharing of regulatory networks between schizophrenia and other pathologies may explain higher cardiovascular mortality and lower odds of glioma previously reported in psychiatric patients.

Keywords: miRNA, schizophrenia, miR-208b-3p, miR-208a-3p, miR-494-5p, gliomagenesis, quaking, heart disease

INTRODUCTION

Schizophrenia, a common psychiatric condition characterized by abnormal social behavior and failure to understand reality, affects up to 1% of the human population and causes substantial morbidity and mortality (Barnett, 2018). It is a complex disorder with an estimated heritability of around 80% and an unclear mode of genetic transmission (Hilker et al., 2018). There are many risk genes for schizophrenia, and there is a very small risk attributed to each one (Winchester et al., 2014; Li et al., 2016). In the largest multi-stage genome-wide schizophrenia association study to date, with 34,241 cases, 45,604 controls, and 1,235 affected parent–offspring trios, a total of 128 independent associations spanning 108 conservative loci were identified (Ripke et al., 2014), many of which were consistent with leading pathophysiological hypotheses of schizophrenia development.

It is worth noting that schizophrenia rarely results from the disruption of an individual gene, or even a contiguous chromosomal region. On the contrary, this condition is commonly attributed to the concerted and stable dysregulation of a complex genetic network or a set of networks (Gilman et al., 2012). Because of that, the dysregulation of master regulatory molecules, such as miRNAs, is expected to play a crucial role in the pathogenesis of schizophrenia. Indeed, altered levels of miRNA in the brain, in peripheral blood mononuclear cells, and in serum are found in patients with schizophrenia (Xu et al., 2010; Wang et al., 2014). Consequently, miRNAs that systemically regulate the genes contributing to the risk of schizophrenia may be of particular importance to its pathophysiology.

In this study, we investigated the miRNA-target gene set associated with schizophrenia with a goal of pinpointing potential master miRNA regulators of the gene networks associated with this disorder. To do that, we selected all experimentally confirmed miRNA–target interactions (MTIs) previously collected in a manually curated miRTarBase (Huang et al., 2020), and we then linked them to a schizophrenia-related tissue context through performing a MAGMA analysis (de Leeuw et al., 2015) of confirmed genes rather than miRNAs itself. The finding was validated by PPI network building and an analysis of secondary GWAS datasets concerning differential antipsychotic treatment responses. Our study prioritizes three miRNAs, miR-208a, miR-208b, and miR-494, as potential high-level regulators of schizophrenia phenotypes.

METHODS AND MATERIALS

Experimentally Confirmed Pairs of miRNA With Their Target Genes

In order to get the most reliable connection, only miRNA–target pairs supported by strong experimental evidence (reporter assay or Western blot) were retrieved from miRTarBase 7.0. (http://mirtarbase.mbc.nctu.edu.tw/php/download.php) (Chou et al., 2017).

Competitive Gene Set Association and Literature-Based Pathway Analysis

A Multi-marker Analysis of GenoMic Annotation (MAGMA) based on a multiple linear principal components regression model was previously designed to analyze the gene set association involved in genome-wide association studies (GWAS) data (de Leeuw et al., 2015). For each miRNA, its target genes were treated as a gene set, and then the competitive MAGMA-based gene-set analysis was utilized to test the association of each gene set using the summary statistics from the PGC2 GWAS (Ripke et al., 2014). The European samples from the 1,000 Genomes data (http://www.1000genomes.org) were used as reference data sets for the summary statistics gene analysis. Potentially confounding effects of gene size and gene density were treated as covariates in a generalized regression model. Multiple comparisons were corrected by a threshold of the false discovery rate (FDR) < 0.05. Then, significantly associated miRNA target sets were validated using the summary result of a GWAS of antipsychotic treatment responses in 2,413 schizophrenia patients (Yu et al., 2018). East Asian samples from the 1,000 Genomes data (http://www.1000genomes.org) were used as reference data sets for the summary statistics gene analysis.

The literature-based pathway analysis has been conducted using Pathway Studio (www.pathwaystudio.com), which allowed us to explore potential functional connections of miRNAs, their targets and schizophrenia by providing high-quality coverage of these connections with evidence extracted from full-text scientific reports.

RESULTS

miRNA–Target Gene Regulating Relationships

A total of 8,496 unique miRNA–target pairs were retrieved from miRTarBase, involving 740 miRNAs and 2,853 target genes (**Supplementary Table S1**). After exclusion of all miRNAs with only one target gene each, a total of 539 miRNAs with two or more targets each were subjected to a gene set association analysis. For each miRNA, its target genes formed a gene set (N = 539). Taken together, all gene sets were comprised of 2,726 unique genes defined in PGC2 genotype data (**Supplementary Table S2**). The statistics describing miRNA–target gene regulations are shown in **Table 1**.

Novel miRNAs Contributing to the Risk of Schizophrenia

Competitive gene set association analysis conducted by MAGMA identified three miRNAs as significantly associated with schizophrenia, namely, hsa-miR-208b-3p (miR-208b) (p = 2.04E-10, FDR = 1.10E-7), hsa-miR-494-5p (miR-494) (p = 1.72E-4, FDR = 0.031), and hsa-miR-208a-3p (miR-208a) (p = 1.75E-4, FDR = 0.031). An analysis of expression for these miRNAs was performed in a comprehensive miRmine dataset (Panwar et al., 2017) that was comprised of 304 high-quality microRNA

TABLE 1 | miRNA–target gene sets associated with schizophrenia.

miRNA	nGenes	Beta	S.E.	*p*	FDR
miR-208b-3p	4	3.8	0.607	2.04E-10	1.10E-07
miR-494-5p	3	2.53	0.708	1.72E-04	0.031
miR-208a-3p	8	1.43	0.4	1.75E-04	0.031
miR-146b-5p	17	0.886	0.275	6.52E-04	0.088
miR-599	2	2.73	0.881	9.81E-04	0.106
miR-4782-3p	3	2.13	0.812	4.40E-03	0.364
miR-466	2	2.68	1.05	5.37E-03	0.364
miR-21-5p	131	0.233	0.092	5.89E-03	0.364
miR-126-3p	43	0.389	0.155	6.08E-03	0.364
miR-29c-5p	4	1.32	0.541	7.30E-03	0.369
miR-33a-5p	31	0.425	0.179	8.68E-03	0.369
miR-153-5p	4	1.41	0.592	8.70E-03	0.369
miR-10a-5p	20	0.574	0.242	8.90E-03	0.369

nGenes: the number of target genes for the miRNA; Beta, the regression coefficient for target gene set analysis; S.E., the standard error of the regression coefficient; FDR, the false discovery rate.

sequencing experiments. Two of the three miRNAs studied, namely, miR-208b-3p and miR-494-5p, were expressed in various brain tissues at substantial levels. Notably, the expression pattern of miR-208b-3p was restricted to brain and plasma, while miRNA miR-208a-3p was specific to serum, plasma, and placenta. While neither heart nor muscle has been covered by the miRmine dataset, a body of work has demonstrated the importance of miR-208a-3p and miR-208b-3p as myoMiRs, expressed in heart tissues along with their myosin heavy chain encoding genes MYH6 and MYH7, respectively (Siddique et al., 2016).

Table 2 presents a list of target genes regulated by these three miRNAs. Interestingly, all three highlighted miRNAs directly target CDKN1A, pointing to its possible function as a hub gene in the pathology of schizophrenia.

Multiple Functional Pathways Link the Three miRNAs to Schizophrenia

A Pathway Studio (www.pathwaystudio.com) analysis provided evidence for multiple functional pathways that link miR-208b-3p (**Figure 1A**), miR-208a-3p (**Figure 1B**), and miR-494-5p (**Figure 1C**) to schizophrenia. The relation types and the reference information are presented in **Supplementary Table S3**. Notably, all three schizophrenia-implicated networks regulated by miRNA included BCL2, a well-known regulator of apoptosis and mitochondrial dynamics, and a calcium voltage-gated channel subunit alpha1 C (CACNA1C), one of the L-type calcium channels (LTCCs) defining the calcium influx into cells, and these are critical for normal brain development and plasticity (**Figure 1**).

Protein–Protein Interaction Among Target Genes of the Three miRNAs

A protein–protein interactions (PPIs) analysis was conducted to study the relationship between the target genes of the three miRNAs (miR-208a, miR-208b, and miR-494), as shown in **Figure 2**. The relation data shown in **Figure 2** were acquired from STRING v10.0 (Szklarczyk et al., 2017) and plotted using Cytoscape (Shannon et al., 2003).

As shown in **Figure 2**, the three miRNAs connect to each other through a complex but relatively compact network through

TABLE 2 | Experimentally confirmed target genes of the three miRNAs contributing to the risk of schizophrenia.

miRNA	Target	Experiments	PMID
miR-208a-3p	CACNA1C	Luciferase reporter assay	27545043
miR-208a-3p	CACNB2	Luciferase reporter assay	27545043
miR-208a-3p	CDKN1A	qRT-PCR//Luciferase reporter assay//Western blot	20190813
miR-208a-3p	CDKN1A	Luciferase reporter assay//Western blot	26754670
miR-208a-3p	ETS1	Luciferase reporter assay//Microarray//qRT-PCR//Western blot	20576608
miR-208a-3p	MED13	Luciferase reporter assay//qRT-PCR//Western blot	17379774
miR-208a-3p	PDCD4	Luciferase reporter assay//qRT-PCR//Western blot	27634902
miR-208a-3p	QKI	Luciferase reporter assay	28283792
miR-208a-3p	SOX6	Luciferase reporter assay//Western blot	25023649
miR-208b-3p	CACNA1C	Luciferase reporter assay	27545043
miR-208b-3p	CACNB2	Luciferase reporter assay	27545043
miR-208b-3p	CDKN1A	qRT-PCR//Luciferase reporter assay//Western blot	20190813
miR-208b-3p	CDKN1A	Luciferase reporter assay//Microarray//qRT-PCR//Western blot	26044724
miR-208b-3p	QKI	Luciferase reporter assay	28283792
miR-494-5p	CXCR4	Luciferase reporter assay//qRT-PCR//Western blot	25955111
miR-494-5p	DPYD	GFP reporter assay//qRT-PCR//Western blot	25873402
miR-494-5p	PTEN	Luciferase reporter assay//qRT-PCR//Western blot	26045065

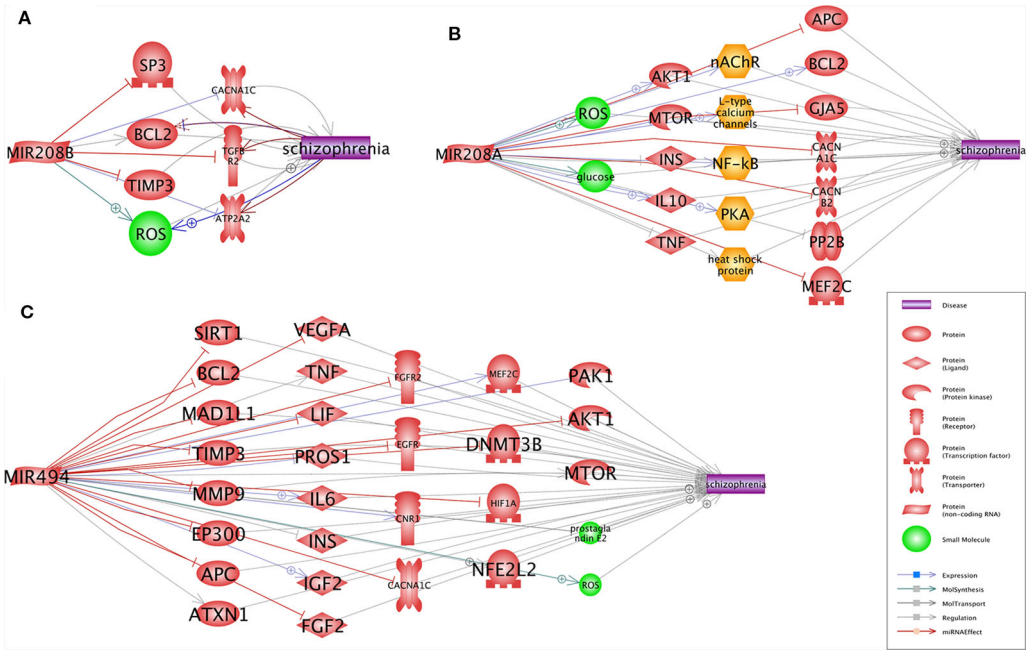

FIGURE 1 | Pathways that link each of three miRNAs, miR-208b **(A)**, miR-208a **(B)**, and miR-494 **(C)**, to schizophrenia. Presented pathways were generated using Pathway Studio (www.pathwaystudio.com) based on known relations mined from existing literature. Each relation has been supported by one or more references summarized in **Supplementary Table 3**.

multiple common target-binding proteins. Moreover, many protein components of this network are known to interact with each other, suggesting that this network is not random.

Validation by Association With the Response to the Treatment With Antipsychotic Drugs

To validate the association of miR-208a-3p, miR-208b-3p, and miR-494-5p and their target sets with schizophrenia, a GWAS of antipsychotic treatment response in 2,413 psychiatric patients (Yu et al., 2018) was mined to detecting enrichment. As shown in **Table 3**, the gene set regulated by miR-494-5p was associated with the drug treatment response of patients with schizophrenia.

DISCUSSION

Accumulating evidence suggests that post-transcriptional gene expression regulators, known as microRNAs (miRNAs), play a crucial role in many physiological and pathophysiological processes in human brain. In particular, various areas of the brain and the serum of individuals with schizophrenia were studied for the cellular and extracellular content of miRNA molecules as well as the widespread alterations of their levels reported (Moreau et al., 2011; Santarelli et al., 2011; Banigan et al., 2013). In their typical biomarker discovery design, these and other studies have not aimed at differentiating causal or consequential relationships between the change in the levels of certain miRNA and the development of psychiatric conditions. Nevertheless, the miRNAs encoded by these genes, for example, miR-137 (Kuswanto et al., 2015), were found to harbor the single nucleotide polymorphisms (SNPs) for an increased risk of schizophrenia.

This study highlights three additional miRNAs, hsa-miR-208b-3p, hsa-miR-494-5p, and hsa-miR-208a-3p, as potential contributors to schizophrenia and as the master regulators for the genes previously implicated in this disorder. An analysis of their gene expression showed that these miRNA species were expressed in the brain tissue, the plasma/serum/placenta, or in a combination of these at relatively high levels. Current evidence suggests that the blood–brain barrier does not block the passage of miRNAs between CSF and blood, even if brain-derived miRNAs are somewhat more diluted in blood (Stoicea et al., 2016). While the data on the penetration of miRNA from peripheral tissues to the brain are limited, one can assume that this transfer is highly possible, especially during embryonic development when brain tissue and its compartmentalization is not yet fully formed. Moreover, recent experiments performed in two different rodent models has shown that, in certain conditions, such as during hypoxia, miRNAs actively contribute to an increase in the penetrability of the blood–brain barrier through the inhibition of genes encoding tight junction proteins (Ma et al., 2017; Burek et al., 2019). The role of prenatal and perinatal factors contributing to the risk of schizophrenia was well documented (Kelly and Murray, 2000; Davis et al., 2016). Thereby, one may surmise that the molecular underpinning of this connection may be dependent on plasma miRNAs being carried to the brain in the course of hypoxia or other types of fetal stress, and it may also possibly be dependent

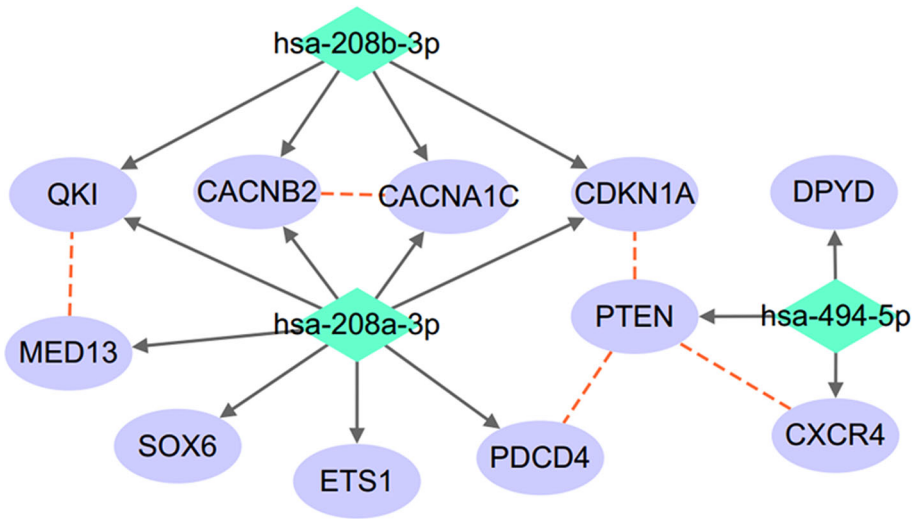

FIGURE 2 | miRNA–target regulatory network connecting miR-208a-3p, miR-208b-3p, and miR-494-5p. Red dashed lines denote protein–protein interactions; solid arrowed lines denote miRNA–target bindings.

TABLE 3 | miRNA–target gene set analyses with schizophrenia in validation dataset.

miRNA	nGenes	Beta	S.E.	*p*
hsa-miR-494-5p	3	0.971	0.506	0.030
hsa-miR-208a-3p	8	0.097	0.349	0.391
hsa-miR-208b-3p	4	-0.133	0.48	0.608

on the pathophysiological pairing between miRNAs and mRNAs in non-target tissue.

The accurate identification of miRNA targets remains a formidable challenge. As the output generated by commonly used microRNA–mRNA interaction-predicting software fails to pinpoint experimentally confirmed microRNA-binding regions correctly (Willgoose, 1981; Plotnikova and Skoblov, 2018), we had resorted to limiting our study by investigating only experimentally validated microRNA–mRNA interactions with a subsequent anchoring of them to schizophrenia-related targets by leveraging the data generated over the course of the largest schizophrenia-dissecting GWAS performed to date. Further support for our findings was obtained by the Pathway Studio guided analysis, which allowed us to perform a systems analysis of the molecular pathways engaged by these miRNAs.

Two functional molecules, BCL2 and CACNA1C, were commonly shared between all three miRNA-coordinated "Shortest Path" networks. Notably, both of these molecules were implicated in schizophrenia in numerous previous studies. *CACNA1C*, which encodes for the $Ca_v1.2$ α1 subunit of L-type calcium channels (LTCCs), is one of the best-supported risk loci for schizophrenia and bipolar disorder since it harbors variants with consequences on neural processing and connectivity (Gurung and Prata, 2015; Kabir et al., 2017). For BCL2, the connections to schizophrenia are at the level of cellular processes rather than genetic ones. In the astroglia and the neurons, BCL2 regulates autophagy, which maintains the balance between the synthesis, degradation, and recycling of mitochondria and other cellular components (Aouacheria et al., 2017) as well as prevents apoptosis (Almeida, 2013). The networks we built for schizophrenia risk miRNAs imply the disease-associated deregulation of BCL2/BAX and the resultant enhancement in cell susceptibility to apoptosis, which possibly involves an increase in the production of reactive oxygen species (Wu et al., 2013).

If increases in respective miRNA signals are defined genetically, their observed effects should be systemic rather than brain specific. In this light, it is important to note that the primary fibroblasts collected form antipsychotic-naïve patients with first-episode schizophrenia have greater apoptotic susceptibility, higher caspase-3 activity, and lower BCL2 expression than healthy controls (Gassó et al., 2014). Increased expression of hsa-miR-208b-3p, hsa-miR-494-5p, and hsa-miR-208a-3p may augment susceptibility to schizophrenia by simultaneously conferring susceptibility to apoptosis and altering neural processing and connectivity through the suppression of *BCL2* and *CACNA1C*, respectively.

Importantly, all three schizophrenia-contributing miRNA molecules are far from novel. Cardiomyocyte molecules miR-208a-3p and miR-208b-3p belong to the miR-208 family, which participates in ventricular remodeling (Liu et al., 2016) by promoting myocardial fibrosis (Shyu et al., 2015) and apoptosis of cardiomyocytes (Shannon et al., 2003; Luo et al., 2004; Moreau et al., 2011; Tsai et al., 2013; Huang et al., 2016).

Both miR-208a-3p and miR-208b-3p reduce the expression of the RNA-binding protein Quaking, encoded by gene *QKI*, which inhibits the apoptosis of cardiomyocytes under ischemia/reperfusion condition (de Bruin et al., 2017; Wang F. et al., 2017). Peculiarly, the dysmyelinating mouse mutant shaking (shk), a model of schizophrenia, is a quaking (qk) allele consisting of a 105-nucleotide insertion in the qk regulatory region that decreases the transcription of qk (Chaverneff et al., 2015). Downregulation of the QKI gene was also noted in the brains of schizophrenic patients (Haroutunian et al., 2006). It was hypothesized that deregulation of QKI underlines the defects of oligodendrocyte differentiation and in myelination detected in schizophrenia (Rosenbluth and Bobrowski-Khoury, 2013) as well as in—as described in a separate study—at least some cases of intellectual disability (Darbelli and Richard, 2016). Moreover, in yet another model tissue, auditory nerves, function of both QKI and its protein product substantially decreases in response to noise exposure, leading to demyelination and hearing deficiency (Panganiban et al., 2018). When QKI-regulating molecules of the miR-208 family are overexpressed, their effects are similar to the decrease in the transcription of QKI and should promote the development of the myelination defects. Remarkably, at clinically relevant concentrations of Haloperidol, the expression levels of QKI-encoding mRNA may be restored (Jiang et al., 2009), which would, in turn, alleviate demyelination-related symptoms.

There is no doubt that miR-208-regulated QKI defines the phenotypic plasticity of the vascular smooth muscle cells (van der Veer et al., 2013; Cochrane et al., 2017). These functional pieces of evidence of the involvement of *QKI* into the development of cardiovascular conditions are also supported by the GWAS, which pointed at *QKI* as a contributor to coronary heart disease (Dehghan et al., 2016). Patients with schizophrenia are known to have higher mortality rates for all major cardiovascular diagnoses (Wu et al., 2015; Westman et al., 2018). It is tempting to speculate that the connection between these two major disabilities may be, at least in part, explained by the sharing of regulatory networks, particularly ones connecting miRNAs of the miR-208 family and *QKI*.

Another pathophysiological process characterized by alterations in *QKI* is the development of gliomas. This gene serves as a tumor suppressor that promotes endolysosome-mediated degradation and suppresses the display of receptors essential for maintaining the self-renewal of neural stem cells outside their niche (Shingu et al., 2017). Consequently, the *QKI* gene tends to be eliminated in gliomas, either through a complete deletion or through a disruption by translocation (Bandopadhayay et al., 2016). While the roles for upstream regulators of *QKI*, hsa-miR-208b-3p and hsa-miR-208a-3p, in glioma have not yet been described, another miRNA that affects schizophrenia risk, hsa-miR-494-5p, is a definite glioma suppressor (Li et al., 2015; Zhang et al., 2015; Xu et al., 2018). Importantly, in the case of the latter miRNA, protection against the development of the tumors comes at a cost of elevated susceptibility to neurotoxicity, after exposure to ischemia/reperfusion for example. Notably, knockdown of hsa-miR-494-5p reverses the neurotoxic phenotype in multiple models (Song et al., 2017; Deng et al., 2019; Zhao et al., 2019a; Zhao et al., 2019b). Hsa-miR-494-5p-dependent antagonistic relationships between gliomagenesis and neurotoxicity are intriguing, as they support previously noted decrease in odds of the development of brain tumors in patients with schizophrenia (Grinshpoon et al., 2005; Levav et al., 2007; Wang Y. et al., 2017).

CONCLUSION

In summary, the presented results point at an orchestrating role of miRNAs in the pathophysiology of schizophrenia. Cellular effects of risk-associated miRNAs, namely, hsa-miR-208b-3p, hsa-miR-494-5p, and hsa-miR-208a-3p, align with the primary etiological hypotheses of schizophrenia and suggest that the three molecules, as well as their target genes, should be investigated for possible pharmacological interventions. The sharing of regulatory networks between schizophrenia and other pathologies may explain higher cardiovascular mortality and lower odds of glioma previously reported in psychiatric patients. Molecular tools for manipulating miRNA activity, including miRNA sponges, are already being developed for cancers (Jung et al., 2015; Fang et al., 2017) and for cardiovascular disease (Bernardo et al., 2018). There is a hope that similarly designed therapeutic interventions may find their utility in the treatment of schizophrenia and other life-long psychiatric illnesses.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the **Supplementary Material**.

AUTHOR CONTRIBUTIONS

HC, DL, and FZ developed the study design. FZ, WY, HY, ZZ, HC, and AB analyzed the data. FZ, HC, and AB drafted and then edited the original paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00149/full#supplementary-material

SUPPLEMENTARY TABLE S1 | miRNA-target gene pairs retrieved from MiRTarBase.

SUPPLEMENTARY TABLE S2 | Enrichment of miRNA Target Set in PGC2.

SUPPLEMENTARY TABLE S3 | References supporting the literature-based pathway linking SCZ and the three miRNAs.

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Integrated Analysis of Summary Statistics to Identify Pleiotropic Genes and Pathways for the Comorbidity of Schizophrenia and Cardiometabolic Disease

Hao Liu[1,2,3], Yang Sun[1,2], Xinxin Zhang[1,3], Shiyang Li[1,2], Dong Hu[1,2], Lei Xiao[1,2], Yanghui Chen[1,2], Lin He[3] and Dao Wen Wang[1,2]**

[1] Division of Cardiology, Department of Internal Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2] Hubei Key Laboratory of Genetics and Molecular Mechanisms of Cardiological Disorders, Wuhan, China, [3] Bio-X Institutes, Key Laboratory for the Genetics of Developmental and Neuropsychiatric Disorders (Ministry of Education), Collaborative Innovation Center for Genetics and Development, Shanghai Mental Health Center, Shanghai Jiaotong University, Shanghai, China

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University of Oslo, Norway

***Correspondence:**
Lin He
helinhelin123@yeah.net
Dao Wen Wang
dwwang@tjh.tjmu.edu.cn

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Genome-wide association studies (GWAS) have identified abundant risk loci associated with schizophrenia (SCZ), cardiometabolic disease (CMD) including body mass index, coronary artery diseases, type 2 diabetes, low- and high-density lipoprotein, total cholesterol, and triglycerides. Although recent studies have suggested that genetic risk shared between these disorders, the pleiotropic genes and biological pathways shared between them are still vague. Here we integrated comprehensive multi-dimensional data from GWAS, expression quantitative trait loci (eQTL), and gene set database to systematically identify potential pleiotropic genes and biological pathways shared between SCZ and CMD. By integrating the results from different approaches including FUMA, Sherlock, SMR, UTMOST, FOCUS, and DEPICT, we revealed 21 pleiotropic genes that are likely to be shared between SCZ and CMD. These genes include *VRK2*, *SLC39A8*, *NT5C2*, *AMBRA1*, *ARL6IP4*, *OGFOD2*, *PITPNM2*, *CDK2AP1*, *C12orf65*, *ABCB9*, *SETD8*, *MPHOSPH9*, *FES*, *FURIN*, *INO80E*, *YPEL3*, *MAPK3*, *SREBF1*, *TOM1L2*, *GATAD2A*, and *TM6SF2*. In addition, we also performed the gene-set enrichment analysis using the software of GSA-SNP2 and MAGMA with GWAS summary statistics and identified three biological pathways (MAPK-TRK signaling, growth hormone signaling, and regulation of insulin secretion signaling) shared between them. Our study provides insights into the pleiotropic genes and biological pathways underlying mechanisms for the comorbidity of SCZ and CMD. However, further genetic and functional studies are required to validate the role of these potential pleiotropic genes and pathways in the etiology of the comorbidity of SCZ and CMD, which should provide potential targets for future diagnostics and therapeutics.

Keywords: schizophrenia, cardiometabolic disease, comorbidity, pleiotropic, GWAS, eQTL

INTRODUCTION

Schizophrenia (SCZ) is a serious mental illness, with approximately 10–20 years life expectancy reduced compared with the general population (1). The most common cause of premature death in people with *SCZ* is cardiovascular disease (CVD), which results in three-fold higher mortality and 10 years shorter life expectancy for patients with *SCZ* than the general population (2). While the increased risk of CVD morbidity and mortality in SCZ can be explained by several factors (*e.g.,* smoking, poor diet, and sedentary behavior) (3), it is now established that cardiometabolic disease (CMD) including body mass index (BMI), coronary artery diseases (CAD), type 2 diabetes (T2D), low-density lipoproteins (LDL), high-density lipoproteins (HDL), total cholesterol (TC), and triglycerides (TG), accounts for the majority of the incidence of CVD-related death in schizophrenic patients (4).

Historically, the high prevalence of CMD among schizophrenic patients has been primarily attributed to unhealthy lifestyle factors and the side effects of antipsychotic medications (5). However, recent evidences have suggested that genetic basis and common biological pathways shared between SCZ and CMD (6–8). For example, Andreassen et al. using genetic-pleiotropy-informed methods detected 10 loci associated with both SCZ and CVD risk factors, which include waist-to-hip ratio, systolic blood pressure, BMI, LDL, HDL, and TG (6). So et al. performed polygenic risk scores, linkage disequilibrium score regression, and Mendelian randomization analysis and showed that genetic basis shared between SCZ and BMI, the causal relationship between SCZ and TG, and common biological pathways (e.g., aldosterone synthesis and secretion, neuronal system, and insulin secretion) shared between SCZ and CMD (8). These evidences provide the foundation for the genetic factors contribute to the comorbidity of SCZ and CMD.

Despite the fact that abundant genetic variants have been reported to be associated with the comorbidity of SCZ and CMD, understanding the functional consequences of genetic variation and identifying the pleiotropic genes and pathways are challenging in human genetics. First, the linkage disequilibrium (LD), a correlation structure exists across genetic variation of different loci (9). The top associated variant at a locus is often not the causal variant (10). Second, the complexity of gene regulatory. As genetic variants can affect phenotype through distal regulation of gene expression, the nearest gene to the genome-wide association studies (GWAS) top signal is often not the causal gene (10). Additionally, genetic variation affects gene expression in a tissue-specific manner (11). The complexity of LD and gene regulatory hinder the identification of pleiotropic genes and pathways for the comorbidity of SCZ and CMD.

In this study, we utilized different approaches and strategies to translate the genetic risk loci into potential candidate genes and pathways for SCZ and CMD, respectively, and then investigated the pleiotropic genes and pathways underlying the comorbidity between them (**Figure 1**). Firstly, we used positional mapping to functionally annotate of traits-associated genetic variants from GWAS summary statistics of SCZ and CMD. We then integrated the GWAS summary statistics of SCZ and CMD, and tissue-specific expression quantitative trait loci (eQTL) data to predicate the causal genes for SCZ and CMD. Finally, we performed gene-set enrichment analysis with GWAS summary statistics to identify the potential biological pathways for SCZ and CMD. This landscape of potential pleiotropic genes and biological pathways will help us to understand clearly the increased risk of CVD morbidity and mortality in SCZ.

MATERIALS AND METHODS

GWAS Summary Datasets

The GWAS summary statistics analysis included in this study were obtained from the following publicly available databases:

i. The GWAS data of SCZ was obtained from Psychiatric Genomics Consortium (PGC) (12), which systematically meta-analyzed of the genome-wide genotypes from 49 independent samples (46 of European and 3 of Asian ancestry, including 35,476 SCZ cases and 46,839 controls). Genotype data was processed by the PGC using unified quality control procedures followed by imputation of SNPs and insertion-deletions using the 1000 Genomes Project reference panel. Around 9.5 million variants after quality control were included in the dataset and used in this study.
ii. The largest-scale GWAS meta-analysis summary data of BMI was performed by Genetic Investigation of ANthropometric Traits (GIANT) (13), which conducted with a total sample of 322,154 individuals of European descent. This GWAS examined the phenotype of BMI as determined from measured or self-reported weight and height, and identified 77 loci reaching genome-wide significance ($P < 5 \times 10^{-8}$).
iii. The summary data of genetic variants associated with CAD was performed by the Coronary ARtery DIsease Genome wide Replication and Meta-analysis plus The Coronary Artery Disease Genetics (CARDIoGRAMplusC4D) Consortium (14), which assembled 60,801 cases and 123,504 controls from 48 studies. The majority (77%) of the participants were of European ancestry; 13% and 6% were of South Asian and East Asian ancestry, respectively, with smaller samples of Hispanic and African Americans. The results of association analysis from an additive model and a recessive model were used in this study.
iv. Genetic variations associated with T2D were obtained from DIAbetes Genetics Replication And Meta analysis (DIAGRAM) Consortium (15). This study is a meta-analysis from 32 GWAS, including 898,130 individuals (74,124 cases and 824,006 controls) of European ancestry. More than 200 loci reaching genome-wide significance ($P < 5 \times 10^{-8}$) in the BMI-unadjusted analysis and 152 loci BMI-adjusted analysis. The GWAS summary statistics with BMI adjustment was considered for our analyses.
v. The GWAS summary statistics of dyslipidemia were accessed from the Global Lipids Genetics Consortium (GLGC) (16),

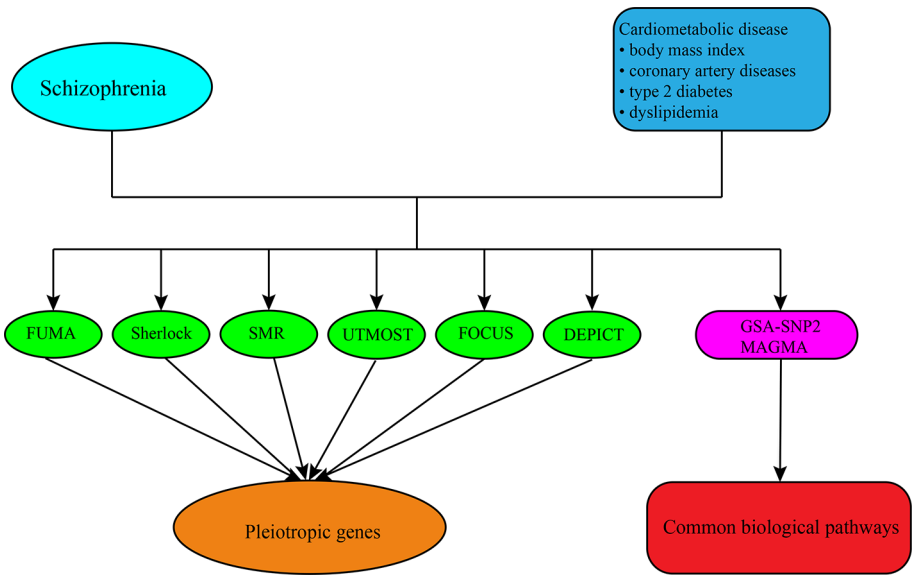

FIGURE 1 | The overall analysis conducted in this study. First, we obtained the summary-level GWAS datasets of SCZ and CMD from public GWAS databases. Then different approaches including FUMA, Sherlock, SMR, UTMOST, FOCUS, and DEPICT were conducted to predicate the candidate genes for them and identified the pleiotropic genes shared between them. Finally, we performed the gene-set enrichment analysis with GWAS summary datasets by the software of GSA-SNP2 and MAGMA to explore the biological pathways shared between them.

which provides the meta-analysis results on four phenotypes: HDL, LDL, TC, and TG. These results are based on GWAS results from 46 cohorts comprising of more than 100,000 individuals of European ancestry (N_{HDL} = 99,900, N_{LDL} = 95,454, N_{TC} = 100,184, and N_{TG} = 96,598).

All GWAS summary statistics used in this study are based on the hg19 human assembly and rsIDs were mapped to dbSNP build 151 using MySQL local database if necessary. We excluded the genetic variants in extended major histocompatibility complex (MHC) region (chr6:25–35MB), due to the complexity of haplotype and LD structure. The major samples in the GWAS summary datasets came from populations of European ancestry. The GWAS summary datasets used in this study were downloaded from publicly available resources listed in **Supplementary Table S1**. More detailed information about sample recruitment and diagnosis, genotyping, quality control, and statistical analysis can be found in the original paper (12–16).

eQTL Datasets

To evaluate the possible effect of genetic variants on transcriptional activity, we applied different eQTL datasets including Schadt et al. (17), Myers et al. (18), Westra et al. (19), Lloyd-Jones et al. (20), Qi et al. (21), Battle et al. (22), and V7 release summary data of the Genotype-Tissue Expression (GTEx V7) project (23). Concisely, Schadt et al. profiled more than 39,000 transcripts and genotyped 782,476 unique single nucleotide polymorphisms (SNPs) in 427 human liver samples of Caucasian individuals to characterize the genetic architecture of gene expression in human liver (17). Myers et al. carried out whole-genome genotyping and expression analysis on a series of 193 neuropathologically normal human brain cortex samples from the individuals of European descent (18). Westra et al. performed a meta-analysis of eQTL in non-transformed peripheral blood from 5,311 samples with replication in 2,775 individuals (19). Lloyd-Jones et al. analyzed the mRNA levels for 36,778 transcript expression traits to investigate the genetic architecture of gene expression and degree of missing heritability for gene expression in peripheral blood in 2,765 European individuals (20). Qi et al. meta-analysed cis- eQTL between brain and blood to identify putative functional genes for brain-related complex traits and diseases (21). Battle et al. sequenced RNA from whole blood of 922 genotyped the European ancestry individuals from the Depression Genes and Networks cohort for understanding the consequences of regulatory variation in the human genome (22). The GTEx V7 was established to characterize human transcriptomes and has created a reference resource of gene expression levels from non-diseased tissues, including genotype, gene expression, and histological data for 449 human donors across 44 tissues (23). More details about sample description, genotyping, expression quantification, and statistical analyses can be found in the corresponding original paper (17–23).

Considering that genetic variants may affect gene expression in a tissue-specific manner, we used brain and whole blood eQTL for SCZ; subcutaneous adipose, visceral omentum adipose and whole blood eQTL for BMI; left ventricle, atrial appendage, aorta artery, coronary artery, tibial artery, subcutaneous adipose, visceral omentum adipose, liver, and whole blood eQTL for

CAD; subcutaneous adipose, visceral omentum adipose, skeletal muscle, liver, pancreas, and whole blood eQTL for T2D; subcutaneous adipose, visceral omentum adipose, liver, and whole blood eQTL for HDL, LDL, TG, and TC.

Identifying Causal Genes Using Positional Mapping (FUMA)

Functional annotation of genetic variants from GWAS summary statistics was performed using FUMA (24), which incorporates 18 biological data repositories to process GWAS summary statistics. In particular, positional mapping in FUMA was performed by the ANNOVAR annotations of specifying the maximum distance between SNPs and genes, and using Combined Annotation Dependent Depletion (CADD) scores (25) to predict the functional consequences of SNPs on genes. The CADD scores predict how deleterious the effect of an SNP is likely to be for a protein structure or function, with higher scores referring to higher deleteriousness. In this method, we chose the default distance 10kb as the maximum distance, and performed SNPs filtering based on CADD score. The threshold for significance is CADD scores ≥ 12.37 with SNP P-value ≤ 5×10^{-8}.

Integration of GWAS and eQTL Datasets (Sherlock)

Considering that genetic variants may affect the disease through regulation of gene expression, we applied the method named Sherlock (26) to integrate GWAS and eQTL data with the aim to identify causal genes for diseases. Its underlying principle is that any genetic variants perturbs expression levels of risk genes is also likely to influence the risk of disease. Sherlock uses a Bayesian model and the information of SNPs in GWAS and eQTL data to calculate the SNP-level Bayes factor for estimating the association of the SNPs with the expression of gene and the disease, respectively. For the SNPs overlap between eQTL for a gene and the significant SNPs loci associated with the disease, it is straightforward to combine the SNP-level Bayes factor to obtain the Bayes factor for the gene and yielding a single per-gene score to test whether the expression change of this gene has any impact on the risk of disease. Statistical significance was determined using a Bonferroni corrected with P-value < 0.05/the total number of genes.

Integration of GWAS and eQTL Datasets (SMR)

Summary databased Mendelian randomization (SMR) (27) was used to predict causal genes by integrating the summary-level data from GWAS and data from eQTL studies. The principle of SMR analysis is to use a genetic variant as an instrumental variable to test for the causative effect of the gene expression (the exposure) on the phenotype of interest. The method including two tests: SMR test and heterogeneity in dependent instruments (HEIDI) test. SMR uses the simulation analysis to evaluate the effect of genetic variant on gene expression, genetic variant on phenotype, and gene expression on phenotype, respectively (SMR test). To test whether gene expression and phenotype are affected by the same causative variant, it uses multiple SNPs in a cis-eQTL region to distinguish pleiotropy from linkage (HEIDI test). The gene is considered to be plausible causal gene if pass the SMR (Bonferroni corrected P-value < 0.05) and HEIDI tests (P-value ≥ 0.05).

Predicting Causal Genes Using Tissue Expression Data (UTMOST)

In contrast to Sherlock and SMR, unified test for molecular signatures (UTMOST) (28) is a powerful approach to studying the genetic architecture of complex traits by using multi-task learning method to jointly impute gene expression in tissues. Briefly, the UTMOST framework includes three main steps. First, it trains a cross-tissue expression imputation model by using the genotype information and matched expression data. Next, it tests the association between the trait of interest and imputed gene expression in each tissue. Finally, a cross-tissue test is performed for each gene to summarize single-tissue association statistics into a powerful metric that quantifies the overall gene–trait association. Statistical significance was determined using a Bonferroni corrected with P-value < 0.05/ the total number of genes.

Predicting Causal Genes Using Expression Weights Data (FOCUS)

To identify potential causal genes involved in complex traits and diseases, we apply the approach of fine-mapping of causal gene sets (FOCUS) (29). This approach is a probabilistic framework that models correlation among transcriptome-wide association study signals to assign a probability for every gene in the risk region to explain the effect of SNPs on a trait. By integrating the GWAS summary data, expression prediction weights (as estimated from eQTL reference panels), and LD among all SNPs in the risk region, it identifies causal gene to be included in a 90%-credible set and give a posterior probability (PIP) to for estimating the causality in relevant tissue types. In this work, we used the recommend eQTL reference panel weight database, which combines GTEx weights with the Metabolic Syndrome in Men study (adipose, n = 563) (30, 31), the Netherlands Twins Registry (NTR; blood, n = 1,247) (32), the Young Finns Study (YFS; blood, n = 1,264) (33, 34), and the CommonMind Consortium (dorsolateral prefrontal cortex, n = 452) (35) weights into a single usable database for FOCUS. The setting of significance threshold is the genes in a 90%-credible set with PIP ≥ 0.5.

Identifying Causal Genes Using Gene Prioritization Analysis (DEPICT)

We used Data-driven Expression Prioritized Integration for Complex Traits (DEPICT) (36) to prioritize genes at associated loci based on predicted gene functions. Briefly, DEPICT prioritizes genes based on the assumption that truly associated genes should share functional annotations. By using co-regulation data from 77,840 microarrays and publicly available datasets, DEPICT accurately predicts gene function and generated 14,461 "reconstituted" gene sets. Integrating these precomputed gene functions and the GWAS summary data,

DEPICT prioritizes genes that share predicted functions with genes from the other associated loci more often than expected by chance. As it has been widely used in previous studies for gene prioritization of SCZ (37, 38) and CMD (39–41), we included their results into our study. We only use this method to prioritizes genes for TC. We chose P-value < 1.0×10^{-5} as the GWAS significance threshold, which was recommended by the developers of the DEPICT software. The Benjamini–Hochberg procedure with a threshold of a false discovery rate (FDR) < 0.05 was regarded with statistical significance.

Pathway Enrichment Analyses

KEGG pathway enrichment analyses were carried out using clusterProfiler package (42) as implemented in R. The significance P-values of the KEGG pathways were corrected by the Benjamini–Hochberg procedure with FDR < 0.05.

Gene-Set Enrichment Analysis

In order to explore the biological pathways shared between SCZ and CMD, enriched pathways were identified for each trait using GSA-SNP2 software (43), which only requires the P-values of the SNPs in GWAS data and retains SNPs with 20 kb upstream or downstream of a gene. In this study, we used gene set databases of canonical pathways, KEGG, BioCarta and Reactome, which were downloaded from the Molecular Signatures Database (MSigDB) in GSEA (http://software.broadinstitute.org/gsea/msigdb/index.jsp). The Benjamini–Hochberg method is used for the multiple testing correction. The minimum P-values of the pathway was chosen, and the P-values of pathways with FDR < 0.05 were regarded as statistical significance.

To validate the significant finding, the common pathways identified by GSA-SNP2 were investigated by MAGMA (44) with the same parameter settings (retaining SNPs with 20 kb upstream or downstream of a gene). Unlike GSA-SNP2, we used a nominal P-value threshold of P < 0.05.

RESULTS

To reveal the potential candidate gene for SCZ and CMD, we utilized six different approaches including FUMA, Sherlock, SMR, UTMOST, FOCUS, and DEPICT. To estimate the LD structure, we used the reference data from the European population of 1000 Genomes Project phase 3 (45). All analyses were carried out using the default parameters recommended by the developers if not mentioned in the methods section. A gene may represent a potential candidate gene if it is predicted by two or more than two approaches.

Candidate Genes Identified for SCZ

Functional annotation of the GWAS summary statistics of SCZ was performed using positional mapping in FUMA, and 110 causal genes associated with SCZ was identified (**Supplementary Table S2**). Through integrating the genetic variations associated with SCZ and tissue-specific eQTL data from brain and whole blood, Sherlock, SMR, UTMOST, and FOCUS identified 198, 50, 236, and 109 causal genes associated with SCZ, respectively (**Supplementary Table S2**). In addition, causal genes prioritized by DEPICT were obtained from Pers et al. (37) and Li et al. (38), which predicted 106 causal genes for SCZ (**Supplementary Table S2**). In total, we identified 553 causal genes whose expression level change may contribute to SCZ risk. KEGG pathway enrichment analysis showed that these causal genes were enriched in dopaminergic synapse (corrected P = 3.3×10^{-2}) and adrenergic signaling in cardiomyocytes (corrected P = 4.3×10^{-2}) (**Supplementary Table S10**).

Through integrating these causal genes predicted by the six different approaches, we identified 150 potential candidate genes associated with SCZ (**Supplementary Table S2**). Among the 150 genes, only *ABCB9* was predicted by all approaches, which has been reported to be correlated with the risk of SCZ (46). There are seven genes (*ARL6IP4*, *C2orf47*, *GATAD2A*, *GNL3*, *NT5C2*, *PCCB*, and *SNX19*) predicted by five approaches, two genes (*C2orf47* and *PCCB*) of which have not been reported in the literature yet.

Candidate Genes Identified for BMI

Through integrating the results from FUMA, Sherlock, SMR, UTMOST, and FOCUS, and causal genes predicated by DEPICT for BMI obtained from Võsa et al. (41), we identified 654 causal genes linked to BMI (**Supplementary Table S3**). KEGG pathway enrichment analysis showed that these causal genes were also enriched in dopaminergic synapse (corrected P = 6.8×10^{-4}) and adrenergic signaling in cardiomyocytes (corrected P = 2.5×10^{-4}) (**Supplementary Table S10**), which were identified to be associated with SCZ.

Among the 654 causal genes, most of them are only predicted by DEPICT, and 79 potential candidate genes were identified (**Supplementary Table S3**). Ten genes (*ZNF668*, *NEGR1*, *KAT8*, *SH2B1*, *BCKDK*, *POC5*, *MAP2K5*, *C18orf8*, *NPC1*, and *C1QTNF4*) were at least predicted by four different approaches, thus represent the most promising candidate genes for BMI. The ten promising candidate genes include six genes (*NEGR1*, *KAT8*, *POC5*, *MAP2K5*, *C18orf8*, and *C1QTNF4*) which were previously reported as causal genes in the original study (13), two genes (*SH2B1* and *NPC1*) have been reported to be associated with BMI by other studies (47, 48), and two genes (*ZNF668* and *BCKDK*) were novel.

Candidate Genes Identified for CAD

Using the GWAS summary statistics of CAD, we identified 273 causal genes associated with CAD. In detail, FUMA, Sherlock, SMR, UTMOST, and FOCUS identified 41, 73, 85, and 48 causal genes associated with CAD, respectively (**Supplementary Table S4**). Causal genes predicated by DEPICT for CAD obtained from Võsa et al. (41), which predicted 123 causal genes for CAD. Overall, 53 potential candidate genes were identified to be associated with CAD (**Supplementary Table S4**), and 13 candidate genes (*CARF*, *FAM177B*, *GGCX*, *FAM117B*, *TDRD10*, *SWAP70*, *SUSD2*, *RP1-257A7.5*, *VAMP5*, *SPECC1L*, *RGL3*, *KANK2*, *SLC22A1*) can be considered as novel genes.

Candidate Genes Identified for T2D

Using different approaches (including FUMA, Sherlock, SMR, UTMOST, and FOCUS) to prioritize the causal genes for T2D, we identified 190, 188, 63, 327, and 178 causal genes associated with T2D, respectively (**Supplementary Table S5**). Causal genes predicated by DEPICT for T2D obtained from Scott et al. (40), which predicted 29 causal genes for T2D. Through integrating the results from these approaches, 171 potential candidate genes were identified to be associated with T2D (**Supplementary Table S5**). Among the 171 potential candidate genes, there are eight genes (*ABCB9*, *PABPC4*, *ITFG3*, *ANK1*, *CALR*, *CEP68*, *GCDH*, and *ZZEF1*) predicted by five approaches, five genes (*PABPC4*, *ITFG3*, *CEP68*, *GCDH*, and *ZZEF1*) of which have not been reported in the literature yet.

Candidate Genes Identified for Dyslipidemia

Through integrating the results from FUMA, Sherlock, SMR, UTMOST, and FOCUS, and causal genes predicated by DEPICT for HDL, LDL, and TG obtained from Bentley et al. (39), we identified 104, 74, 101, and 71 potential candidate gene associated with HDL, LDL, TC, and TG, respectively (**Supplementary Tables S6–9**). Among these potential candidate genes, there are six genes (*CETP*, *APOB*, *TMEM258*, *FADS2*, *FADS1*, and *PVRL2*) associated with all phenotypes of dyslipidemia. Similar to our results, all of the six genes were previously reported to be associated with at least one phenotype of dyslipidemia. Overall, 245 potential candidate genes were identified to be associated with dyslipidemia.

Similar to our results, genetic variants in *NT5C2* were previously reported to be associated with the comorbidity of SCZ and BMI (6), and genetic variants in or around *MPHOSPH9* were reported to be increased risk of T2D in SCZ (49). The remaining genes are considered as novel genes associated with the comorbidity of SCZ and CMD.

Pleiotropic Genes Identified for the Comorbidity of SCZ and CMD

Through integrating the potential candidate genes identified for SCZ and CMD, we identified 21 potential pleiotropic genes

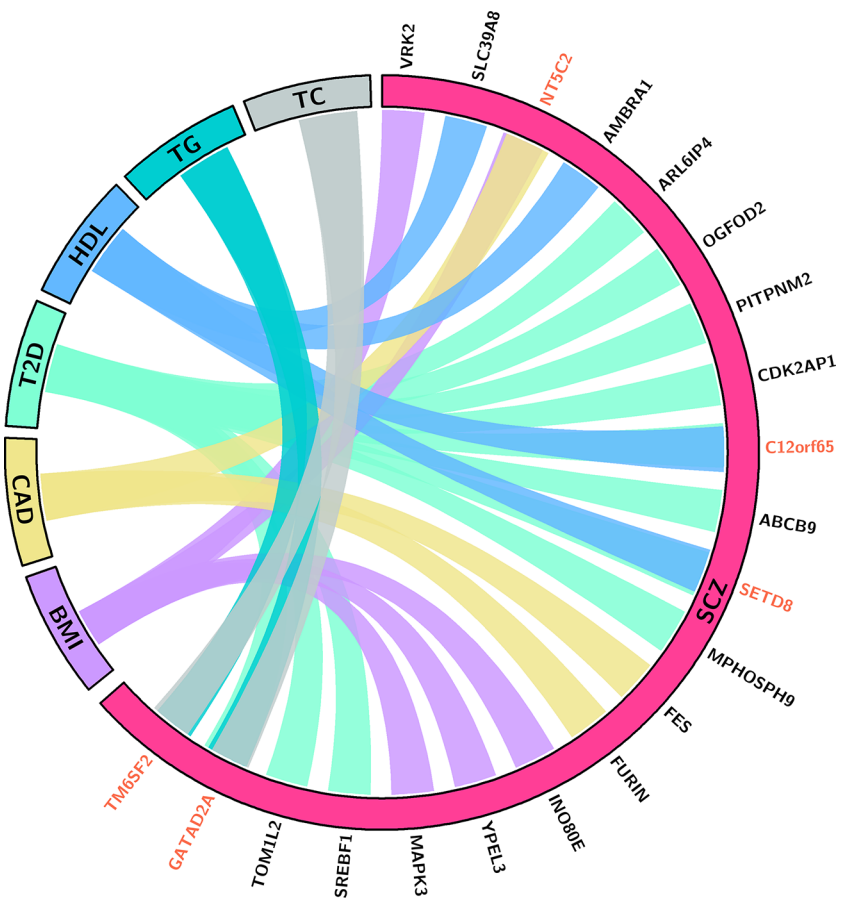

FIGURE 2 | This chord (50) diagram depicts the potential pleiotropic genes shared between SCZ and CMD. Connections indicate that the pleiotropic genes for SCZ and specific phenotype of CMD. Red indicates the gene is associated with SCZ and multiple phenotypes of CMD. BMI, body mass index; CAD, coronary artery diseases; T2D, type 2 diabetes; HDL, high-density lipoproteins; TC, total cholesterol; TG, triglycerides.

TABLE 1 | The potential pleiotropic genes shared between SCZ and CMD are identified in this study.

Gene symbol	Chromosome and position	Diseases (prediction methods)
VRK2	ch2: 257907651-58164001	SCZ (FUMA/UTMOST), BMI (UTMOST/DEPICT)
SLC39A8	ch4:102251041-102345498	SCZ (FUMA/FOCUS), HDL (FUMA/Sherlock)
NT5C2	ch10: 103088017-103193306	SCZ (FUMA/Sherlock/SMR/UTMOST/FOCUS), BMI (Sherlock/FUMA), CAD (Sherlock/SMR/UTMOST)
AMBRA1	ch11: 46396412-46594069	SCZ (FUMA/DEPICT), HDL (UTMOST/DEPICT)
ARL6IP4	ch12: 122980060-122982913	SCZ (FUMA/Sherlock/SMR/UTMOST/FOCUS), T2D (FUMA/Sherlock)
OGFOD2	ch12: 122974703-122980041	SCZ (FUMA/UTMOST/FOCUS), T2D (FUMA/UTMOST)
PITPNM2	ch12: 122983480-123150015	SCZ (FUMA/Sherlock/SMR/FOCUS/DEPICT), T2D (FUMA/FOCUS)
CDK2AP1	ch12: 123260970-123272316	SCZ (Sherlock/UTMOST), T2D (FUMA/Sherlock/UTMOST)
C12orf65	ch12: 123233297-123257959	SCZ (FUMA/UTMOST), T2D (FUMA/Sherlock/UTMOST), HDL (Sherlock/DEPICT)
ABCB9	ch12: 122917324-122975160	SCZ (FUMA/Sherlock/SMR/UTMOST/FOCUS/DEPICT), T2D (FUMA/Sherlock/SMR/UTMOST/FOCUS)
SETD8	ch12: 123384116-123409356	SCZ (FUMA/Sherlock/UTMOST/DEPICT), T2D (Sherlock/UTMOST/DEPICT), HDL (UTMOST/FOCUS/DEPICT)
MPHOSPH9	ch12: 123152324-123244014	SCZ (FUMA/UTMOST), T2D (FUMA/SMR/UTMOST/FOCUS)
FES	ch15: 90884421-90895776	SCZ (FUMA/Sherlock/SMR/UTMOST), CAD (Sherlock/SMR/UTMOST/DEPICT)
FURIN	ch15: 90868592-90883458	SCZ (FUMA/SMR/UTMOST/FOCUS), CAD (SMR/DEPICT)
INO80E	ch16: 29995690-30005794	SCZ (FUMA/Sherlock/UTMOST/FOCUS/DEPICT), BMI (Sherlock/UTMOST/DEPICT)
YPEL3	ch16: 30092314-30096216	SCZ (Sherlock/UTMOST/DEPICT), BMI (Sherlock/DEPICT)
MAPK3	ch16: 30114105-30123309	SCZ (Sherlock/SMR/UTMOST/FOCUS/DEPICT), BMI (Sherlock/DEPICT)
SREBF1	ch17: 17811349-17837017	SCZ (Sherlock/SMR/UTMOST/FOCUS), T2D (SMR/UTMOST/FOCUS)
TOM1L2	ch17: 17843508-17972470	SCZ (Sherlock/UTMOST/FOCUS/DEPICT), T2D (SMR/UTMOST/FOCUS)
GATAD2A	ch19: 19385803-19508932	SCZ (FUMA/Sherlock/SMR/UTMOST/FOCUS), T2D (FUMA/Sherlock/UTMOST/DEPICT), TC (Sherlock/UTMOST), TG (Sherlock/UTMOST)
TM6SF2	ch19: 19264365-19273265	SCZ (Sherlock/FOCUS), TC (Sherlock/DEPICT), TG (Sherlock/DEPICT)

SCZ, schizophrenia; CMD, cardiometabolic disease; BMI, body mass index; CAD, coronary artery diseases; T2D, type 2 diabetes; HDL, high-density lipoproteins; TC, total cholesterol; TG, triglycerides.

shared between them (**Figure 2**, **Table 1**). Specifically, *VRK2*, *NT5C2*, *INO80E*, *YPEL3*, and *MAPK3* are the common candidate genes of SCZ and BMI; *NT5C2*, *FES*, and *FURIN* are the candidate genes for both SCZ and CAD; *ARL6IP4*, *OGFOD2*, *PITPNM2*, *CDK2AP1*, *C12orf65*, *ABCB9*, *SETD8*, *MPHOSPH9*, *SREBF1*, *TOM1L2*, and *GATAD2A* are associated with the comorbidity of SCZ and T2D; *SLC39A8*, *AMBRA1*, *C12orf65*, and *SETD8* are associated with the comorbidity of SCZ and HDL; *GATAD2A* and *TM6SF2* are the common candidate genes of SCZ, TC, and TG.

Common Pathway Identified for the Comorbidity of SCZ and CMD

We conducted KEGG pathway enrichment analysis for the causal genes of SCZ and CMD, however, there is no significant pathway enrichment shared between SCZ and CMD. To further explore whether there are some pathogenic pathways shared between SCZ and CMD, we performed gene set enrichment analysis using GSA-SNP2 with GWAS summary datasets. The results show that there are nine pathways shared between SCZ and CMD (**Table 2**, **Supplementary Table S11**). Among these significant pathways, there are two pathways including CXCR4

TABLE 2 | The potential biological pathways shared between SCZ and CMD are identified in this study.

Pathway	GSA-SNP2							MAGMA		
	BMI	CAD	T2D	LDL	TC	TG	SCZ	BMI	T2D	SCZ
M13494	0.377	**3.49E–03**	0.679	0.295	0.329	0.652	**0.044**	0.177	0.639	**0.02**
M882	**0.018**	**0.038**	0.134	**0.018**	0.056	0.585	**0.041**	0.148	0.134	0.258
M16811	**0.042**	0.264	1.0	**0.043**	0.069	0.193	**6.19E–03**	0.411	**0.018**	**3.24E–03**
M9043	0.175	0.269	**7.36E–03**	**1.22E–03**	**8.92E–03**	0.246	**0.044**	0.803	**0.012**	**0.018**
M255	0.081	**0.013**	**4.4E–03**	0.862	0.487	0.898	**0.04**	0.103	**3.71E–05**	0.2358
M270	**6.76E–04**	0.182	1.0	0.771	0.719	0.898	**0.013**	**6.54E–03**	0.096	**0.022**
M756	**6.05E–03**	0.948	1.0	0.833	0.799	**0.027**	**0.02**	0.117	0.714	0.069
M1921	**0.011**	0.540	**1.04E–04**	0.873	0.799	0.769	**0.036**	0.08	**5.01E–04**	**0.041**
M910	**0.018**	0.220	0.768	0.777	0.763	0.898	**0.036**	0.141	0.223	0.065

The Black body indicates the significance of each approach for diseases. M13494, biopeptides pathway; M882, CXCR4 pathway; M16811, ERK5 pathway; M9043, growth hormone signaling; M255, PID-HIF1 pathway; M270, MAPK-TRK pathway; M756, peptide hormone biosynthesis; M1921, regulation of insulin secretion; M910, synthesis, secretion, and inactivation of glucose-dependent insulinotropic polypeptide; LDL, low-density lipoproteins.

pathway and growth hormone pathway shared between SCZ and three phenotypes of CMD.

To further validate the significant finding, the common pathways identified by GSA-SNP2 were investigated by MAGMA. However, only one pathway (MAPK-TRK pathway) shared between SCZ and BMI, and two pathways including growth hormone signaling and regulation of insulin secretion signaling shared between SCZ and T2D were confirmed by MAGMA (**Table 2**, **Supplementary Table S12**). Among the three pathways, regulation of insulin secretion signaling was previously reported to be associated with the comorbidity of SCZ and CMD (8). The other pathways can be considered as novel.

DISCUSSION

As the key risk factor for CVD, CMD was getting more prevalent among patients with SCZ which is chiefly responsible for increasing risk of CVD morbidity and mortality in SCZ. Although unhealthy lifestyle factors and the side effects of antipsychotic medications have been primarily attributed to the high prevalence of CMD in SCZ, shared genetics between SCZ and CMD might also be of importance. Previous studies have shown that both SCZ and CMD are high heritable and polygenic (51, 52). Recent studies have identified numbers of risk loci that are associated with the comorbidity of SCZ and CMD (6). These evidences provide the foundation for the genetic factors contribute to the comorbidity of SCZ and CMD. In this study, by utilizing several well-characterized methods of integrating the GWAS summary statistics of SCZ and CMD, and tissue-specific eQTL data and gene set database to translate the genetic risk loci into potential causal genes and pathways for them, we systematically predicted the candidate genes and biological pathways for SCZ and CMD. Through integrating the results from different approaches, we first revealed 21 potential pleiotropic genes and three biological pathways that are likely to be shared between SCZ and CMD.

Among the 21 potential pleiotropic genes, there are five genes associated with the comorbidity of SCZ and multiple phenotypes of CMD. *NT5C2* encodes a cytosolic purine 5′-nucleotidase (cytosolic 5′-nucleotidase II) involved in cellular purine metabolism (53), which is associated with SCZ, BMI, and CAD. Previous studies have shown that loss of function of *NT5C2* gene reduced body weight gain, improved glucose tolerance, reduced plasma insulin and triglyceride in high-fat diet mice (54). However, a recent study showed that knockdown of neuronal CG32549 in D. melanogaster, which is similar to NT5C2 protein in human, is associated with impaired motility behavior (55). These results strongly suggest that *NT5C2* may play a key role in SCZ and CMD. *C12orf65* is associated with SCZ, HDL, and T2D. The *C12orf65* gene encodes a mitochondrial matrix protein participating in the process of mitochondrial translation (56). Although multiple phenotypes have been shown to be associated with mutations in *C12orf65* gene, including early-onset optic atrophy, encephalomyopathy, peripheral neuropathy, intellectual disability, and spastic paraparesis (56–58), the function of *C12orf65* gene remains largely unknown. *SETD8* (*SET8*/*Pr-SET7*/*KMT5A*) is the causal gene for SCZ, HDL, and T2D. As a member of methyltransferase family specifically targeting Histone H4 Lys20 for methylation, *SETD8* plays an important role in cellular senescence, proliferation and apoptosis (59–61). Consistent with our results, multiple experiments indicated that changed expression level of *SETD8* may affect insulinoma cell proliferation (62), hyperglycemic memory (63), and lipid metabolism (64). One study has shown that reducing the expression levels of *SETD8* may contribute to altered hippocampal cellular composition, impaired neurodevelopment, and subsequent neurocognitive impairment (65), which are associated with the phenotype of SCZ. *GATAD2A* is a subunit of the nucleosome remodeling and histone deacetylase (NuRD) complex, which is generally associated with embryonic development, cellular differentiation, and the repression of transcription (66). Although our results showed that *GATAD2A* is associated with SCZ, T2D, TC, and TG, the pathogenesis is still unclear. Recent studies showed that NuRD complex is involved in neuronal development (67), cardiac and skeletal muscle structural and metabolic (68). These results indicated that *GATAD2A* may contribute to the comorbidity of SCZ and CMD. *TM6SF2* is associated with SCZ, TC, and TG. The *TM6SF2* gene encodes a multi-pass membrane protein localized in the endoplasmic reticulum and the ER-Golgi intermediate compartment (69). Several studies *in vivo* and *in vitro* have proved that *TM6SF2* is closely related to abnormal metabolism of blood lipids, especially plasma TC and TG (70, 71). However, the detailed mechanisms contributing to SCZ are still poorly understood.

Beyond these genes associated with the comorbidity of SCZ and multiple phenotypes of CMD, there are 16 potential pleiotropic genes associated with SCZ and one phenotype of CMD. In detail, *VRK2*, *INO80E*, *YPEL3*, and *MAPK3* are the common candidate genes for SCZ and BMI. *FES* and *FURIN* are the candidate genes for both SCZ and CAD. *ARL6IP4*, *OGFOD2*, *PITPNM2*, *CDK2AP1*, *ABCB9*, *MPHOSPH9*, *SREBF1*, and *TOM1L2* are associated with the comorbidity of SCZ and T2D. *SLC39A8* and *AMBRA1* are associated with the comorbidity of SCZ and HDL. Except that two genes (*FURIN* and *SREBF1*) have strongly suggested association with the phenotype of SCZ and CMD, the role of other genes in the pathogenesis of both SCZ and corresponding phenotype of CMD remains unknown. *FURIN* encodes a protein of the proprotein convertases family, which processes proproteins through limited proteolysis and convert them into bioactive proteins and peptides (72). Accumulating evidence suggests that *FURIN* plays a critical role in atherosclerosis through regulation of lipid metabolism and vascular inflammation (73). Recent studies also showed that overexpression of *FURIN* in monocyte/macrophage cell promoted migration, increased proliferation, and reduced apoptosis (74), which might contribute to atherogenesis. Intriguingly, studies of the function of *FURIN* in brain have shown that knockdown of *FURIN* decrease head size, and inhibit human neural progenitor cells migrate (35). Overexpression of *FURIN* enhances long-term potentiation and spatial learning and

memory performance (75). Further study is needed to state the role of *FURIN* in SCZ and CAD. *SREBF1* is a transcription factor participates in lipogenesis (76), insulin resistance (77), and inflammatory response (78), which may contribute to the development of T2D. Intriguingly, a recent study showed that *SREBF1* is associated with multiple subphenotypes of SCZ, such as hyperlocomotor activity in dark, depression-like and aggressive behaviors, and social deficits (79). These evidences suggest that *SREBF1* is likely associated with the comorbidity of SCZ and CMD. Although most of these genes have been confirmed to be related to the occurrence and development of SCZ and CMD, there is no experiment to confirm whether they are related to the comorbidity of SCZ and CMD.

To identify the potential biological pathways for SCZ and CMD, we conducted KEGG pathway enrichment analysis for all significant causal genes of SCZ and CMD. The results show that dopaminergic synapse and adrenergic signaling in cardiomyocytes are likely shared between SCZ and BMI. However, when we performed KEGG pathway enrichment analysis for the potential candidate genes of SCZ and CMD, there is no significant pathway enrichment. This may be caused by the function of some candidate genes are still unclear. To further explore the potential biological pathways shared between SCZ and CMD, we performed gene set enrichment using GSA-SNP2 and MAGMA. Our results showed that MAPK-TRK pathway shared between SCZ and BMI, growth hormone signaling and regulation of insulin secretion signaling shared between SCZ and T2D. The MAPK-TRK pathway is mainly regulated by neurotrophic factor ligands (e.g. brain-derived neurotropic factor, nerve growth factor) binding to tropomyosin-related kinase (Trk) receptor, which was associated with neuronal survival and morphogenesis, hippocampal long-term potentiation, and synaptic plasticity (80, 81). Loss of Trk signaling also has been linked with food intake regulation and body weight (82, 83). These evidences strongly suggest that the MAPK-TRK pathway may be related to the comorbidity of SCZ and BMI. The insulin and growth hormone signaling are closely related to the occurrence and development of T2D, and the biological effects of insulin and growth hormone are involved in lipid metabolism, carbohydrate metabolism, and glucose metabolism (84–86), which are potential therapeutic effectiveness for T2D. Intriguingly, a recent clinical research showed that insulin and growth hormone signaling were associated with the development of SCZ (87). Further research is worthwhile to explore the insulin and growth hormone signaling in the comorbidity of SCZ and CMD.

There were some limitations of the current study. First, the major samples in the GWAS summary datasets came from populations of European ancestry, and it is worthwhile to validate in other ethnic groups. Second, to generate highly credible candidate genes for the comorbidity of SCZ and CMD, the causal gene for a disease was chosen if it is predicated by two or more than two approaches. Although these genes are promising candidate genes for SCZ and CMD, genes supported by individual prediction approach may also have a role in disease. Third, the eQTL datasets used in this study mostly came from normal human tissues, which may miss the candidate gene for diseases. Lastly, though this study identified potential candidate genes shared between SCZ and CMD, further biological experiments are needed to demonstrate the role of these genes in the comorbidity of SCZ and CMD.

In summary, we first characterized the landscape of potential pleiotropic genes and biological pathways that are likely to be shared between SCZ and CMD. Through integrating the GWAS summary statistics, tissue-specific eQTL data and gene set database, we identified some potential candidate genes and biological pathways for SCZ and CMD (including BMI, CAD, T2D, HDL, LDL, TC, and TG), respectively. In total, we revealed 21 potential pleiotropic genes and three biological pathways shared between SCZ and CMD, which will enable us to better understand the etiology for the comorbidity of SCZ and CVD.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. The links to download GWAS datasets can be found in **Supplementary Table S1**. Other data including eQTL datasets and the code of software for data analysis could be obtained from the resource described in *Materials and Methods*.

AUTHOR CONTRIBUTIONS

HL contributed to data analysis and wrote the manuscript. YS and XZ were responsible for technical support and revised the manuscript. SL and DH contributed to data and method preparation. LX and YC contributed to plot pictures and tables. LH and DW were responsible for the study design and supervised the whole study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.00256/full#supplementary-material

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STUDY PROTOCOL
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Study Protocol for a Prospective Longitudinal Cohort Study to Identify Proteomic Predictors of Pluripotent Risk for Mental Illness: The Seoul Pluripotent Risk for Mental Illness Study

Tae Young Lee[1], Junhee Lee[2], Hyun Ju Lee[2], Yunna Lee[3], Sang Jin Rhee[2], Dong Yeon Park[4], Myung Jae Paek[5], Eun Young Kim[2], Euitae Kim[6], Sungwon Roh[7], Hee Yeon Jung[8], Minah Kim[2], Se Hyun Kim[2], Dohyun Han[2], Yong Min Ahn[2], Kyooseob Ha[2] and Jun Soo Kwon[2]*

[1] Department of Neuropsychiatry, Pusan National University Yangsan Hospital, Yangsan, South Korea, [2] Department of Neuropsychiatry, Seoul National University Hospital, Seoul, South Korea, [3] Department of Neuropsychiatry, Kosin University Gospel Hospital, Pusan, South Korea, [4] Department of Psychiatry, National Center for Mental Health, Seoul, South Korea, [5] Department of Psychiatry, The Armed Forces Capital Hospital, Seongnam, South Korea, [6] Department of Neuropsychiatry, Seoul National University Bundang Hospital, Seongnam, South Korea, [7] Department of Neuropsychiatry, Hanyang University Hospital, Seoul, South Korea, [8] Department of Psychiatry, SMG-SNU Boramae Medical Center, Seoul, South Korea

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Edited by:
Shaohua Hu,
Zhejiang University, China

Reviewed by:
Massimo Tusconi,
University of Cagliari, Italy
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***Correspondence:**
Kyooseob Ha
kyooha@snu.ac.kr

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Background: The Seoul Pluripotent Risk for Mental Illness (SPRIM) study was designed to identify predictors leading to mental illness in help-seeking individuals by securing sufficient statistical power through transdiagnostic approaches. The SPRIM study aims to examine the clinical characteristics of high-risk individuals for mental illness and to identify proteomic biomarkers that can predict the onset of mental illness.

Methods: This paper describes the study protocol of the SPRIM study. We aim to recruit 150 participants who meet the criteria for high risk for major mental illness, 150 patients with major psychiatric disorders (schizophrenia, bipolar disorder, and major depressive disorder), and 50 matched healthy control subjects for 2 years. Clinical evaluations, self-report measures, and proteomic analyses will be implemented. The assessment points are at baseline, 6, 12, 18, and 24 months.

Conclusions: In the present study, we introduced the study protocol of the SPRIM study, which is the first prospective cohort study of transdiagnostic high-risk concepts using proteomic biomarkers. This study has a paradigm that encompasses various diseases without aiming at predicting and preventing the development of a specific mental illness in help-seeking individuals. The transdiagnostic high-risk concept could be extended to provide a perspective for people with various psychopathological tendencies below a threshold, such that they do not meet the existing diagnostic criteria of mental illnesses, to determine what may lead them to a specific disease and help identify appropriate preventative interventions.

Keywords: bipolar disorder, high-risk for mental illness, major depressive disorder, pluripotential, proteomics, schizophrenia, transdiagnostic

INTRODUCTION

For the past 20 years, the concept of ultra/clinical high-risk for psychosis (CHR-P) has been proposed and developed for the early detection and prevention of the transition to psychosis (1–4). Previous studies have shown good predictability compared with other medical diseases, and it was expected that effective interventions for early psychosis would soon be available (5). However, as time went on, the incidence of psychotic transition in CHR-P was found to gradually decrease in several cohort studies, and the reasons, such as lead-time bias due to early referral, high rate of false positives, and prevention through effective treatments, have been discussed (6–8). Recent studies have revealed that CHR-P is not pluripotent but highly specific for psychosis, and the problem of comorbidity is not significantly affected (9, 10), however since the necessity of specialized clinics with trained experts, we realized that the current high-risk concept covers only a fraction of the total schizophrenia incidence (11, 12). This epidemiologic inadequacy is also supported by the fact that psychosis still develops, although at a low rate, in clinical high-risk for non-psychotic mental disorders (13). Lack of the pragmatic transdiagnostic ability of the CHR-P designation and modest statistical power due to the recruitment difficulties and low conversion rate do not given much latitude in high-risk research (14, 15). As a way to overcome the limitation of statistical power, a method of expanding to a broad range of disorders has been proposed instead of limiting the outcome to schizophrenia. Recently, McGorry and his colleagues presented the CHARMS approach, a pioneering new concept, which is a transdiagnostic cohort program (16). This program is a high-risk program that covers a variety of outcomes, including psychotic disorder, bipolar disorder, depressive disorder, etc., unlike the existing CHR cohort program aimed at the onset of psychosis. The CHARMS approach is somewhat different from the current CHR concept that targets only schizophrenia and more closely reflects the clinical staging model that distinguishes a distress that needs only a little help or a stage that requires clinical attention just before transitioning over the threshold on the continuum of mental illness (17, 18). Despite the fact that there are already other high-risk approaches such as the bipolar prodrome, it is expected that the high-risk studies adopting this clinical staging perspective will provide a transdiagnostic understanding of mental illnesses (19–21). Although there have been many studies on the transition and remission in high-risk populations, few studies have been conducted using the definition of transdiagnostic high risk and investigated the long-term outcomes from this approach (22–24). However, this approach is a more common practice in the community because it is more practical for addressing mental illness that can be performed under normal circumstances and does not need a specialized clinic. The cohort program for a broad range of mental disorders is expected to provide greater flexibility in finding biomarkers to predict the development of mental disorders based on higher statistical power by recruiting a wider range of subjects.

The identification of biomarkers in mental illness plays an essential role in the differential diagnosis of disease and prediction of prognosis (25, 26). In psychiatry, which is based on phenotypic classification, biomarker research has a very long history (27, 28). In high-risk for psychosis, meta-analytical sequential testing simulations showed that probabilistic risk assessment using the information from clinical assessment has a greater predictive accuracy compared with that of the phenotypical model only (29). Moreover, considering phenotypical nondisjunction and biotypical reclassification of schizophrenia spectrum disorders or schizophrenia and bipolar disorder, applying the current high-risk concepts based on the existing classification systems of mental illness makes it more difficult to predict outcomes (30, 31). Therefore, a transdiagnostic approach with biomarkers would be a prerequisite for more accurate diagnosis and prognosis prediction. Recently, biomarker studies using proteomics have gained more attention (32, 33). Proteomics generally refers to the large-scale experimental analysis of proteins and proteomes; it has the advantage of being able to identify alterations in certain steps of the biochemical pathways of mental illness because it can distinguish the overexpression of specific proteins (34). Proteomic analyses are used to identify disease-specific alterations, including those for schizophrenia, bipolar disorder, and major depressive disorder (35). Furthermore, proteomics profiling has revealed that inflammatory biomarkers may serve as predictors of antidepressant treatment response (36). Besides, data-driven proteomic analysis using feature selection is expected to provide a new basis for the transdiagnostic approach (37, 38).

Since proteomic analysis is performed through blood sampling, it can be performed more easily than other biomarker studies, including neuroimaging or electrophysiology. However, no studies have used proteomics analyses in a high-risk group before the onse of the disease. Therefore, studies using proteomic biomarkers in a high-risk population before a transition to mental illnesses will enable further classification and prediction at the molecular level.

In this prospective cohort study, we have three goals. First, we will examine the clinical characteristics and clinical outcomes for subjects who do not meet the diagnostic criteria for mental illness, and these subjects will be classified as high-risk individuals for specific psychiatric disorders (schizophrenia, bipolar disorder, major depressive disorder, and undifferentiated). Second, we will identify proteomic biomarkers that differentiate (1) patients with mental illnesses and healthy individuals, and (2) patients with schizophrenia, bipolar disorder, and major depressive disorder. Third, we will attempt to explore the proteomic markers that predict the transition of the high-risk group to mental illness.

METHODS

Design

The Seoul Pluripotent Risk for Mental Illness (SPRIM) study is a prospective cohort study and aims to recruit 150 participants

who meet the criteria for high risk for major mental illness for 2 years. To verify disease-specific proteomic profiles and to use them as templates for classifying and predicting the course of high-risk individuals, we will also recruit 150 patients with major psychiatric disorders (schizophrenia, bipolar disorder, major depressive disorder) and 50 matched, healthy control subjects.

Participants are being recruited from the Seoul National University Hospital, Seoul National University Bundang Hospital, SMG-SNU Boramae Medical Center, Hanyang University Hospital, Inha University Hospital, National Mental Health Center, Korean Armed Forces Capital Hospital, and Gwanak-gu Public Health Center. All institutions are located in Seoul, covering community, military service, and general hospital populations. All participants are referred to the Seoul Youth Clinic or Mood Disorders Clinic of Seoul National University Hospital and evaluated by experienced psychiatrists.

Sample

Potential participants are high-risk individuals for major mental disorders aged 15–45 who were recruited and referred to Seoul National University Hospital. High-risk subjects participating in the SPRIM study are classified into four categories. The inclusion criteria for each group are shown in **Table 1**. Patients with major mental disorders (schizophrenia, bipolar disorder, and major depressive disorder) who had been diagnosed within the previous 5 years are also being recruited from the Seoul National University Hospital. The exclusion criteria are as follows: the presence of mental retardation, a pervasive developmental disorder, neurological disorders, a history of head trauma with loss of consciousness, pregnancy, medical conditions that may cause mental illness, and substance abuse.

Procedure

The first recruitment of high-risk subjects started in September 2017 and is expected to be completed in September 2019, and the follow-up evaluations will be continued until September 2021. After obtaining informed consent, a baseline interview is scheduled. If the subject is a minor, informed consent from their guardian is also obtained. A flow chart of the study procedure is shown in **Figure 1**. For the high-risk participants, they receive prospective follow-up evaluations, and the assessment points are at baseline, 6, 12, 18, and 24 months. A long-term follow-up investigation will continue at 6-month intervals if desired by the subjects, even if the 24-month

TABLE 1 | SPRIM study criteria.

Subgroup	Description
Psychosis risk group	• 15–45 years old • Attenuated psychosis group. Meeting all of the following criteria: (1) At least one SOPS score rated 3–5 points from PSP1 to PSP5; (2) Symptoms occurred during the past years, and the current score is higher than 12 months ago; (3) Symptoms appear at least once a week in the past month. • Brief limited intermittent psychotic symptoms group. Meeting all of the following criteria: (1) At least one of SOPS score of 6 points was obtained from PSP1 to PSP5; (2) Symptoms have started within the last 3 months; (3) Symptoms now appear more than once a month, at least several minutes per day. • Exclusion criteria: antipsychotics are prescribed for more than 30 days.
Bipolar risk group	• 15–45 years old • Common inclusion criteria: Meeting criteria for at least one group from the below in the last 12 months. • Subthreshold mania group: manic episodes lasting more than two days, less than four days. • Depression + cyclothymic features group: Meeting all of the following criteria: (1) at least a week, there is depressed mood or loss of interest or pleasure; (2) two or more items from diagnostic criteria A of major depressive disorder in DSM; (3) multiple episodes with subthreshold manic symptoms not meeting group I criteria and numerous episodes with depressive symptoms. • Depression + genetic risk group: The criteria for depression listed above AND the presence of first-degree relatives with bipolar disorder. • Cyclothymic features and genetic risk group: Meeting all of the following criteria: (1) Numerous episodes with subthreshold manic symptoms, not meeting group I criteria and numerous episodes with depressive symptoms; (2) the presence of first-degree relatives with bipolar disorder. • Subthreshold mixed episode group: Meeting all of the following criteria: (1) Subthreshold mania; (2) depressed mood nearly every day but less than five consecutive days. • Mood swings group: There is a recent onset of mood instability. • Exclusion criteria: (1) history of psychosis that lasted more than seven days in SIPS evaluation regardless of treatment; (2) history of mood stabilizer treatment for six weeks or more; (3) history of antipsychotic treatment for more than three weeks, and (4) history of bipolar I disorder. • Extended bipolar risk group: Participants who have been diagnosed with the following mental illness before the age of 25 and has not yet passed 5 years; bipolar II disorder, cyclothymia, bipolar disorder NOS, recurrent MDD (recurrent MDD, regardless of the duration after diagnosis in case of recurrent MDD).
Depression risk group	• 15–45 years old • Meeting all of the following criteria in the last 12 months: (1) at least 1 week of a depressed mood or loss of interest or pleasure; (2) two or more items on diagnostic criteria A for major depressive disorder from the DSM. • Exclusion criteria: major depressive episodes lasting more than two weeks.
Undifferentiated risk group	• 15–45 years old • Subjects who complained of psychotic or mood symptoms not meeting the criteria above but meeting all of the following criteria: (1) symptoms experienced by the subject are causing distress and help-seeking, (2) cares where the clinician judges that the follow-up observation is necessary due to the possibility of future mental illness. • Extended undifferentiated risk group. Meeting either of the following criteria: (1) Those who have been diagnosed with anxiety disorders during the last 5 years and (2) those with a family history of psychosis, bipolar disorders or anxiety disorders.

SOPS, Scale of Prodromal Symptoms; NOS, not otherwise specified; MDD, major depressive disorder.

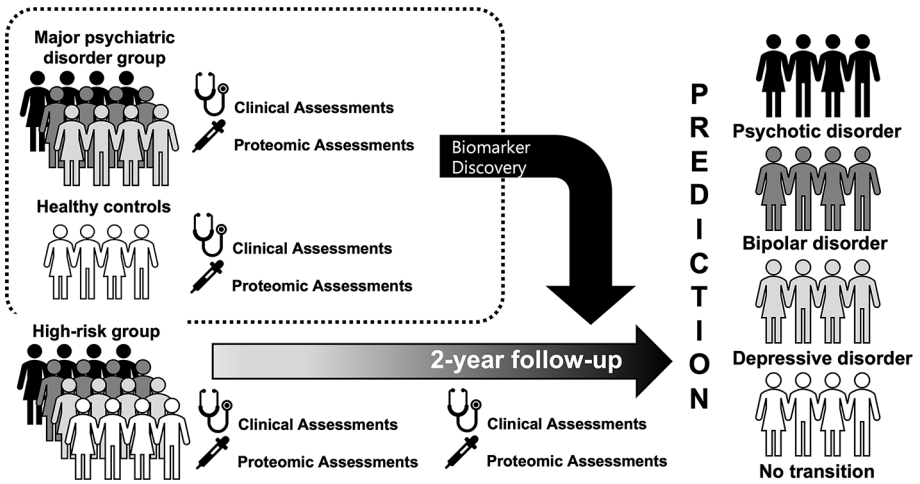

FIGURE 1 | Flow chart showing the recruitment process for the patients and high-risk groups.

follow-up period is over. All high-risk participants will receive case management and supportive psychotherapy on a bimonthly basis and will receive an intensive assessment within the 6-month intervals if they are expected to be converted or remitted. The conversion to a major mental illness and remission from the current high-risk status is determined by an intensive assessment and a consensus meeting. The definition of the transition was defined based on the diagnostic instruments 39, 40, and full remission was defined as no positive symptom item that met the severity corresponding to the criteria for a high-risk group and attribution criteria > 6 months. The patients with major mental disorders are also evaluated at baseline.

Clinical Assessments

At baseline, the high-risk participants are interviewed and diagnosed by a psychiatrist who has had many years of experience through the following diagnostic instruments: Structured Clinical Interview for the DSM-IV (SCID-I, II); Structured Interview for Prodromal Syndrome (SIPS) (39); and Bipolar Prodrome Symptom Scale-Prospective (BPSS-P) (40). To assess the clinical status and function at baseline, the following scales are performed: Positive and Negative Syndrome Scale (PANSS); Hamilton Depression Rating Scale (HAM-D) (41); Hamilton Anxiety Rating Scale (HAM-A) (42); Young's mania rating scale (YMRS) (43); Biological Rhythms Interview of Assessment in Neuropsychiatry (BRIAN) (44); Clinical Global Impression-Severity (CGI-S); Global Assessment of Functioning (GAF) (45); and Global Functioning: Social and Role Scales (GF) (46). The high-risk participants are also interviewed at the 6-, 12-, 18-, and 24-month follow-ups using the same instruments. For the patients with major mental disorders, they are also interviewed at baseline by the psychiatrist using the following diagnostic instruments: SCID-I, II; HAM-D; HAM-A; YMRS; CGI-S; GAF; and Brief Psychiatric Rating Scale (BPRS).

Self-Report Measures

At baseline, the high-risk participants complete the following self-report measures: Barratt Impulsiveness Scale (BIS) (47); Childhood Trauma Questionnaire (CTQ) (48); Morningness-Eveningness Questionnaire (MEQ) (49); WHO Quality of Life-BREF (WHOQOL-BREF) (50); Connor-Davidson Resilience Scale (CD-RISC) (51); Prodromal Questionnaire-Brief (PQ-B) (52); Quick Inventory of Depressive Symptomatology (QIDS) (53); Seasonal Pattern Assessment Questionnaire (SPAQ) (54); and State-Trait Anxiety Inventory (STAI) (55). The high-risk participants also complete the same self-report instruments at the 6-, 12-, 18-, and 24-month follow-up evaluations.

Proteomics Analysis

To elucidate potential blood biomarkers of (1) the disease-specific alterations in schizophrenia, bipolar disorder, and major depressive disorder and (2) the transition to major psychiatric disorders in high-risk groups, mass spectrometry-based proteomics analysis is performed in both the high-risk participants and the patients with major mental disorders. For protein identification and label-free quantification, mass spectra were processed, and peptide lists were searched against the human UniProt FASTA database (version 2014.10). After the plasma collection, sample preparations including protein digestion and peptide purification are performed as described above. Target proteins that are discovered in the major psychiatric disorder cohort are analyzed in the individual samples by liquid chromatography-tandem mass spectrometry (LC-MRM-MS) run using triple quadrupole (QQQ) mass spectrometry. These results are used as a template for each disease to validate the proteomic profiles in high-risk subjects. For proteomic analysis of blood biomarkers in a prospective cohort of transdiagnostic high-risk participants, targeted quantification is performed using multiple reaction monitoring (MRM) (56).

Data Analyses

Descriptive statistics will be used to show the demographic and clinical characteristics of the participants at baseline and to examine the baseline characteristics in relation to the outcomes of the subjects. The Kaplan-Meier survival analysis will be used to examine the transition or remission in each high-risk group and the high-risk group as a whole. Cox regression will be used to determine the difference in hazard ratios between each high-risk group and in the high-risk group as a whole.

DISCUSSION

In the present study, we introduced the study protocol of the SPRIM study, which is the first prospective cohort study of transdiagnostic high-risk concepts using proteomic biomarkers. This study, an extension of studies of high-risk patients with psychosis that has lasted for 20 years, has a paradigm that encompasses various diseases without the aim of predicting and preventing the development of specific mental illnesses in help-seeking individuals. McGorry and his colleagues have already proposed a pluripotent prospective program that reflects this concept. The CHARM study encompasses schizophrenia, mood disorder, and borderline personality disorder outcomes and implements a clinical staging model along the mental health continuum (57). The CHARM study will also provide predictive and discriminant validity of the proposed criteria 16. Our study, on the other hand, focused on identifying the clinical characteristics in an Asian sample, and further look at predictors for blood biomarkers. Therefore, it is expected that the clinical usefulness of the transdiagnostic approach at an early stage will be tested. The transdiagnostic high-risk concept could be extended to provide a perspective for people with various psychopathological tendencies below a threshold, such that they do not meet the existing diagnostic criteria of mental illnesses, to determine what may lead them to a specific disease and help identify appropriate preventative interventions. Since the pluripotent high-risk group encompasses most mental illnesses, studies with large sample sizes will be needed to avoid false-negative consequences. In addition, further studies of other biomarkers that are receiving attention from psychosis will be needed (58–60). Further research into the pathophysiology leading to a risk for a specific mental illness in the pluripotent risk state and more common treatments such as cognitive behavioral therapy or anti-inflammatory therapy should be undertaken.

ETHICS STATEMENT

The study was designed in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board of Seoul National University Hospital (no. 1704-075-846). Written informed consent will be obtained from the participants, or their parents when required.

AUTHOR CONTRIBUTIONS

All authors were responsible for the design of the whole study and wrote the protocol. Author TL wrote the manuscript. All authors supported the manuscript preparation and approved the final manuscript.

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Analysis of the Gut Microbiota and Inflammatory Factors in mGluR5-Knockout Mice

Guohong Cai[1†], Yuanyuan Zhu[1†], Jing Chen[2†], Suo Zhao[1], Liying Wang[1], Mengmeng Wang[1], Jing Huang[1] and Shengxi Wu[1*]*

[1] Department of Neurobiology, School of Basic Medicine, Fourth Military Medical University, Xi'an, China, [2] Department of Anatomy, School of Basic Medicine, Fourth Military Medical University, Xi'an, China

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***Correspondence:**
Jing Huang
jinghuang@fmmu.edu.cn
Shengxi Wu
shengxi@fmmu.edu.cn

[†]These authors have contributed equally to this work

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Introduction: Accumulating evidence indicates that the glutamatergic system plays an important role in the development of depression. Notably, the antidepressant effect of metabotropic glutamate receptor 5 (mGluR5) modulation is inconsistent across studies. Here, we attempted to identify the involvement of the gut microbiota and inflammation in mGluR5$^{-/-}$ mice.

Methods: mGluR5$^{-/-}$ mice and their wild-type littermates were used in our study. We used the open field (OF) and elevated plus maze (EPM) tests to assess anxiety-like behaviors, and we used the two-day forced swim test (FST) and tail suspension test (TST) to test despair-like behaviors. 16S rDNA was used to analyze the gut microbiota. Enzyme-linked immunosorbent assays (ELISAs) were used to measure the levels of inflammatory factors. Western blotting was used to detect the levels of various proteins.

Results: mGluR5$^{-/-}$ mice had no significant increase or decrease of despair-like behavior in the absence of stress exposure. However, mGluR5$^{-/-}$ mice exhibited despair-like behaviors following stress exposure. No significant changes in other glutamate receptors or representative synaptic proteins were detected in the prefrontal cortex (PFC) or hippocampus of mGluR5$^{-/-}$ mice. Very similar bacterial groups were observed in mGluR5$^{-/-}$ mice and wild-type controls. In addition, there was no significant difference in the α-diversity of the microbiota between mGluR5$^{-/-}$ mice and wild-type controls. The levels of all measured cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, and TNF-α) did not change significantly in the PFCs or colons of mGluR5$^{-/-}$ mice.

Conclusion: In conclusion, we deduced that mGluR5$^{-/-}$ mice are susceptible to despair-like behavior. The systemic knockout of mGluR5 did not affect the gut microbiota or inflammatory factors in mice.

Keywords: depression, gut microbiota, inflammation, mGluR5, prefrontal cortex

INTRODUCTION

In the nervous system of vertebrate, glutamate is the most abundant neurotransmitter (1). The glutamatergic system plays an important role in the development of depression and is an essential target of antidepressant drugs (2). Recently, more attention has been focused on the potential roles of metabotropic glutamate receptors (mGluRs) (3–5). The mGluRs are classified into three groups: group I includes mGluR1 and mGluR5, group II includes mGluR2 and mGluR3, and group III includes mGluR4, mGluR6, mGluR7, and mGluR8 (6, 7). Among them, group I mGluRs are distributed primarily at postsynaptic excitatory synapses and are related to many neuropsychiatric diseases, such as anxiety, stress disorders, neurodegeneration, and depression (3, 5, 8).

It is noticeable, however, that the antidepressant-like effect of mGluR5 modulation is still controversial. Some studies showed that the antagonists of mGluR5 can effectively alleviate depression-like behaviors in rodents (9) and that the basal immobility of mGluR5$^{-/-}$ mice decreased in the forced swim test (FST) (9) and tail suspension test (TST) (1). However, Shin et al.'s study showed that in many stress-induced models of depression, mGluR5$^{-/-}$ mice exhibited increased depression-like behaviors which could be reversed by rescue of mGluR5 in the shell of the nucleus accumbens (NAc) (10).

In addition, many studies have shown that the gut microbiota and inflammation play a major role in the pathophysiological process of depression (11–13). Do gut microbiota and inflammation also affect the mGluR5-related mouse model? Here, we attempted to identify the involvement of the gut microbiota and inflammation in mGluR5$^{-/-}$ mice, a controversial model linked to depression.

METHODS

Animals

mGluR5$^{-/-}$ mice and wild-type littermates were purchased from Nanjing BioMedical Research Institute of Nanjing University (NBRI). All experimental mice were housed in groups in a room with controllable temperature and humidity and a 12/12-h light/dark cycle. mGluR5$^{-/-}$ mice were divided into three groups, group one (n = 7) for the open field (OF) and FST, group two (n = 7) for elevated plus maze EPM and TST, and group three (n = 8) for gut microbiota, protein, and inflammatory factor analysis.

Behavioral Testing

Open Field Test

The experimental process is as described in the previous study (14). Briefly, mice were tested in a white plastic box measuring 40 cm × 40 cm × 40 cm under full-light conditions (1,000 lux). At the beginning of the experiment, the mouse was placed in the center of the arena. The duration of each video recording was 5 min. Mice were taken to their home cages after video recording. We used an automated analysis system (SMART 3.0, Panlab S.L.U.) to analyze the time spent in the center of the arena, which was used to evaluate anxiety levels.

Elevated Plus Maze Test

The experimental process is as described in the previous study (14). Briefly, mice were tested in the black plastic equipment with four arms measuring 50 cm × 5 cm each, which were rested on a platform 1 m from the ground. There are two closed arms with 15-cm-high walls and two open arms without walls. At the beginning of the experiment, the mouse was placed in the center of a platform facing one of the open arm. The duration of each video recording was 5 min, after which the mouse was taken to its home cage. We used an automated analysis system (SMART 3.0, Panlab S.L.U.) to analyze the time spent in the open arms, which was used to evaluate anxiety levels.

Two-Day Forced Swim Test

The experimental process is as described in the previous study (14). Briefly, mice were tested in a glass cylinder with 24°C water. The duration of each video recording was 6 min, and afterwards the mouse was taken from the tank into an individual cage for recovery for 90 min. The last 4 min of the FST video was used to assess immobility of the mouse. Twenty-four hours later, the mice were repeatedly tested under the same conditions.

Two-Day Tail Suspension Test

The experimental process is as described in the previous study (15). Briefly, mice were suspended 50 cm above the floor acoustically and visually isolated by adhesive tape, which was placed one-third of the way from the tip of the tail. The last 4 min of the 6-min period was used to analyze the immobility time. Mice were suspended by the tail on 2 consecutive days.

Gut Microbiota Analysis

DNA Extraction and Detection

The fecal samples were collected before behavioral testing, placed in 1.5 ml tubes and stored at −80°C. For mGluR5$^{-/-}$ mice and wild-type mice, fecal samples were collected before behavioral testing. The genomic DNA of each sample was extracted by Beijing Genomics Institute Tech Solutions Co., Ltd. (Shenzhen, Guangdong, China). The microplate reader and agarose gel electrophoresis were used to analyze DNA concentration and integrity.

16S rDNA Compositional Sequencing

The 16S rDNA compositional sequencing process is as described in the previous study (16). Once the DNA sample was received, a quality test was performed first, and then a library was constructed of all the qualified DNAs. The T4 DNA polymerase, Klenow fragment, and T4 polynucleotide kinase were used to convert the jagged ends of PCR products into blunt ends. Then, we added an 'A' base to each 3′ end to make it easier to add adapters. We used fusion primers with dual indexes and adapters for PCR. In both cases, we only used the qualified libraries for sequencing, and the next bioinformatic analysis was based on the results of the sequencing.

Bioinformatic Analysis

The raw data were filtered to obtain clean reads by eliminating adapter pollution and low-quality reads, and then paired-end reads with overlaps were merged to tags, which were clustered into OTUs (operational taxonomic units) at 97% sequence

similarity (17). The Ribosomal Database Project (RDP) Naïve Bayesian Classifier v.2.2. was used to assign taxonomic ranks to OTU-representative sequences. The tag numbers of each taxonomic rank or OTU in different samples were summarized in a profiling table or histogram, which was drawn with the software R (v3.1.1) (18). The different species screenings and α-diversity were analyzed based on OTU and taxonomic ranks.

Brain and Colon Tissue Cytokine Detections

Brain (prefrontal cortex, PFC) and colon tissue of mGluR5$^{-/-}$ mice and wild-type littermates (group three) were collected. Samples were analyzed using the MSD V-Plex Custom Mouse Cytokine kit (4A Biotech Co., Ltd, China) per the vendor instructions.

Western Blot Analysis

The experimental process is as described in the previous study (19). PFC and hippocampal tissues of mGluR5$^{-/-}$ mice and wild-type littermates (group three) were collected and lysed in 100–300 µl of Radio Immunopreciptation Assay (RIPA) lysis buffer (10 mM Tris, 1 mM EDTA, 0.5% NP-40, 150 mM NaCl, and 1% Triton X-100 at pH 7.4). The RIPA lysis buffer contained a 1:100 (v/v) ratio of a protease inhibitor cocktail and a phosphatase inhibitor cocktail (Roche). The BCA protein assay (Pierce) was used to quantify the total protein samples (20–40 µg), and then the samples were resolved *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The primary antibodies were as follows: anti-β-actin (1:1,000, Cell Signaling Technology); anti-mGluR5 (1:1,000, Abcam); anti-NR2A (1:1,000, Cell Signaling Technology); anti-NR2B (1:1,000, Cell Signaling Technology); anti-PSD95 (1:1,000, Abcam); anti-homer1 (1:1,000, Abcam); and anti-Erk1/2 (1:1,000, Cell Signaling Technology). The enhanced chemiluminescence (ECL) detection method (Advansta) was used to visualize all Western blots. Image J software (version 1.47) was used to quantify the scanned images.

Statistical Analyses

All data except for data on the microbiota are expressed as means ± SEM. The unpaired t-test, two-sided t-test, or one- or two-way ANOVA was used to test the statistical significance according to the experimental design. The significant differences in microbiota compositions were assessed by the Mann–Whitney U test. Multiple comparisons were corrected by *P*-values using the Benjamini–Hochberg (BH) correction [false discovery rate (FDR) < 0.05].

RESULTS

mGluR5$^{-/-}$ Mice Exhibited Despair-Like Behaviors After Stress Exposure

Depression is often accompanied by anxiety-like symptoms, therefore, we tested for anxiety-like symptoms in mGluR5$^{-/-}$ mice using the OF test and EPM test. The time spent in the central area of the OF test and the open arms of the EPM test made no significant difference between mGluR5$^{-/-}$ mice and wild-type mice (**Figures 1A, B**).

Although antidepression-like symptoms of mGluR5$^{-/-}$ mice were found by using FST in a previous study (9), our results did not show any difference between wild-type control mice and mGluR5$^{-/-}$ mice in a one-day FST or TST. We then performed two-day FST to test depressive behaviors. Under the two-day FST and TST, wild-type mice and mGluR5$^{-/-}$ mice showed no substantial differences in immobility time on day 1, whereas on day 2, mGluR5$^{-/-}$ mice spent more time immobile than wild-type mice (**Figures 1C, D**). These results suggest that after the first day of stress stimulation, the mice exhibit despair-like symptoms the next day.

mGluR5$^{-/-}$ Mice Showed No Changes in Other Glutamate Receptors or Synaptic Proteins

Previous studies suggest that mGluR5 in the PFC and hippocampus may play key roles in the pathological process of depression (20). In the Western blot analysis, mGluR5$^{-/-}$ mice showed normal levels of synaptic scaffold proteins (homer1 and PSD-95), ionotropic glutamate receptors (NR2A and NR2B), and extracellular regulated protein kinases (Erk1/2) in the PFC and hippocampus (**Figure 2**).

Knockout of mGluR5 Did Not Change the Gut Microbiota

For the controversial mGluR5$^{-/-}$ model, the detection of intestinal microflora may be used to identify the key issues

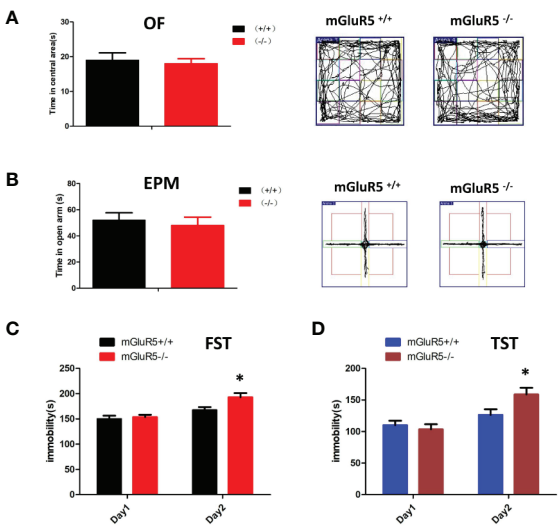

FIGURE 1 | (A, B) mGluR5$^{-/-}$ mice exhibit despair-like behaviors after stress exposure. **(A)** Anxiety-like behaviors assessed in OF tests. **(B)** Anxiety-like behaviors assessed in EPM tests. **(C)** The immobility in FST was measured during the last 4 min of the 6-min video. Two-sided t-test, $^*P < 0.05$. **(D)** The immobility time of TST was measured during the last 4 min of the 6-min video. Two-sided t-test, $^*P < 0.05$. n = 7 mice for mGluR5$^{+/+}$ and 7 for mGluR5$^{-/-}$.

underlying the disease. Therefore, we examined the intestinal microflora of mGluR5$^{-/-}$ mice before stress stimulation. MiSeq sequencing was a total of 1,970,656 raw reads, ranging from 188,224 to 205,284. Paired-end reads were spliced into tags based on the overlap between reads. There were a total of 822,680 tags among all samples, with an average of 82,268 samples and an SD of 705. The stitched tags were optimized to cluster them into OTUs for species classification at 97% similarity. The abundance of the OTUs preliminarily illustrated the species richness of the sample. A total of 476 OTUs were generated from the 10 samples, which were assigned to the taxa from the phylum level to the genus level. The OTU statistics for each sample are shown in **Supplementary Table 1** and **Supplementary Figure 1**.

The taxonomic-composition distribution histograms of each sample are shown at the phylum, class, order, family, genus, and species levels separately. The ratio of the species in each sample is displayed using the histograms. No significant changes of taxonomic-composition distribution in the bacterial groups were observed in the mGluR5$^{-/-}$ mice (**Figure 3**).

α-Diversity is used to analyze the complexity of species diversity by using multiple indices (21), including observed species, Chao, Ace, Shannon and Simpson indices. No significant difference was observed in the α-diversity of microbiota between mGluR5$^{-/-}$ mice and wild-type mice (**Figure 4**).

Cytokines in the PFC Did Not Change Significantly in mGluR5$^{-/-}$ Mice

Cytokines in the PFC, including IL-1β, IL-2, IL-4, IL-6, IL-10, and TNF-α, were assessed as markers of inflammation. The levels of these cytokines did not change significantly in the PFC of mGluR5$^{-/-}$ mice (**Figures 5A–F**).

Cytokines in the Colon Did Not Change Significantly in mGluR5$^{-/-}$ Mice

Colonic levels of inflammatory cytokines play a key role in some patients with depression (22). In our study, the levels of cytokines in the colon, including IL-1β, IL-2, IL-4, IL-6, IL-10, and TNF-α were assessed as markers of inflammation. The levels of these cytokines did not change significantly in the colons of mGluR5$^{-/-}$ mice (**Figures 6A–F**).

DISCUSSION

The mGluR5$^{-/-}$ mouse is a promising genetic tool for studying psychiatric diseases. Our results suggest that mGluR5$^{-/-}$ mice had no significant despair-like or antidepressant behavior in the absence of stress exposure. Furthermore, there was no significant change in the intestinal flora and the levels of inflammatory factors in the PFC and colon. Notably, mGluR5$^{-/-}$ mice exhibited despair-like behaviors following stress exposure.

Previous studies have indicated that mGluR5 was involved in many neuropsychiatric disorders (4, 5). For example, Wijetunge et al. used mGluR5$^{-/-}$ mice to research the role of mGluR5 in pattern formation (23). Carvalho et al. demonstrated that the cortex and striatum of mGluR5$^{-/-}$ mice showed reduced number of neurons at 12 months of age and provided evidence that mGluR5 plays an important role in brain aging through modulating multiple cell types (8). Most recently, a study showed that mGluR5$^{-/-}$ mice have some translationally relevant abnormalities associated with schizophrenia (24).

In fact, the depression-related behaviors of mGluR5$^{-/-}$ mice are still controversial. In 2006, Li et al. demonstrated for the first time that the mGluR5-knockout mouse exhibits antidepressant-like behavior (9). Subsequently, Chen et al. found that mGluR5$^{-/-}$ mice had increased number of spine densities, which may partly explain the hyperexcitability observed in mGluR5$^{-/-}$ mice (25). Recently, Liu et al. verified the antidepressant-like effects of mGluR5 in whole-body knockout mice. In addition, transplanting bone marrow from mGluR5$^{-/-}$ mice to wild-type mice can also induce depression-like behaviors (1).

However, in the present study, we did not detect significant depression-like or antidepressant behavior in mGluR5$^{-/-}$ mice,

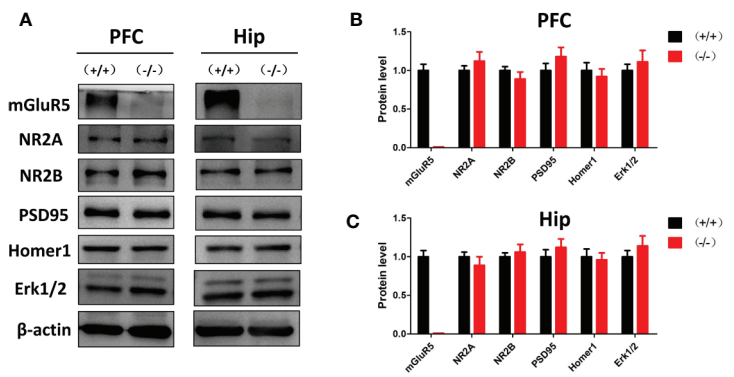

FIGURE 2 | (A) Western blot analysis of the prefrontal cortex and hippocampus of mGluR5$^{+/+}$ and mGluR5$^{-/-}$ mice. **(B, C)** mGluR5$^{-/-}$ mice showed normal levels of NR2A, NR2B, PSD-95, homer1 and extracellular regulated protein kinases (Erk1/2) in the prefrontal cortex and hippocampus. The experiment was successfully repeated three times.

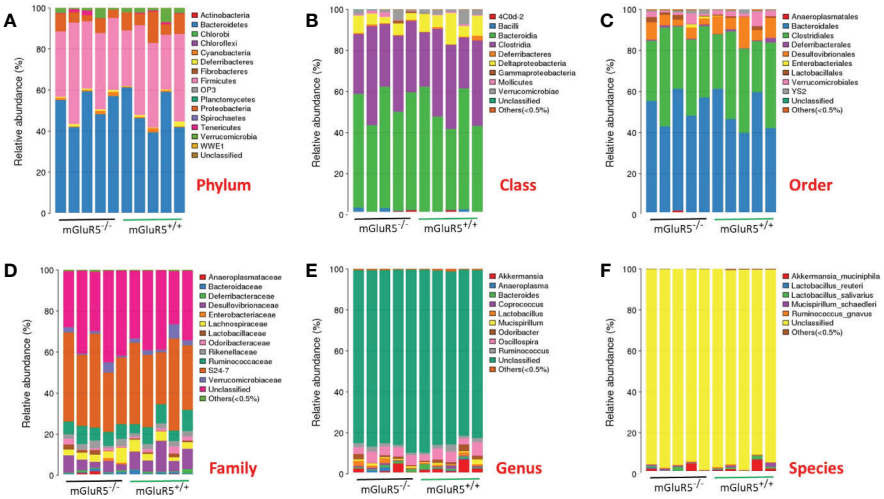

FIGURE 3 | Composition of the gut microbiota at different taxonomic levels. The gut microbiota did not change significantly in mGluR5$^{-/-}$ mice. The taxonomic-composition distribution histograms of each sample are shown at the phylum **(A)**, class **(B)**, order **(C)**, family **(D)**, genus **(E)**, and species **(F)** levels separately. The ratio of each species in a certain sample is displayed by the histogram. The species whose abundances were less than 0.5% in all samples were classified into 'others' in sub-phylum ranks.

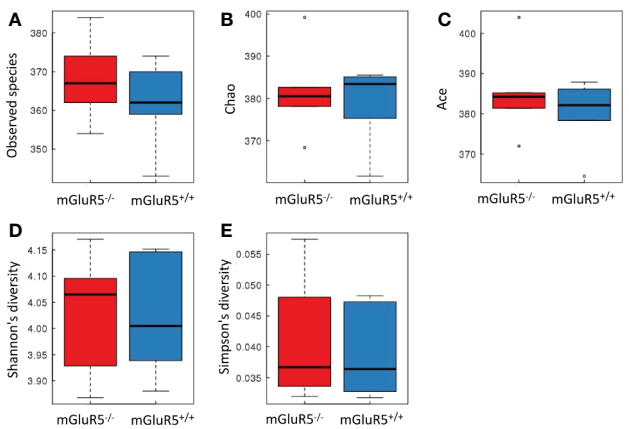

FIGURE 4 | There was no significant difference in the α-diversity of microbiota between groups. Bacterial α-diversity was tested by observed species **(A)**, Chao **(B)**, Ace **(C)**, Shannon **(D)**, and Simpson indices **(E)**. The Wilcoxon rank-sum test was used for comparisons between two groups. The boxplot of α-diversity was drawn, and the analysis above was performed using R software (v3.1.1).

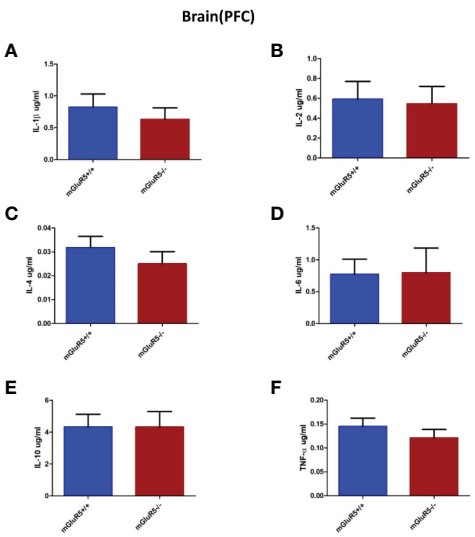

FIGURE 5 | Brain (PFC) cytokine analysis in two groups of mice (mGluR5$^{+/+}$ and mGluR5$^{-/-}$). The levels of all IL-1β **(A)**, IL-2 **(B)**, IL-4 **(C)**, IL-6 **(D)**, IL-10 **(E)**, and TNF-α **(F)** did not change significantly in the PFC of mGluR5$^{-/-}$ mice.

which is similar to the previous study by Shin et al. (10). There are several possible reasons why our findings are inconsistent with previous findings. First, mGluR5 KO mice were constructed differently. For example, the congenic global mGluR5 KO mice used by Liu et al. were based on embryonic stem cell gene targeting technology (1). In our study, the Grm5$^{flox/flox}$ mice were crossed with B6.C-Tg (CMV-cre) mice (NBRI, China) to generate the mGluR5 KO mice. Second, the differences in the process of despair-like behavioral tests may also affect the results. For example, every test session of the TST in Liu et al.'s study lasted for 5 min, and the whole period was scored for immobility (1). In our study and Shin et al.'s study (10), the immobility time of TST was analyzed during the last 4 min of the 6-min period.

In addition to analyzing behavior, we analyzed the gut microbiota, which plays a major role in the pathophysiological processes of depression (11–13). The gut microbiota in stress-induced depression models changes significantly (26–29).

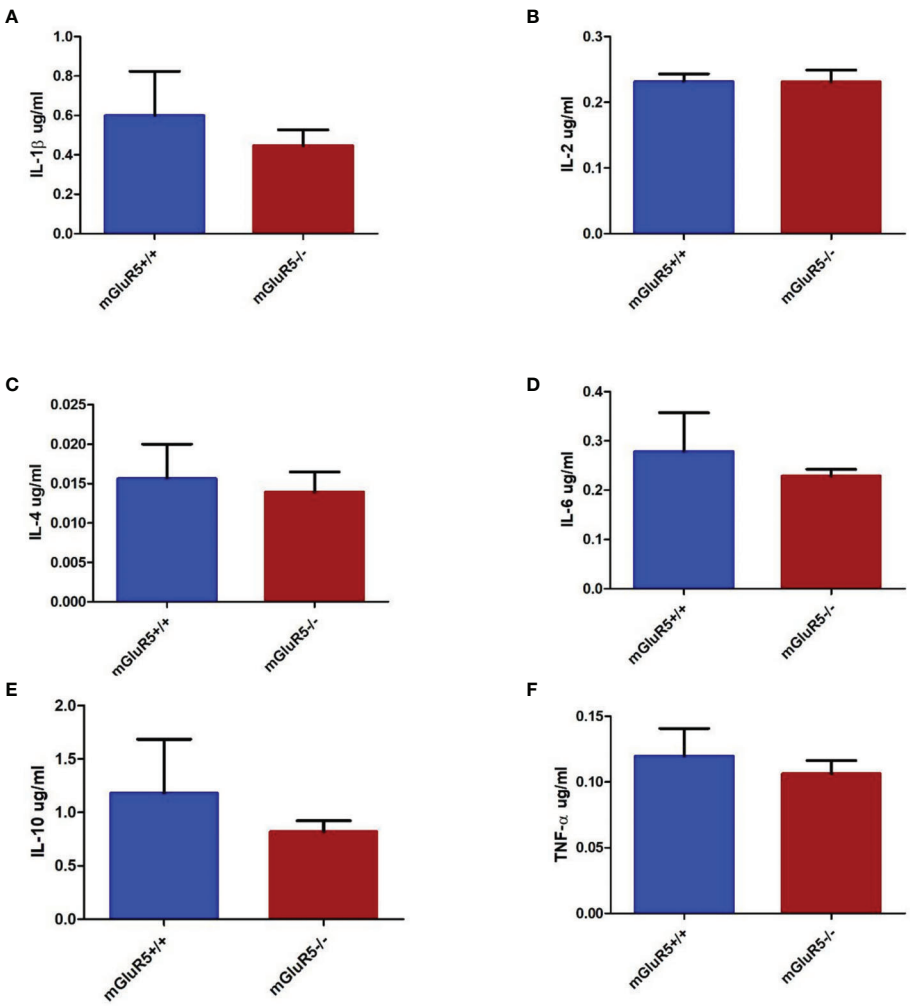

FIGURE 6 | Colon cytokine analysis in two groups of mice (mGluR5$^{+/+}$ and mGluR5$^{-/-}$). The levels of all IL-1β **(A)**, IL-2 **(B)**, IL-4 **(C)**, IL-6 **(D)**, IL-10 **(E)**, and TNF-α **(F)** did not change significantly in the colons of mGluR5$^{-/-}$ mice.

However, no study has been conducted to observe changes in the gut microbiota in mGluR5 KO mice. Consistent with our behavioral results, we did not detect abnormalities in the gut microbiota. A recent study showed that there is no significant correlation between gut microbiome and genetic ancestry, and that host genetics play a secondary role in determining microbiome composition (30). Over 20% of the interpersonal microbiome variability is related to factors associated with drugs, diet, and anthropometric measurements (30). Our results further confirm that the effect of host gene changes on the intestinal microflora may be less pronounced than that of the external stimuli.

Accumulating evidence suggests that inflammation is involved in depression (11, 31). A meta-analysis published in 2010 showed significantly higher levels of proinflammatory cytokines, such as IL-6 and TNF-α, in depressed groups than in control groups (32). In addition, a more recent systematic review concluded that antidepressant treatment significantly decreases IL-6 and TNF-α levels (31). Interestingly, Norman et al. showed that the expression of IL-1β gene increased in the frontal cortex of a depression mouse model and that depression-like behavior is blocked by the injection of an IL-1 receptor antagonist (33, 34), which implicates neuroinflammatory activity in the brain as an underlying mechanism of depression. In the present study, we measured the cytokines in the PFC and colon. The results showed that the levels of the measured cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, and TNF-α) did not change in mGluR5$^{-/-}$ mice, which further suggests the reliability of our behavioral results.

In one previous study, Shin et al. found that mGluR5$^{-/-}$ mice exhibited depression-like behaviors after stress exposure, which can be rescued by increasing mGluR5 expression in the nucleus accumbens (10). The limitations of FST and TST for detecting the resilience state have been documented by several studies using specific gene knockout animal models (10, 35). In our study, the 2 d-FST and 2 d-TST were used instead of the 1 d-FST and 1 d-TST. Consistent with published results, mGluR5$^{-/-}$ mice exhibited despair-like behavioral phenotype after stress exposure in the present study. Although mGluR5$^{-/-}$ mice showed no difference in the expression levels of NR2A, NR2B, PSD-95, homer1 and Erk1/2 in the PFC and hippocampus, the systemic knockout of mGluR5 still caused problems in mood regulation. The mechanism that specifically leads to enhanced susceptibility to despair-like behaviors requires more research.

Our research had some limitations. First, despair-like behavior testing in mice is not comprehensive, and there are many detection methods that have not been applied, such as the novelty-suppressed feeding test, sucrose preference test, nesting test and splash test, *etc*. Second, research on inflammation levels in mGluR5$^{-/-}$ mice requires evidence from other tissues. Third, the sample size of our study was small, and the conclusion we draw needs to be verified by future studies.

In conclusion, we deduced that mGluR5$^{-/-}$ mice are susceptible to despair-like behavior. The systemic knockout of mGluR5 did not affect the gut microbiota or inflammatory factors in mice.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the BioProject ID : PRJNA605506.

ETHICS STATEMENT

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Fourth Military Medical University and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH). All efforts were made to minimize animal suffering and to reduce the number of animals that were used.

AUTHOR CONTRIBUTIONS

SW conceived and designed the experiments. GC and YZ performed most of the experiments and analyzed the data. GC and JC wrote and refined the article. MW participated in the animal modeling and behavioral experiments. LW and SZ assisted in laboratory work and figure preparation. JH supervised the acquisition of results.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.00335/full#supplementary-material

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REVIEW
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Associations Among Monoamine Neurotransmitter Pathways, Personality Traits, and Major Depressive Disorder

Xiaojun Shao[1] *and Gang Zhu*[1,2]*

[1] Department of Psychiatry, The First Affiliated Hospital of China Medical University, Shenyang, China, [2] Central Laboratory, The First Affiliated Hospital of China Medical University, Shenyang, China

Major depressive disorder (MDD) is a complex psychiatric disease requiring multidisciplinary approaches to identify specific risk factors and establish more efficacious treatment strategies. Although the etiology and pathophysiology of MDD are not clear until these days, it is acknowledged that they are almost certainly multifactorial and comprehensive. Monoamine neurotransmitter system dysfunction and specific personality traits are independent risk factors for depression and suicide. These factors also demonstrate complex interactions that influence MDD pathogenesis and symptom expression. In this review, we assess these relationships with the aim of providing a reference for the development of precision medicine.

Keywords: personality traits, mood disorder, major depressive disorder, monoamine neurotransmitters, mechanism

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Dubravka Svob Strac,
Rudjer Boskovic Institute, Croatia

***Correspondence:**
Gang Zhu
gzhu@cmu.edu.cn

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INTRODUCTION

Major depressive disorder (MDD) is the most prevalent mood disorder and the most common disabling psychiatric disease across the globe. In the United States, the lifetime prevalence of MDD is 20.6% (1), and the associated healthcare and economic burdens are surpassed only by cardiomyopathy (2). The most clinically significant symptom of MDD is suicidality (3, 4). Over the years, MDD has been explained in genetic, biological, psychosocial, personality and other terms. No definite explanation accounts for the mechanism of MDD, however. Reducing the morbidity and mortality associated with MDD requires a more complete understanding of disease pathophysiology. Evidence accrued over many decades strongly implicates dysregulation of monoamine neurotransmitter systems in MDD development. Further, there is compelling evidence that MDD risk is strongly associated with certain personality traits. In this review, we expound the underlying relationships among monoamine neurotransmitter systems, personality traits, and MDD.

A biological basis for MDD risk is strongly supported by genetic studies demonstrating moderate heritability (ranging from about 37% and 45%) (5–9). Thus, gene–environment interactions are likely crucial to disease etiology, such as stressful life events (10, 11), childhood maltreatment (including emotional abuse, sexual abuse, emotional neglect, and physical neglect) (12, 13), and in fact these interactions result in an underestimation of the overall genetic influence (14). Kendler et al. reported a genetic correlation for liability to major depression of 0.63 in both males and

females (9), and a similar estimate was reported in a population-based twin study (0.55) (15), consistent with several earlier studies suggesting that genetic risk factors are not sex-specific (16–19). However, the largest-sample twin study reported greater heritability in females (0.49, 95%CI = 0.31—0.56 vs. 0.41, 95% CI = 0.21—0.49), as well as 0.36 (95%CI = 0.31—0.38) in full siblings and 0.51 (95%CI = 0.51—0.53) in half-siblings (20). Several other studies have found a similarly elevated genetic propensity in females (9, 21, 22). These observed differences in MDD heritability between males and females are particularly interesting because recent neuroimaging and molecular genetic studies have also shown potential biological differences in MDD etiology between men and women. Edvardsen et al. reported a higher monozygotic/dizygotic ratio among male twins compared to female twins (8). Alternatively, a sex-limitation model suggested that the same genes influence MDD in males and females (19), although others have found that different genes impacted depressive illness (23). Thus, there is still no consensus on sex differences in the genetics of MDD.

MONOAMINE NEUROTRANSMITTERS AND MDD

Multiple studies have implicated the monoamine neurotransmitters 5-hydroxytryptamine (5-HT or serotonin), dopamine (DA), and norepinephrine (NE) as the primary contributors to MDD etiology. In the mammalian central nervous system (CNS), the major sources of the three monoamines are the raphe nuclei (24), substantia nigra and ventral tegmentum area (VTA) (25), and locus coeruleus, respectively.

Raphe serotonergic neurons project to the caudate, putamen, pallidus, amygdala, limbic forebrain, and neocortex, where 5-HT signaling contributes to motivation, emotion stress processing (26), and regulation of other limbic functions (27). Acute depletion of the 5-HT precursor tryptophan (acute tryptophan depletion, ATD) markedly influences affective experience and emotional regulation in subjects with a family history of MDD (28). Challis et al. reported sensitization of inhibitory GABAergic neurons within the dorsal raphe nuclei and concomitant inhibition of serotonergic activity following social defeat in mice (29). Collectively, human and animal studies of tryptophan depletion (30) and associated serotonergic signaling deficiency strongly implicate 5-HT in mood regulation and MDD pathogenesis. Such insufficient 5-HT signaling may result from both reduced release and lower postsynaptic sensitivity as MDD patients demonstrate both decreased plasma and platelet levels of 5-HT, as well as blunted prefrontal cortical responses to 5-HT (31). Barton et al. reported elevated brain serotonin turnover before antidepressant therapy and markedly reduced turnover after antidepressant therapy and condition improvement, suggesting brain serotonin turnover as a potential biomarker for MDD (32). Further, a recent positron emission tomography (PET) study found reduced binding potential of the 5-HT_{1A} receptor subtype in MDD patients relative to controls, and the authors suggested that lower 5-HT_{1A} activity may result in "decreased engagement of the cognitive control network and impaired resolution of interfering cognitive stimuli" (33). Also consistent with a major contribution of 5-HT signaling dysfunction to MDD, elevated brain turnover of 5-HT is strongly influenced by 5-HT transporter (5-HTT) genotype (32), which in turn is associated with MDD risk. The urine serotonin/dopamine ratio may also be a useful diagnostic indicator for patients with MDD (34). Alternatively, selective serotonergic reuptake inhibitors (SSRIs) like fluoxetine, fluvoxamine, paroxetine, sertraline, and citalopram can enhance brain serotonin levels and are considered the first-line therapies for MDD patients based on demonstrated efficacy in the majority of placebo-controlled clinical studies (35). Growing evidence supports the hypothesis that epigenetic mechanisms, such as DNA methylation, play an important role in psychiatric diseases (36) such as MDD and personality disorders (37, 38), where epigenetic factors bridge the environmental and genetic mechanisms. A multitude of reports have considered the DNA methylation of the serotonin transporter gene (SLC6A4), located on chromosome 17 (39), as the major research target in investigation and evaluation in depression (**Table 1**). In summary, 5-HT is the biogenic amine most strongly associated with depression, as evidenced by the negative influence of 5-HT depletion on mood, the antidepressant efficacy of SSRIs, the perturbed 5-HT turnover and neuronal sensitivity in MDD patients and animal models, and the numerous associations between 5-HT pathway gene polymorphisms and MDD (**Table 1**).

Changes in 5-HT signaling may also predict suicidality. Patients with suicidal impulses exhibited lower cerebrospinal fluid (CSF) concentrations of the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIIA) and fewer 5-HT uptake sites on platelets (92, 93). Weissmann et al. reported increased editing of the 5-HT_{2C} receptor (5-HT_{2C}R) mRNA in cortical areas of depressed suicides compared to non-psychiatric controls, suggesting that region-specific changes in 5-HT_{2C}R function may contribute to MDD etiology (94). Further, altered activities of the major 5-HT biosynthetic enzymes tryptophan hydroxylase 1 and 2 (TPH 1 and TPH 2) (95), of 5-HTT (96), and of serotonin receptors, especially HTR_{1A} (97), HTR_{2A} (98), and HTR_{2C} (99), are associated with suicidal impulses and violent suicidal behavior. However, contradictory findings have been reported (98, 100, 101), possibly due to low statistical power or heterogeneity of study populations. Larger-scale studies of different clinical and ethnic populations may resolve these controversies.

In animal models, genetic and pharmacological manipulation of serotonergic signaling can induce acute depression- and anxiety-like behaviors (102). Further, manipulating serotonergic and dopaminergic signaling during development can affect later-life somatosensory, anxiety/depression-like, and aggressive behavior (103). A recent study found generally lower levels of all three monoamines in a Wistar–Kyoto (WKY) animal model of maternal depression compared to matched control Sprague–Dawley (SD) rats (104).

Norepinephrine (NE) secreted from the locus coeruleus (LC) is a critical modulator of neural circuits involved in learning and

TABLE 1 | Serotonergic gene polymorphisms in MDD.

Reference	Candidate gene	Sample size	Main findings
(40)	serotonin transporter (SERT)	30 (15 healthy controls)	Compared to controls, MDD patents showed reduced SERT in brain.
(41)	5-hydroxyindoleacetic acid (5-HIAA)	68 depressed subjects	Lower 5-HIAA predicted suicide attempt in MDD.
(42)	5-HIAA, SERT	10 matched pairs	5-HIAA and SERT deficiency in depression.
(43)	serotonin transporter (5-HTT) and the serotonin-transporter-linked polymorphic region (5-HTTLPR)	220 subjects	Lower 5-HTT binding related to suicide and MDD. 5-HTTLPR related to MDD but not to suicide or 5-HTT binding.
(11)	5-HTT	1,037 subjects	Short allele of the 5-HTT promoter related to depressive symptoms, diagnosable depression, suicide, and stressful life events.
(44)	5-HTT	549 twins	Individuals expressing 2 short (S) alleles most sensitive to the depressogenic effects of stressful life events.
(45)	The intron 2 (STin2) polymorphism of the serotonin transporter	258 (152 controls)	The STin2 variant predicts suicide in MDD.
(46)	STin2 polymorphism of the serotonin transporter	170 (99 healthy controls)	Significant difference in the genotype frequency of STin2.10/10 in MDD.
(47)	5-HTT	66 (43 healthy controls)	Lower 5-HTT binding potential proportional to the number of available transporters in individuals with childhood abuse.
(48)	the serotonin transporter gene (SLC6A4)	98 subjects	Depressed mood during the 2nd trimester of pregnancy negatively correlated with maternal SLC6A4 promoter methylation status.
(49)	SLC6A4	108 depressed subjects	SLC6A4 methylation status related to childhood adversities and MDD.
(50)	SLC6A4	84 twins	Serotonin transporter receptor gene methylation variation in peripheral blood leukocytes positively related to depressive symptom severity.
(51)	SLC6A4	100 (50 healthy controls)	Compared with healthy controls, no significantly differed with MDD.
(52)	SLC6A4	94 depressed subjects	Reduced SLC6A4 expression related to impaired antidepressant treatment response after 6 weeks.
(53)	SLC6A4, and Serotonin 2A receptor (5-HT_{2A}R)	137 depressed subjects	SLC6A4 AA genotype and A-allele related to antidepressant response.
(54)	SLC6A4	43 (24 healthy controls)	No significant associations with MDD.
(55)	SLC6A4	36 depressed subjects	Three candidate genes, including SLC6A4 related to the etiology of MDD and suicide attempts in Chinese.
(56)	SLC6A4	224 (150 healthy controls)	SLC6A4 allelic variations related to suicidal ideation in MDD.
(57)	SLC6A4	370 Parkinson's Disease patients	SS genotype predicts higher depression risk in Parkinson's disease.
(58)	5-HTTLPR	150 depressed subjects	No significant associations with MDD.
(59)	5-HTTLPR	136 (68 healthy controls)	SS genotype and S allele of 5-HTTLPR related to MDD in children.
(60)	5-HTTLPR	1,206 twins	No association between 5-HTTLPR and MDD.
(61)	5-HTTLPR	316 (125 healthy controls)	L^G and S allele positively correlated with MDD in patients experiencing moderate to severe life events.
(62)	5-HTTLPR	4,175 depressed subjects	Significant association between social adversity and MDD prevalence.
(63)	5-HTTLPR	306 males	The 34-item Childhood Trauma Questionnaire (CTQ) score and 5-HTTLPR level are independent risk factors predicting suicide attempt.
(64)	5-HTTLPR	233 depressed subjects	Associations among 5-HTTLPR polymorphisms, comorbid disorders, and sex in MDD.
(65)	5-HTTLPR	103 depressed subjects	5-HTTLPR SS genotype related to poor antidepressant response in females.
(66)	5-HTTLPR	984 subjects	Trauma was a risk factor for depressive symptoms who carries S/S or S/L genotype.
(67)	5-HTTLPR and Serotonin 2A receptor (5-HT_{2A}R)	132 depressed subjects	5-HT_{2A} A-allele associated with MDD, 5-HTTLPR S allele associated with higher irritability score.
(68)	5-HTTLPR	104 depressed subjects	Statistical association between MDD and 5-HTTLPR L allele.
(69)	5-HTTLPR	121 (66 healthy controls)	No significant associations with MDD.
(70)	5-HTTLPR	1,111 subjects	Limited role of 5-HTTLPR in mediating effects of adolescent/parent relationship on depressive symptoms.
(71)	5-HTTLPR	73 (18 healthy controls)	Decreased fractional anisotropy (FA) related to 5-HTTLPR-S′L′in MDD.
(72)	5-HTTLPR	57 (29 healthy controls)	5-HTTLPR genotype related to mean methylation levels in MDD.
(73)	5-HTTLPR	160 depressed subjects	5-HTTLPR polymorphisms related to dysphoria score on Montgomery-Åsberg Depression Rating Scale (MADRS).
(74)	5-HTTLPR	178 depressed subjects	5-HTTLPR genotype predictive of resistance to SSRI treatment.

(Continued)

TABLE 1 | Continued

Reference	Candidate gene	Sample size	Main findings
(75)	Serotonin 2A receptor (5-HT_{2A}R) and 5-HTTLPR	136 (69 healthy controls)	5-HT_{2A} promoter -1438A variant associated with depressive symptoms of seasonal affective disorder.
(76)	Serotonin 1A receptor (5-HT_{1A}R)	263 (134 healthy controls)	Compared to the healthy controls, depressed individuals twice as likely to carry -1019G genotype.
(77)	5-HT_{2A}R	251 (131 healthy controls)	5-HT_{2A}R 102C allele significantly associated with MDD, particularly in patients with suicidal ideation.
(78)	5-HT_{1A}R	24 (8 healthy controls)	Decreased 5-HT_{1A}R binding potential in MDD compared to controls.
(79)	HTR1A, HTR2A, HTR6, TPH1 and TPH2	481 (395 healthy controls)	No significant associations with MDD.
(80)	5-HT_{2A}R	56 depressed subjects	AA genotype of 5-HT_{2A}R -1438 G/A polymorphism related to sexual dysfunction in male MDD patients.
(81)	5-HT_{2A}R and Serotonin 3A receptor (5-HT_{3A}R)	50 (25 healthy controls)	Increased 5-HT_{2A}R mRNA expression in peripheral blood mononuclear cells of MDD patients.
(82)	SERT, 5-HT_{1A}R, and 5-HT_{2A}R	167 depressed subjects	Lower SERT binding associated with MDD. Both greater 5-HT_{1A} binding and 5-HT_{2A} binding associated with MDD.
(33)	5-HT_{1A}R	25 depressed subjects	Reduced 5-HT_{1A}R binding potential in MDD.
(83)	5-HT_{1A}R, 5-HT_{2A}R and SERT	76 brain samples	Lower 5-HT_{2A} receptor binding in Brodmann areas 41/42 of MDD patients.
(84)	HTR_{1A}	800 (400 healthy controls)	5-HTR_{1A} C (–1,019) G polymorphism significantly related to MDD.
(85)	HTR_{2A}	1,282 (325 MDD patients, 155 BP patients and 802 healthy controls)	No significant associations.
(86)	HTR_{1A}	1,135 (804 healthy controls)	No significant associations.
(87)	HTR_{1A}, HTR_{2A}	2,023 depressed subjects	No significantly associated SNP at genome-wide level.
(88)	HTR_{1A}	81 (62 healthy controls)	HTR_{1A} rs6295 genotype related to MDD.
(89)	Tryptophan hydroxylase-2 (TPH2) and 5-HT_{2A}	564 (287 healthy controls)	TPH2/5-HT_{2A} interaction influences MDD susceptibility.
(90)	Serotonin 4 (5-HT_4) receptor	96 (48 depressed subjects, 48 schizophrenia subjects)	Associations between HTR4 polymorphisms and mood disorder.
(91)	5-HT_4	57 healthy subjects, including 26 subjects had a family history of MDD	Association between the family history of MDD and lower striatal 5-HT_4 receptor binding.

memory (105–107), mood, sleep, appetite, and neuroendocrine function (108). Moreover, the antidepressant actions of monoamine oxidase (MAO) inhibitors and non-selective monoamine reuptake blockers suggest that NE plays a major role in the neurobiology of MDD (109). One potential pathogenic mechanism is elevated NE sensitivity of α_2-adrenoceptors, which can inhibit NE release from the LC *via* negative feedback (110, 111). Indeed, elevated density and enhanced activity of α_2-adrenoceptors have been reported in the brain tissues and platelets of MDD patients (112, 113). Elevated α_2-adrenoceptor density has also been found in the frontal cortex and hippocampus of depressed suicides (114, 115). Moreover, Rivero and co-workers found that the elevated α_2-adrenoceptors density in the prefrontal cortex of suicidal depressed subjects was resistant to antidepressant therapy, whereas elevated β_1-adrenoceptor density was reduced by such therapy (116).

The efficacy of selective norepinephrine reuptake inhibitors (SNRIs) provides the strongest evidence for a direct contribution of deficient NE transmission to depression. A recent systematic review concluded that the SNRI duloxetine hydrochloride was effective against MDD as well as panic disorder, obsessive–compulsive disorder, and other psychiatric disorders (117), indicating broad involvement of NE in psychopathology. Another review suggested that duloxetine may be safe for older adults with MDD (118), although this agent has not been suggested for use as first-line acute therapy for MDD (119). Nonetheless, the norepinephrine transporter (NET) is well documented therapeutic target for MDD and like SSRIs (120), nonselective 5-HT/NE reuptake inhibitors such as venlafaxine (121) are widely used for MDD treatment. Many studies have also implicated NET gene polymorphism in MDD pathogenesis (**Table 2**). Abnormalities of noradrenergic function may also be involved in the pathogenesis of suicide (148). Several earlier studies reported upregulation of β-adrenoceptors in the brains of suicides (114, 149, 150), although several others reported the opposite (150, 151). Aside for receptor abnormalities, excessive stress could trigger depletion of NE and the onset of MDD (152).

While 5-HT and NE are the biogenic amines most consistently associated with MDD, abnormalities in DA signaling have also been implicated. For instance, depletion of DA has also been reported in MDD patients (153). The medial part of the VTA projects mainly to the nucleus accumbens and ventral striatum, which are central hubs of the brain reward system (154, 155). Allelic variation of DA-related genes modulate brain circuitry involved in the regulation of negative emotional stimuli (156), and DA system dysfunction has been associated with many symptoms of MDD such as anhedonia and low motivation (157, 158), as well as with cognitive symptoms such as impaired concentration (159, 160).

TABLE 2 | Dopaminergic and noradrenergic gene polymorphisms in MDD.

Reference	Candidate gene	Population/sample size	Main findings
(122)	Norepinephrine transporter (NET)	34 brain tissue samples (19 healthy controls)	Reduced NET in the LC related to MDD.
(123)	NET	179 (74 healthy controls)	No significant associations.
(124)	NET	200 (100 healthy controls)	No significant associations.
(125)	NET	248 (136 healthy controls)	Tendency for lower TT genotype frequency in MDD.
(126)	NET and 5-HTT	96 depressed subjects	T-allele of NET T-182C polymorphism associated with better antidepressant response.
(127)	NET	309 (164 healthy controls)	C/C genotype related to low MDD risk.
(128)	NET	426 (210 healthy controls)	No significant difference.
(129)	NET	776 (388 healthy controls)	Selected NET gene polymorphisms influence MDD risk from negative life events.
(130)	NET and 5- HTTLPR	579 depressed subjects	Both NET and 5-HTTLPR related to MDD, while the interaction between them associated with depression and Hamilton Depression Rating Scale for Depression baseline scores.
(131)	NET, and 5-HTTLPR	252 depressed subjects	No significant associations between selected polymorphisms and antidepressant response.
(132)	the norepinephrine transporter (SLC6A2), HTR_{1A}, and COMT	126 depressed subjects	No significant associations between SLC6A2 polymorphisms and antidepressant treatment response.
(133)	SLC6A2, TPH2	205 depressed subjects	SLC6A2 polymorphism related to MADRS-defined olanzapine+fluoxetine response in MDD.
(134)	SLC6A2	550 (201 with MDD and suicide attempts, 160 with MDD without suicide attempts, and 189 healthy controls)	SLC6A2 polymorphism related to suicide risk in MDD.
(135)	NET	604 (302 healthy controls)	CC genotype of NET gene may reduce risk of depression.
(136)	SLC6A2	243 depressed subjects	Association between SLC6A2 gene variation and remission after venlafaxine treatment in MDD.
(137)	NET	776 (388 healthy controls)	Significant association between T-182C polymorphism and MDD.
(138)	SLC6A4, NET, HTR_{1A}, HTR_{2A}, COMT, and brain-derived neurotrophic factor (BDNF)	53 (27 healthy controls)	No difference in NET polymorphisms between MDD group and controls.
(139)	NET	78 (48 healthy controls)	Significant diagnosis interaction for NET G1287A polymorphism in MDD.
(140)	DRD4, TPH, MAO-A, and 5-HTTLPR	134 nuclear families with mood disorders (58 with MDD)	No significant associations.
(141)	DRD4, MAO-A, 5-HTTLPR, DRD2, and DAT1	United States	DRD4 5-repeat allele related to depressive symptoms among adolescents/young adults.
(142)	DAT1	264 depressed subjects	DAT1 VNTR polymorphism related to antidepressant response.
(143)	DAT1	Russia	DAT1 polymorphism rs40184 related to MDD and suicidal ideation.
(144)	DAT1	Chinese	No significant associations.
(145)	DAT1, COMT	German	9R/9R and Val/Val genotype negatively related to Sadness score.
(146)	DAT1	1,714 subjects	DAT1 related to children's depressive symptoms.
(147)	DAT1 and COMT	Chinese	Interaction of DAT1, COMT, and peer acceptance predictive of adolescent depressive symptoms.

A dopamine deficiency has also been reported in MDD. One study measuring monoamine neurotransmitters and related metabolites in the cortex of rats detected DA only in the control group (161). A multi-data source-based prioritization (MDSP) study by Liu et al. identified 143 depression-related genes, including the DA receptor 4 (DRD4), as well 16 significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, including the 'dopaminergic synapse' as well as the 'serotonergic synapse' and 'glutamatergic synapse'. The neuroactive ligand–receptor interaction list from KEGG pathway analysis also included the dopaminergic synapse (162). Further, a number of dopaminergic gene polymorphisms are associated with MDD (**Table 2**).

Reduced NE, 5-HT, and DA have been identified as significant biomarkers for depression in animal studies (163, 164). Advances in imaging techniques, including PET and single-photon emission computed tomography (SPECT), have also provided valuable insights into the contributions of DA to MDD. For instance, a recent study reported significantly reduced DA transporter (DAT) availability in the bilateral putamen and VTA of patients compared to healthy controls (Cohen d range, −0.62 to −0.71) (158). Moreover, this same study found lowest DAT availability in the VTA of patients reporting the greatest stress-related fatigue (165). While this relationship was replicated (166), the findings of a meta-analysis were contradictory (167).

In summary, the evidence is very strong that dysregulation of NE, DA, and 5-HT signaling contributes to MDD development and symptom expression. However, prospective studies are required to establish causal relationships between these deficiencies and MDD.

PERSONALITY TRAITS AND MDD

Personality can be described as a composite of multiple, relatively stable traits and specific trait profiles, as measured using instruments such as the Neuroticism, Extraversion, Openness Five-Factor Inventory (NEO-FFI) questionnaire, Temperament and Character Inventory (TCI), and Eysenck Personality Questionnaire (EPQ) for associations with MDD risk.

A large-scale longitudinal cohort study using baseline and 2-year follow-up data found that increased neuroticism scores on the NEO-FFI were associated with both anxiety and depressive disorders. Higher agreeableness has also been associated with the occurrence of MDD, while openness demonstrated no association with the occurrence of, or recovery from, any depressive or anxiety disorder (168). In contrast, extraversion trait scores were associated with lower depressive disorder incidence and increased rate of recovery (169). Pair-wise genome-wide association studies (GWASs) have also found that numerous genetic variants overlap between depression and trait neuroticism (170). Further, high trait neuroticism has been confirmed as a dominant risk factor for depression (104). Also, low extraversion scores were a predictor of depression during the remission period of bipolar disorder (BP), the other main subtype of mood disorder (171). A recent resting-state dynamic functional network connectivity analysis found that state 4 was positively correlated with trait extraversion and negatively correlated with neuroticism, as measured by the EPQ, and that MDD patients showed significantly reduced dwell time and fractional time in state 4 compared to healthy controls, with lowest centrality degree in hippocampus and ventral striatum (172).

Neuroticism can improve the ability to cope with negative emotional stimuli (173) and has been linked to panic disorder (174), schizophrenia (175), and obsessive–compulsive disorder (OCD) (176) as well as to MDD. According to twin studies, the heritability of trait neuroticism is approximately 40%, with 15% to 37% caused by single-nucleotide polymorphism (SNP) variations (177). High trait neuroticism is associated with sensitivity to stress and negative emotional experiences, as well as with excessive worry, emotional vulnerability, and increased emotional exhaustion (178), all of which can impact an individual's physical activity (179), perception (180, 181), and emotion (182). An early meta-analysis of GWASs analyzing over 106,000 individuals identified nine neuroticism-associated loci (including the ionotropic kainate 3 glutamate receptor, Kelch-like protein 2, and corticotropin-releasing hormone receptor 1). This same study also found a strong association between neuroticism and MDD (genetic correlation = 0.64), but no sex difference in the heredity of neuroticism (177). Another meta-analysis of GWASs identified the Membrane-associated guanylate kinase inverted repeat member 1 (MAGI1) gene as a novel locus for neuroticism, both among the entire cohort of 63,661 individuals as well as in the combined Netherlands Twin Registry (NTR)/Netherlands Study of Depression and Anxiety (NESDA) cohort, with significant polygenic risk scores associated with MDD for SNP sets at P-value thresholds of 0.01 and 0.05, again providing compelling evidence that higher neuroticism is strongly correlated with MDD (183).

Harm avoidance (HA), a core personality trait defined by Cloninger, reflects a tendency to avoid potential danger, and like neuroticism, is related to traits such as pessimism, anxiousness, insecurity, bashfulness, and unusual susceptibility to fatigue (184). Trait HA has a high degree of stability throughout life (185), and is strongly associated with OCD (186), eating disorders (187), and other psychiatric disorders. High HA scores are also considered predictive of MDD (188). Bipolar disease patients demonstrating high HA scores on the TCI also showed a strong tendency for poor antidepressant treatment response during depressive episodes (189). A meta-analysis focusing on the associations between personality traits and MDD recovery found that patients with high novelty seeking (NS), high self-directedness (SD), and low HA exhibited better antidepressant responses (190). Alternatively, higher HA scores and lower SD scores were significantly correlated with non-remission in MDD patients (191), these findings have been replicated (192–194). Interestingly, a meta-analysis from Zaninotto et al. not only found such correlations, but the team reported the influence of HA in MDD vs healthy subjects was significantly greater than that found in BP vs healthy subjects (195), although there was marked heterogeneity among the included studies. Additional longitudinal studies are needed to confirm the association between HA and MDD.

Personality traits are also the major focus of suicide research. Garcia Herrero et al. concluded that high neuroticism can predict suicidal ideation (196). Similarly, Peters and his colleagues followed a large sample population in the United Kingdom for 10 years and found that neuroticism was related to suicide risk in both males and females and that neuroticism was a major predictor of suicide in females with mood disorders (197). An earlier study also found that neuroticism and openness were risk factors for suicide specifically in females, while extraversion and conscientiousness reduced the risk in males (198).

A recent study using the TCI to assess personality traits found that higher HA increased the risk of suicidal ideation in depression (199). Eric et al. also reported significantly higher HA scores, as well as low SD scores in subjects with suicidal ideation (192). Further, several studies have found that higher HA and NS scores are significant risk factors for suicidal behavior (200–202), while others have linked lower SD and higher self-transcendence (ST) to suicidality (203, 204).

Mood state may also impact personality traits, at least as measured at specific times, which complicates these association results. Nonetheless, the relatively consistent relationships between specific traits and MDD, including suicidal MDD, and the overlap between several trait-related and MDD-related genes suggest that investigations of the genetic and physiological attributes underlying specific traits may provide additional clues to the pathophysiology of MDD.

MONOAMINE NEUROTRANSMITTERS AND PERSONALITY TRAITS

Twin, family, and genomic studies have shown that personality traits are strongly influenced genetics, with estimated heritability ranging from 40% to 60% (205–208). Cloninger's Tridimensional Personality Questionnaire (TPQ) traits NS, HA, and reward-dependence (RD) have all been associated with monoamine functions (209, 210), as have the so called "the Big Five" personality traits assessed by NEO, NEO-PI-R, and NEO-FFI (neuroticism, extraversion, openness to experience, agreeableness, and conscientiousness) (211) and the three personality traits of the EPQ (psychoticism, extraversion, and neuroticism) (212).

Extraversion, a higher-order personality trait, has been linked to reward system function in several studies (213–215). Furthermore, evidence strongly suggests that DA modulation is involved in both reward system function and extroversion (216). Smillie et al. (208) and co-workers reported that subjects with the DA receptor 2 (DRD2) gene A1-allele had significantly higher extroversion scores. In contrast, however, a functional magnetic resonance imaging (fMRI) study reported that A1-allele carriers exhibited lower extraversion scores, although the difference between carriers and non-carriers was not significant (217). A cross-national study of personality differences by Fischer et al. found a positive correlation between dopaminergic brain function index score and extraversion as well as a negative association between dopaminergic function and neuroticism score in those under high stress (218). A meta-analysis also found a relationship between self-consciousness (one facet of neuroticism) and the domain receptor 1 (DDR1) gene (219). Again, these relationships may be complicated by covariables. For instance, a previous study reported a negative correlation between neuroticism scores and quality of life in schizophrenia (175).

The opponent interactions between serotonin and DA makes the relationship between serotonin and personality traits was interesting and complex (220). Several studies have looked at the relationship, but the results have been inconsistent (**Table 3**). For example, most evidence to date support a link between the serotonin-transporter-linked polymorphic region (5-HTTLPR) and neuroticism (252, 286), meanwhile the different result were obtained using NEO-FFI (225). Interesting, in Swedish cohort study, they observed openness was significantly associated with 5-HTTLPR, while they also found that the positive association between openness and childhood adversity in the gene-environment model regardless of 5-HTTLPR genotype (225). Paaver et al. demonstrated S allele carriers with adverse family relations were related to higher thoughtlessness, disinhibition and impulsivity using the Barratt Impulsiveness Scale 11 (BIS-11) solely among girls (254), they also indicated that, in agreement with other studies, the influence of 5-HTTLPR genotype on affect is related to environmental adversity (61, 66). Indeed, environmental adversity, such as childhood adversity, can have a negative effect on child's expectations and present strained interpersonal relationships, which can affect personality or temperature (295), as well as associate with a range of psychopathology, including MDD (11). This factor has not been considered in some studies, which might be one of the fundamental reasons for the inconsistent results. Some studies on children have demonstrated significant association between 5-HTTLPR short (S) allele and higher NS scores (253), and S allele closely related to higher prevalence of substance use (296). In addition, the study of the relationship between personality trait and NE is rather little.

A number of monoaminergic transmitter-related genes are linked to personality traits, such as those encoding catechol-O-methyl-transferase (COMT) (297), monoamine oxidase A (MAOA) (222), and glutathione peroxidase 1 (GP × 1) (268). Furthermore, polymorphisms in monoamine receptors, for example 5-HTTLPR (226) and DRD4 (221), are associated with personality traits (**Table 3**). Recent studies in our laboratory have demonstrated associations between personality traits and Neurotensin receptor 1 (NTR1) (236), Dopamine- and cAMP-regulated phosphoprotein (DARPP-32) (255), and casein kinase 1ε (CK1ε) (246), all of which can affect monoaminergic signaling.

Undoubtedly, it is important that any assessment of the role of monoamines in personality traits should involve precise neural circuits associated with the relevant behavioral processes from the examples provided above (298). However, in many studies, there are some limitations, such as the small sample size with low statistical power, still need more participants to provide high quality evidence in further analysis.

CONCLUSIONS

MDD, therapeutic strategy still remain unclear, is one of the most prevalent medical disorder which causes life-threatening conditions, like suicides tend and suicidal behaviors. Although the precise etiology is not known, several studies support the fact that MDD is the severe mental disease that involves disturbance of chemical neurotransmitters, psychosocial factors, genetic factors, personality traits and other formulations. In our study, numerous strong associations have been identified among monoamine signaling deficits, detrimental personality traits, and major depressive disorder, providing potential clues to disease pathogenesis. And through incredible advancements in medical technology, these independent and interactive dimensions may be promising targets for precision medicine. Suicide is a massive public problem in depressed patients, thus research regarding the prevention and intervenient countermeasures of suicide should be thoroughly investigated in the field of biogenic amines changes and personality traits. Moreover, such studies have identified potential biomarkers for MDD risk that could aid in the early identification of at-risk individuals (299). Clinical programs should focus on early identification and intervention for emotional problems and high-risk behaviors among children and adolescents. Notably, the evidences for the relationship between monoamines, MDD and personality traits are confused and contradictory. Small sample size (significantly drop the accuracy rate and lead bias), unified analyzing methods, differences in tissues, depressive

TABLE 3 | Relationships between monoaminergic system function and personality traits.

Reference	Sample size	Approach	Main findings
(221)	290 (147 males and 143 females)	Zuckerman–Kuhlman–Aluja Personality Questionnaire	Four tagged single-nucleotide polymorphisms (tagSNPs), including DRD4, were related to Neuroticism and the 4 tagSNPs, including DRD2 and DRD4, were associated with Sensation Seeking.
(222)	99 females	NEO	MAOA-u variable number of tandem repeats (VNTR) polymorphism significantly associated with trait Neuroticism. No associations with COMT Val^{158} Met, 5-HTTLPR, or DAT 3'UTR VNTR.
(223)	600 males	NEO-FFI	DRD4 significantly related to extraversion, the DAT1 to agreeability.
(218)	127,685 subjects	NEO-PI-R and Occupational Personality Questionnaire (OPQ)	Dopamine-system only in climatic stress closely related to personality trait Neuroticism and Extraversion. Interaction between dopamine and climatic demands significant for Openness/Intellect on OPQ scores.
(224)	50 males	TCI	5-HT_{1A} receptor binding not associated with ST/SA scores.
(225)	3,112 subjects	Swedish translation of Schafer's FFM rating scale	Openness (to experience) associated with serotonin-transporter-linked polymorphic region.
(226)	1,139 (550 males and 589 females)	Short-form Maudsley Personality Inventory (MPI)	Serotonin transporter polymorphisms (5-HTTLPR and rs25531) associated with Neuroticism in males.
(227)	69 (51 males and 18 females)	NEO	No association between personality traits and 5-HT_4R.
(228)	147 (91 males and 56 females)	NEO-PI-R NEO-FFI,	Neuroticism positively associated with serotonin transporter binding potential in males, negatively associated with serotonin transporter in females.
(229)	44 (22 males and 22 females)	Karolinksa Scales of Personality	Explicit associations between the D2/3R and the trait impulsivity.
(230)	61 (47 males and 14 females)	Buss–Perry Aggression Questionnaire (BPAQ) and Barratt Impulsiveness Scale	Positive correlations of 5-HT_4R with BPAQ total score and BPAQ physical aggression score in males.
(231)	272 females	NEO-FFI	Statistically significant relationship between Openness to experience score and the 5-HTT polymorphism. No significant relationship between NEO-FFI score and MAO-A polymorphism.
(232)	1,576 (675 males and 901 females)	Estonian version of Revised NEO Personality Inventory (NEO-PI-R)	Lower Neuroticism and higher Conscientiousness scores significantly related to tryptophan hydroxylase 2 (TPH2).
(233)	616 (273 males and 373 females)	NEO-FFI	Higher COMT enzymatic activity (GG) related to lower Neuroticism, higher Agreeableness, and higher Conscientiousness scores.
(234)	34 (18 males and 16 females)	Karolinska Scales of Personality	Negative relation between Neuroticism and serotonin 5-HT_{1A} receptor binding.
(235)	16 subjects	TCI	Self-transcendence was associated with serotonin transporter (SERT) availability.
(236)	575 (274 males and 301 females)	TPQ	HA2, HA3 and RD1 scores significantly associated with NTR1 polymorphism rs6090453. HA2 and total RD scores significantly associated with rs6011914. No associations between NS and the selected SNPs.
(237)	12 males	TPQ	Significant correlation between DA synthesis ability in the ventral striatum and NS3.
(238)	46 subjects	Eysenck Personality Questionnaire (EPQ-R)	No significant result.
(239)	599 (341 males and 258 females)	Zuckerman Kuhlman Personality Questionnaire (ZKPQ)	D4R promoter polymorphisms not related to Sensation seeking.
(240)	372 males	TCI and Eysenck personality questionnaire	Significant associations between Sensation seeking and both 5-HTTLPR and 5-HT2CR.
(241)	72 (41 males and 31 females)	NEO PI-R	Openness to experience was related DRD2-mediated transmission.
(242)	2075 subjects	TCI	Positive correlation between 5-HTT BPND and SD score.
(243)	94 (60 males and 34 females)	Buss–Perry Aggression Questionnaire (AQ) and BIS-11	No associations between 5-HT_{2A}R and AQ or BIS-11 total scores.
(244)	418 (104 males and 314 females)	the Formal Characteristics of Behaviour–Temperament Inventory	Significant association between DAT1 polymorphism and sensory sensitivity. Sex/DRD4 interaction impacts the same trait.
(245)	1,084 (407 males and 677 females)	TCI	No significant association between -141C Ins/Del polymorphism or the DRD2/ankyrin repeat and kinase domain containing 1 (ANKK1) Taq1 A polymorphism and personality traits, but an ANKK1 × DRD2 interaction affects TCI scores.
(246)	502 (240 males and 262 females)	TPQ	No significant association between CK1ϵ and TPQ scores.
(247)	1,091 subjects	EPQ	No significant result.
(248)	20 males	NEO	Significant associations between low 5-HTT in the dorsal raphe nucleus and both straight forwardness and trusting personality.
(249)	21 (8 males and 13 females)	TCI	HA score negatively correlated with D2/3 receptor availability.

(Continued)

TABLE 3 | Continued

Reference	Sample size	Approach	Main findings
(250)	652 (222 males and 430 females)	Eysenck Personality Inventory (EPI) and Temperament and Character Inventory-125 (TCI-125).	Significant effects of ANKK1/DRD2 Taq1A on Neuroticism and of dopamine transporter gene (SLC6A3) rs27072 on Persistence in both sexes. Significant association between ANKK1/DRD2 Taq1A A2/A2-genotype and higher NS and lower RD in males. Significant association between SLC6A3 10R*G-haplotype and higher Persistence in females.
(251)	289 (123 males and 166 females)	TCI	No significant associations with TCI scores.
(252)	94 (14 males and 80 females)	Dutch personality questionnaire (DPQ)	5-HTTLPR S-allele increases affective reactivity to examination stress independent of trait Neuroticism.
(253)	216 (129 males and 87 females)	TPQ and Buss–Durkee Hostility Inventory	S allele of 5-HTTLPR was related to higher NS scores.
(254)	483 (222 males and 261 females)	BIS-11 and Adaptive and Maladaptive Impulsivity Scale	S allele of 5-HTTLPR was associated with high maladaptive impulsivity.
(255)	502 (240 males and 262 females)	TPQ	Significant associations between rs12601930C/T and the trait NS. Both rs879606A/G and rs3764352A/G associated with HA.
(256)	16 (8 males and 8 females)	Swedish universities Scales of Personality	Social desirability negatively correlated with D2-receptor availability in striatum.
(257)	21 (10 males and 11 females)	TCI	The different regions of 5-HT_{2A} affects Persistence independent of sex.
(258)	50 (35 males and 15 females)	NEO PI-R	Negative correlation between Openness to Experience and *in vivo* cerebral 5-HTT binding.
(259)	1,114 subjects	TCI	DRD2 related to Novelty seeking in childhood.
(260)	83 (52 males and 31 females)	NEO PI-R	Positive correlation between 5-HT_{2A} binding and Neuroticism.
(261)	549 (304 males and 245 females)	TCI	Monoamine oxidase A (MAOA-VNTR) gene high-activity allele exhibited significant higher P scores than low-activity gene in females.
(262)	301 subjects	EPQ and TCI	5-HTT gene S Tin2.10 allele associated with Neuroticism and HA.
(263)	31 subjects	NEO	Positive correlation between neuroticism and 5-HTT binding in the thalamus.
(264)	42 (19 males and 23 females)	Maudsley personality inventory	Lie scale related to striatal dopamine D2/D3 receptor availability.
(265)	324 subjects	TCI	Significant associations between monoamine oxidase A polymorphism and both NS and RD.
(266)	256 subjects	NEO PI-R	No significant interaction among three functional polymorphisms in the tyrosine hydroxylase, monoamine oxidase A, and COMT genes on personality traits.
(267)	370 females	TPQ	MAOA-uVNTR gene related to HA of TPQ, and the HA4 got the highest score.
(268)	149 (65 males and 84 females)	TCI and NEO-PI-R	Association between rs1050450 polymorphism and Openness on NEO. No association was found using TCI.
(269)	219 females	TCI	No significant associations between monoamine oxidase A promoter polymorphism and personality traits.
(270)	33 subjects	Karolinska Scales of Personality	High scores on somatic anxiety and muscular tension and irritability significantly associated with reduced [^{18}F] fluorodopa uptake in the caudate.
(271)	15 males	TCI	5-HT_{1A} receptor binding potential (BPND) negatively correlated with ST/SA.
(272)	115 subjects	NEO-FFI	DRD4 exon III and -521C/T not related to any personality trait.
(273)	101 females	TCI	Association between DRD4 variants of DRD4 and both NS and P personality traits.
(274)	149 (57 males and 92 females), and 252 (103 males and 149 females)	TPQ	COMT gene polymorphism related to higher HA scores in females, with Met158/Met158 genotype most strongly associated.
(275)	66 males	TPQ and EPQ	EPQ correlated with [^{11}C]WAY-100635 binding of 5-HT_{1A} receptors.
(276)	71 (33 males and 38 females)	NEO-FFI	Significant interaction of sex and DRD4 polymorphisms (-616 and -521C) related to Extraversion scores.
(277)	11 (8 males and 3 females)	TPQ	Cerebral cortex 5-HT_{2A} receptors associated with HA.
(278)	371 (206 males and 165 females)	Karolinska Scales of Personality, Scandinavian Universities Scales of Personality, Health-Relevant 5-Factor Personality inventory, TCI and NEO-PI-R	No association between MAOA promoter region and personality traits in Swedish population.
(279)	16 males	TCI	Significant relation between dopamine D2 receptor (D2R) and personality trait of HA.
(280)	24 males	TCI	NS scores negatively correlated with D2R.
(281)	19 (11 males and 8 females)	NEO-PI-R	Negative correlation between Neuroticism and cortical 5-HT_{1A} receptor.
(282)	577 subjects	TPQ	COMT and 5-HTTLPR significantly related to RD2 scores by grouping.

(Continued)

TABLE 3 | Continued

Reference	Sample size	Approach	Main findings
(283)	18 (10 males and 8 females)	Karolinska Scales of Personality	Negative correlation between dopamine transporter and detachment personality scores, especially in the right hemisphere.
(284)	86 subjects	TCI	DRD4 exon III -521C/T polymorphism significantly associated with NS, with higher scores for C/C genotype.
(285)	256 subjects	NEO PI-R	No association between extraversion and DRD4 polymorphisms.
(286)	902 (505 males and 397 females)	NEO-PI-R	Higher NEO Neuroticism related to 5-HTTLPR polymorphism.
(287)	69 females	TCI	Significant association between DRD4 exon III long allele and NS scores.
(288)	119 males	TPQ	Young males with all three minor DRD2 alleles and the DRD4 7R allele show the most significant difference in NS scores.
(289)	341 (204 males and 137 females)	TPQ	No significant difference between D4 dopamine-receptor (DRD4) and the trait NS.
(290)	126 subjects	Karolinska Scales of Personality	DRD4 polymorphisms not related to personality traits.
(291)	153 females	TCI	Dopamine D4 receptor (D4DR) polymorphic exon III related to NS subscale of Exploratory Excitability.
(292)	124 subjects	TPQ	Association between NS scores and D4DR polymorphisms.
(293)	115 subjects	TCI	Norepinephrine transporter T-182C gene polymorphism was associated with personality trait RD in Koreans.
(294)	270 subjects (117 males and 153 females)	NEO-FFI	NET gene polymorphisms related to extraversion.

phenotypes, ethnicities, and others may lead to these inconsistent data. These factors should be considered in future studies.

AUTHOR CONTRIBUTIONS

GZ planned and directed the paper, and XS wrote it.

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Altered Effective Connectivity in Schizophrenic Patients With Auditory Verbal Hallucinations: A Resting-State fMRI Study With Granger Causality Analysis

Jie Gao[1†], Dongsheng Zhang[1†], Lei Wang[2], Wei Wang[3], Yajuan Fan[3], Min Tang[1], Xin Zhang[1], Xiaoyan Lei[1], Yarong Wang[2], Jian Yang[2] and Xiaoling Zhang[1*]*

[1] Department of MRI, Shaanxi Provincial People's Hospital, Xi'an, China, [2] Department of Radiology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China, [3] Department of Psychiatry, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China

Purpose: Auditory verbal hallucinations (AVH) are among the most common and prominent symptoms of schizophrenia. Although abnormal functional connectivity associated with AVH has been reported in multiple regions, the changes in information flow remain unclear. In this study, we aimed to elucidate causal influences related to AVH in key regions of auditory, language, and memory networks, by using Granger causality analysis (GCA).

Patients and Methods: Eighteen patients with schizophrenia with AVH and eighteen matched patients without AVH who received resting-state fMRI scans were enrolled in the study. The bilateral superior temporal gyrus (STG), Broca's area, Wernicke's area, putamen, and hippocampus were selected as regions of interest.

Results: Granger causality (GC) increased from Broca's area to the left STG, and decreased from the right homolog of Wernicke's area to the right homolog of Broca's area, and from the right STG to the right hippocampus in the AVH group compared with the non-AVH group. Correlation analysis showed that the normalized GC ratios from the left STG to Broca's area, from the left STG to the right homolog of Broca's area, and from the right STG to the right homolog of Broca's area were negatively correlated with severity of AVH, and the normalized GC ratios from Broca's area to the left hippocampus and from Broca's area to the right STG were positively correlated with severity of AVH.

Conclusion: Our findings indicate a causal influence of pivotal regions involving the auditory, language, and memory networks in schizophrenia with AVH, which provide a deeper understanding of the neural mechanisms underlying AVH.

Keywords: schizophrenia, auditory verbal hallucination, magnetic resonance imaging, resting state, effective connectivity

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Lin Liu,
Peking University Health Science Centre, China
Ning Hua Wang,
Xijing Hospital, Fourth Military Medical University, China
Manli Huang,
Zhejiang University, China

***Correspondence:**
Xiaoling Zhang
zxl.822@163.com
Jian Yang
yj1118@mail.xjtu.edu.cn

[†]These authors have contributed equally to this work

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INTRODUCTION

Auditory verbal hallucinations (AVH) are the most prominent and burdensome symptoms of schizophrenia, and comprise sensory experiences wherein voices are heard without a causative external stimulus. The prevalence of AVH exceeds 60% in patients with schizophrenia worldwide (1, 2). In 30% of patients with AVH, the hallucinations are not affected by clinical treatments (3), and cause functional disability and therefore lower quality of life. Clinical options to treat such patients are currently limited. Since the neurobiological mechanisms involved in AVH remain unclear; insights into the pathophysiology of AVH could facilitate the development of novel strategies to treat patients who do not respond to currently used medications.

The mechanism by which AVH occurs spontaneously from the brain's intrinsic activity is of great clinical interest. Resting-state functional magnetic resonance imaging (fMRI) can show spontaneous brain activity *in vivo*, and can therefore provide invaluable insights into the psychopathology of AVH. Based on time series derived from resting state fMRI data, functional connectivity (FC) analysis can be used to calculate the temporal correlations between a blood-oxygenation-level dependent in any two regions, offering an effective method to study alterations in brain connectivity within multiple brain networks associated with AVH in schizophrenia.

In previous models including the "memory intrusion", "self-monitoring", "two hit bottom-up and top-down", and hybrid models, language, auditory, and memory brain networks were closely associated with AVH in patients with schizophrenia (4). The superior temporal gyrus (STG) (auditory cortex), Broca's area (speech production), Wernicke's area (language comprehension), hippocampus (memory retrieval), and putamen (initiating language representations) are the key areas responsible for AVH in schizophrenia, and showed functional alterations in previous brain imaging studies (5–18). Although aberrant FC associated with AVH has been reported in these regions in many resting-state fMRI studies (6, 7, 15, 16, 19–25), the interactions between these regions have not been described completely. Furthermore, the information flow within these networks has not been completely characterized.

Effective connectivity (EC) analyses using data on time-lagged relationships between cerebral regions, offers additional information on the directionality of information flow within brain networks (26, 27). Granger causality analysis (GCA) is a special method used to study EC, and could yield deductive networks based on hypothetical seed regions without prior knowledge. This model is simple, has low computational complexity, and is not limited by the number of brain regions studied. It is therefore an efficient and convenient method for analyzing fMRI data on resting-state functional organization in various healthy and diseased brain networks (28–30). To our knowledge, this method has not been used to study the information flow within the aforementioned regions in resting-state fMRI studies.

In this study, we used the GCA method on resting-state fMRI data to determine the patterns of effective connections between the bilateral STG, Broca's area, Wernicke's area, hippocampus, and putamen in schizophrenia patients with and without AVH, in order to obtain novel imaging evidence for the elucidation of the neural mechanisms underlying AVH. We first used GCA to study the differences in effective connections between schizophrenia patients with and without AVH to determine the direction of connection changes. We then performed correlation analysis between changes in causal influence and severity of hallucination symptoms to identify the functional disturbances caused by AVH.

MATERIALS AND METHODS

Subjects

This study was conducted in accordance with the Declaration of Helsinki. The experimental protocol was approved by the Ethics Committee of Shaanxi Provincial People's Hospital, and written parental consent was obtained from all participants.

Study participants were recruited from the in-patient ward, and met the following criteria: 1) diagnosed with schizophrenia/schizoaffective disorder by using the Structured Clinical Interview of the DSM-IV (SCID); 2) with the Positive and Negative Syndrome Scale (PANSS) assessment; 3) Han Chinese in origin; 4) right-handed; 5) between 18 and 40 years old; 6) received conventional MRI and resting-state fMRI scans with available images. Exclusion criteria were as follows: 1) history of other psychotic disorders; 2) substance abuse or dependence; 3) severe medical disorders; 4) traumatic brain injury; 5) electroconvulsive therapy within the past 6 months; 6) intellectual disability or neurological impairment. Patients who presented with AVHs at least once a day for the past four weeks, with a PANSS P3 score ≥4 were assigned to the AVH group, and patients who did not experience hallucinations or had a P3 score of ≤3 belonged to the non-AVH group (31, 32).

All clinical data were double reviewed by a senior researcher, who verified the credibility of the patient statements. Information of usage of antipsychotics (name, dosage, and duration of drugs used before MRI scanning) was also collected, and the converted antipsychotic doses to chlorpromazine (CPZ) milligram equivalent units were calculated. No significant correlation was found between granger causality (GC) and illness duration or CPZ equivalent in either AVH group or non-AVH group; thus, they were not taken as covariates in further statistics.

MRI Acquisition

All MRI data were performed within 3 days after the PANSS. Conventional MRI and resting-state fMRI were acquired on a 3.0T scanner (Signa HDxt, General Electric Medical System, Milwaukee, WI, USA) with an eight-channel phase array radio-frequency head coil. Head motion was minimized by positioned with restraining foam pads. Earplugs were used to decrease the effects of scanner noise. The total scan time was <20 min. Three-dimensional fast-spoiled gradient-recalled echo T1-weighted images (repetition time/echo time, 10/4.6–14 ms) and fast-spin echo T2-weighted images (repetition time/echo time, 4,200/116.4–124.0 ms) were obtained. Before fMRI scanning, the patients were instructed to stay awake, relax, keep their eyes closed, and refrain from moving. Blood oxygen level-dependent

(BOLD) fMRI was acquired using the following parameters: 185 volumes, echo time = 30 ms, repetition time = 2000 ms, flip angle = 90°, 40 axial slices, slice thickness = 4 mm; field of view, 240 × 240 mm^2; matrix = 64×64. The acquisition time was 6 min and 30 s.

Data Preprocessing

Preprocessing of fMRI data was performed using SPM12 software (http://www.fil.ion.ucl.ac.uk/spm). Functional images were subjected to slice-timing correction and were then realigned to the first volume to correct for head motions. The data with head movement > 1.5 mm and/or rotation angle >1.5° were excluded. The realigned images were then spatially normalized to the MNI EPI template and resampled to a voxel size of 3 × 3 × 3 mm^3. A band-pass filter between 0.01 and 0.08 Hz was subsequently applied to the data to remove the effects of very-low-frequency drift and high-frequency noise. Finally, spatial smoothing was applied using a 6-mm full-width-at-half-maximum Gaussian kernel. The head-motion parameters, white-matter signals, and cerebrospinal-fluid-signals were regressed out of the BOLD signals.

Regions of Interest (ROIs) Selection

The bilateral STG, Broca's area, Wernicke's area, putamen, and hippocampus were selected as the ROIs. Masks representing each of the ROIs were created by using the Wake Forest University PickAtlas tool with TD-ICBM Human Atlas (TD Brodmann) (33). The fMRI time courses of all the voxels located within the masks for each ROI were extracted.

Granger Causality Analysis

GCA was performed using REST-GCA software (http://www.restfmri.net). By applying an order 2 vector auto-regression model, for any two ROI time series x(t) and y(t), the time domain pairwise GCA components from x(t) to y(t) ($F_{x\to y}$) and from y(t) to x(t) ($F_{y\to x}$) were calculated respectively. Pairwise Granger causal connectivity indicates that neuronal activity of one region is predictive of activity occurring in another region. Causal influence was normalized using the following computing method (34):

$$R_{x\to y} = \left(F_{x\to y} - F_{y\to x}\right) / \left(F_{x\to y} + F_{y\to x}\right)$$

$R_{x\to y}$ is the ratio describing the relative strength and directionality of the causal influences between x and y. A positive $R_{x\to y}$ with a larger absolute value indicates stronger causal influence from x to y, while a negative $R_{x\to y}$ with a larger absolute value denotes stronger causal influence from y to x. Changes in $R_{x\to y}$ were calculated by analyzing the differences between the AVH and non-AVH groups.

Correlation Analysis

The association between changes in causal influence and hallucination were assessed using correlation analyses performed between the normalized $R_{x\to y}$ ratios of the pairwise ROIs and the PANSS P3 score. The Bonferroni correction was applied to correct for multiple comparisons, and $P < 0.005$ (0.05/10) was considered statistically significant.

Statistical Analysis

Statistical analysis was performed on SPSS 17.0 (SPSS, Chicago, IL, USA). The measurement data of normal distribution were presented as means ± standard deviations, and categorical data as frequencies and percentages. The two sample *t*-test or χ^2 test, were used to compare demographic and clinical data between the AVH and non-AVH groups. All statistical tests were two-tailed, and $P \leq 0.05$ was considered significant.

RESULTS

Subjects

This study included 18 patients with AVH and 18 without AVH. The AVH and non-AVH groups did not show significant differences in age, gender, education, smoking, drinking, illness duration, proportion of first episode patients, CPZ equivalent dose, total, negative and general symptom severity PANSS score. Positive PANSS score and PANSS P3 score were significantly higher in the AVH group than in the non-AVH group (**Table 1**).

GCA Results

Compared with the non-AVH group, AVH group showed an increase in GC from the right homolog of Broca's area to the left STG, and a decrease from the right STG to the right hippocampus, from the left putamen to the right hippocampus, from the right putamen to the right hippocampus, and from the right putamen to Broca's area (**Tables 2** and **3**).

The normalized ratios of GC from the left STG to Broca's area and its right homolog, from the right STG to the right homolog of Broca's area, and from the right STG to the right hippocampus were significantly lower in the AVH group than in the non-AVH group. The bi-directional causal values ($F_{x\to y}$ and $F_{y\to x}$) explained the results caused by increased GC from Broca's area and its right homolog to the left STG and from the right homolog of Broca's area to the right STG, and the decreased GC from the right STG to the right hippocampus (**Table 4** and **Figure 1**).

TABLE 1 | Demographic and clinical characteristics of AVH and non-AVH groups.

Items	AVH group	Non-AVH group	*P*
Number	18	18	/
Age (years)	24.33 ± 6.16	24.89 ± 6.73	0.81
Gender (female/%)	11(61.11)	10(55.56)	0.74
right handness (R/L)	18/0	18/0	/
Education level (years)	12.33 ± 3.65	11.89 ± 3.39	0.71
Illness duration (months)	38.78 ± 47.55	14.28 ± 18.48	0.05
Smoking (yes/%)	0/0.00	3/16.67	0.23
Drinking (yes/%)	1/5.56	0/0.00	1.00
First episode patients (yes/%)	9/50.00	10/55.56	0.74
PANSS total score	89.28 ± 19.06	77.89 ± 17.20	0.07
PANSS positive score	28.72 ± 5.28	22.56 ± 3.94	<0.001
PANSS negative score	21.67 ± 9.29	21.56 ± 6.56	0.97
PANSS general psychopathology score	17.94 ± 6.33	15.94 ± 5.10	0.30
P3 score	5.06 ± 0.73	1.89 ± 0.90	<0.001
CPZ	328.61 ± 141.44	283.33 ± 140.46	0.34

AVH, auditory verbal hallucinations; PANSS, Positive and Negative Syndrome Scale; CPZ, chlorpromazine.

TABLE 2 | Comparisons of the pairwise GC values ($F_{x\rightarrow y}$) between AVH and non-AVH groups.

$F_{x\rightarrow y}$	AVH group	Non-AVH group	T	*P*
STG.R - Hipp.R	0.95	1.65	−2.26	0.03*
Putmen.L - Hipp.R	0.93	1.94	−2.74	0.01*

*L, left; R, right; *P < 0.05.*
GC, granger causality; AVH, auditory verbal hallucinations; Hipp, hippocampus; STG, superior temporal gyrus.

TABLE 3 | Comparisons of the pairwise GC values ($F_{y\rightarrow x}$) between AVH and non-AVH groups.

$F_{y\rightarrow x}$	AVH group	Non-AVH group	T	*P*
Hipp.R - Putmen.R	0.94	1.78	−2.46	0.02*
STG.L - Broca.R	2.81	1.64	2.57	0.02*
Broca.L - Putmen.R	1.25	2.23	−2.39	0.02*

*L, left; R, right; *P < 0.05.*
GC, granger causality; AVH, auditory verbal hallucinations; Hipp, hippocampus; STG, superior temporal gyrus; Broca, Broca's area.

The normalized ratios of Granger causality from the right homolog of Broca's area to the right homolog of Wernicke's area, from Broca's area to the left hippocampus, and from Broca's area to the STG were significantly increased in the AVH group (**Table 4** and **Figure 1**). Bi-directional causal values ($F_{x\rightarrow y}$ and $F_{y\rightarrow x}$), indicated that these differences were mainly due to the decreased Granger causality from the right homolog of Wernicke's area to the right homolog of Broca's area and from the left hippocampus to Broca's area, and increased Granger causality from Broca's area to the STG (**Table 4** and **Figure 1**).

Correlation Analysis

The normalized ratios of Granger causality from the left STG to Broca's area, from the left STG to the right homolog of Broca's area, and from the right STG to the right homolog of Broca's area were negatively correlated with PANSS P3 score, and the normalized ratios of Granger causality from Broca's area to the left hippocampus and from Broca's area to the right STG were positively correlated with PANSS P3 score (**Table 5**). However, after the Bonferroni correction, statistically significant correlations were only observed between normalized ratios of Granger causality from the right STG to the right homolog of Broca's area and from Broca's area to the right STG and PANSS P3 score.

DISCUSSION

GCA, an effective connection analysis method, is used to examine the directional interaction and influence between brain regions by calculating the information between two time series. In recent years, GCA has been widely applied in the field of neurocognitive science. Studying alterations in interactive causal influences (driving forces) in brain regions involved in schizophrenia-related hallucinations could improve our understanding of the underlying neurobiological substrates. In this study, we found significant differences in effective connections related to auditory, speech, and memory circuits, involving the STG, Wernicke's area, Broca's area, and hippocampus, in schizophrenia patients with and without AVH by using GCA. These abnormal functional connections could contribute causally to the onset of auditory hallucinations.

The Auditory Processing Circuit

The STG contains the auditory cortex, which is responsible for the perception and processing of sounds. Broca's area is closely associated with the auditory cortex. Previous resting state fMRI studies suggested that abnormal connectivity between Broca's area and the auditory cortex could be responsible for AVH (8, 24, 35). However, evidence of resting connectivity between these two areas is unclear. Sommer et al. (8) reported reduced synchronization between the left STG and inferior frontal gyrus (IFG, close to Broca's area) in patients with chronic AVH (8). Hoffman et al. (35) studied the time course of AVH and FC at the various stages of hallucinations (35), and found an increased coupling just prior to hallucinations between the left IFG and right temporal areas (including the STG and middle temporal gyrus). Diederen et al. (24) also observed elevated connectivity between the left STG and right IFG (24). Thus, the change in connection strength in the two regions is not clear, and possibly not uniform in all patients with schizophrenia. Furthermore, the directionality of abnormal connections related to auditory and language processing regions remains unclear.

Our results from the GCA provide information on the direction of the effective connection between the STG and Broca's area. We observed increased Granger causality from Broca's area and Broca's homolog to the bilateral STG in the AVH group, which was also associated with severity of hallucination. To date, only Baojuan et al. (36) have studied the interaction between the auditory cortex and Broca's area in a dynamic causal model. They reported that a positive correlation was observed between the strength of EC from Broca's area to the auditory cortex and AVH severity, which is consistent with our results. The increased effective connection from the auditory cortex to Broca's area was likely a compensatory change due to the decreased connection from Wernicke's area to Broca's area (7), which was also verified in our study. In addition, we hypothesize that AVHs are derived from auditory cortical activity, and spontaneous activity is very likely caused by the intrusion of internal auditory signals from Broca's area.

The Speech Processing Circuit

Our results showed decreased Granger causality from the right homolog of Wernicke's area to the right homolog of Broca's area. Broca's area is involved in the production of language, and Wernicke's area is involved in the comprehension of written and spoken language. Although the left hemisphere is widely considered the site of speech processing, studies in patients who suffered strokes indicate that the right hemisphere is also capable of basic language production (37, 38). Further studies indicated that the right homologs of Broca's and Wernicke's areas have prominent roles in the processing of emotional information and spoken language tone (39, 40). Our results also clearly indicated functional disturbance in speech processing circuits in patients with AVHs.

TABLE 4 | Comparisons of the pairwise normalized ratios of GC values ($R_{x\to y}$) between AVH and non-AVH groups.

Pairwise ROIs	$R_{x\to y}$			$F_{x\to y}$			$F_{y\to x}$		
	AVH group	Non-AVH group	*P*	AVH group	Non-AVH group	*P*	AVH group	Non-AVH group	*P*
STG.L - Broca.L	–0.15	0.25	0.03*	2.09	1.91	0.74	2.56	1.45	0.06
STG.L - Broca.R	–0.3	0.09	0.04*	2.03	2.16	0.81	2.81	1.64	0.02*
STG.R - Broca.R	–0.35	0.27	0.00**	1.86	2.24	0.38	2.64	1.79	0.06
STG.R - Hipp.R	–0.4	–0.04	0.04*	0.95	1.65	0.03*	1.49	1.93	0.25
Broca.R - Wernicke.R	0.2	–0.14	0.03*	1.93	1.89	0.92	1.58	2.16	0.19
Broca.L - Hipp.L	0.19	–0.22	0.04*	1.35	1.55	0.64	1.01	1.58	0.13
Broca.L - STG.R	0.34	–0.07	0.02*	2.22	1.33	0.05	1.66	1.65	0.99

*L, left; R, right; *P < 0.05; **P < 0.001.*

GC, granger causality; AVH, auditory verbal hallucinations; Hipp, hippocampus; STG, superior temporal gyrus; Broca, Broca's area; Wernicke, Wernicke's area.

Functional alterations in Broca's and Wernicke's areas in patients with schizophrenia who experience AVHs have been widely reported in fMRI studies (4). However, there have been few reports of abnormal resting FC between these areas, and most results have been inconsistent. Vercammen et al. reported reduced FC between the left temporal-parietal junction and the right homolog of Broca's area in schizophrenia patients with AVH. However, patients without AVH were not studied, and symptom correlations for hallucination severity were not presented (23). Hoffman et al. found greater FC between the bilateral Wernicke's area and Brodmann area 45/46 of IFG (Broca's area) in patients with AVH compared to patients without AVH (41); follow-up analysis indicated greater FC along a loop linking Wernicke's area, the IFG, and the putamen compared with patients without AVH and healthy controls. In addition, structural connectivity in the left arcuate fasciculus, which connects Broca's and Wernicke's areas, was lower in patients with schizophrenia with AVH than in patients with non-AVH schizophrenia and healthy controls (42–45). These results also suggested disrupted functional interactions within speech-processing systems.

AVH have been linked to several cognitive mechanisms, including misattribution or impaired self-monitoring during speech generation, which could be due to disrupted connectivity between frontal and temporo-parietal brain regions (46). Misattribution models of AVH posit that auditory hallucination experiences occur because of failure to monitor internal speech and attributing it to an external source (47, 48), and therefore predict abnormal FC between typical speech processing areas, mainly in the left fronto-temporal network (including Broca's and Wernicke's areas), possibly extending to right hemisphere language homolog areas (49). The delayed "corollary discharge" theory (50, 51) proposes that the failure of neural signal transmission between temporal speech perception areas and inferior frontal speech production areas cause precognition disability in inner speech generation, and temporal speech perception areas cannot suppress response intensity in auditory perception areas, which subsequently incorrectly identify inner speech as external speech. However, the directional information of the connection between Broca's and Wernicke's areas is not described clearly.

Ćurčić-Blake et al. (7) primarily investigated EC in the language circuitry by using dynamic causal modeling in schizophrenia patients with and without AVH in an inner speech task. Their results showed diminished connectivity from Wernicke's area to Broca's area and a decreasing trend in connectivity from homologs of Broca's and Wernicke's areas to Broca's area. Our results were obtained in a resting state by using GCA, but also suggested reduced information transmission from temporal to frontal language areas in schizophrenia AVH patients. Thus, neuronal activity of frontal language areas was less restrained by temporal language areas, leading to diminished self-monitoring and subsequent misperception of internal speech. These findings add to the aforementioned theoretical models from the perspective of information flow. However, similar research methods and results have not been reported and the connection changes in language processing mode underlying AVH have not been fully characterized. Further studies to verify these preliminary findings should be conducted.

The Memory Circuit

Increased GC from the left hippocampus to left Broca's area and decreased GC from the right STG to the right hippocampus were observed in this study. The hippocampus is associated with complex memories in humans, and may be a temporary storage site for memories (52). Hippocampal damage was associated with the occurrence of auditory hallucinations in animal models of schizophrenia (53). Several neuroimaging studies have suggested that the hippocampus is involved in auditory hallucinations. Amad et al. (54) showed that functional connections, white matter connections, and hippocampal volume changes were associated with auditory hallucinations. A meta-analysis based on "activation study" demonstrated increased activity in the left hippocampus/parahippocampal region during AVH in patients with schizophrenia (10). This region also connects widely distributed association cortices, including the language areas responsible for hallucinatory experiences. The hippocampus/parahippocampal region could therefore trigger auditory hallucination-related language brain areas, as our findings also suggest.

Based on a resting-state study, some researchers (8) found reduced FC between the left STG and left hippocampus in patients with chronic schizophrenia with auditory hallucinations compared to patients with non-auditory hallucination, which was negatively correlated with the severity of AVH. Baojuan et al. (36) reported a weakened effective connection from the auditory cortex to the hippocampus in patients with schizophrenia with AVH, by using the dynamic causal model,

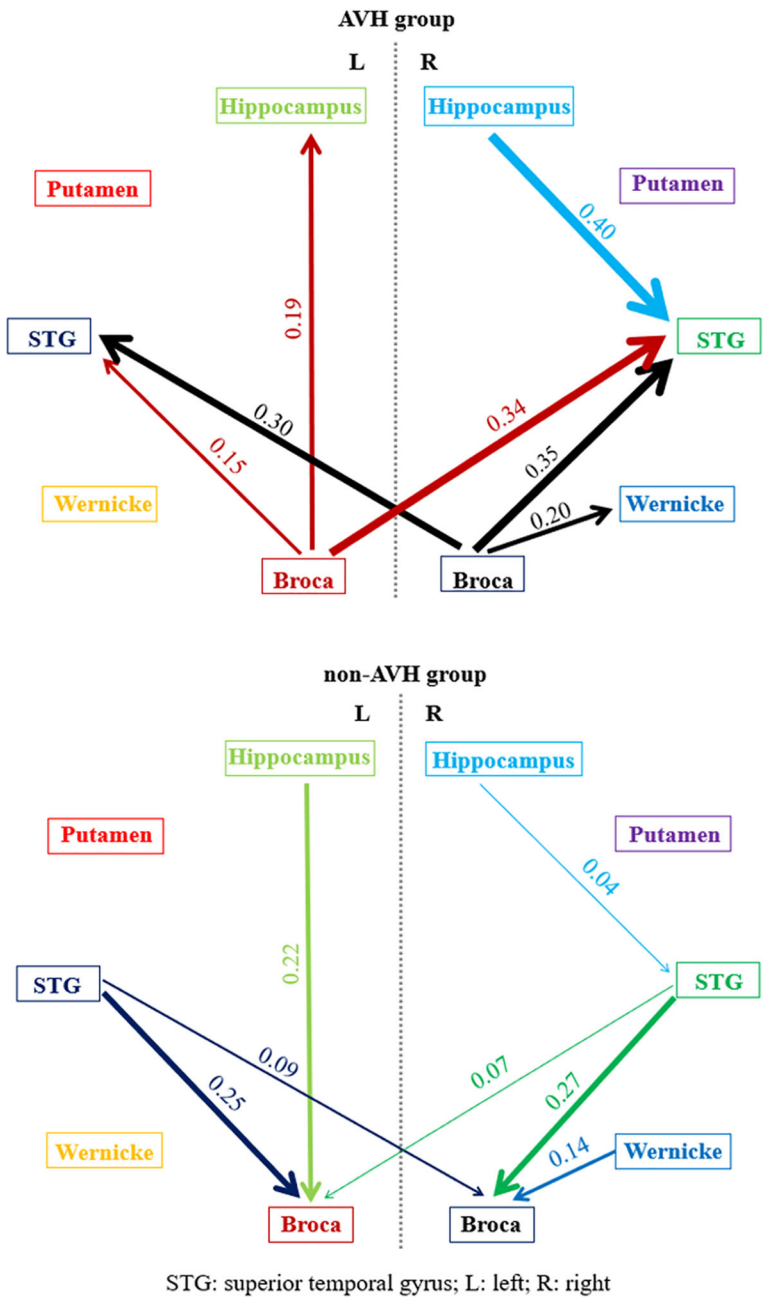

FIGURE 1 | The pairwise normalized ratios of GC values ($R_{x\rightarrow y}$) in AVH and non-AVH groups. The thickness of lines with marked numbers indicated the strength of granger causality. The different color of lines corresponded to different brain regions.

which was consistent with the results of this study. Although no projection fibers have been found between the auditory cortex and hippocampus anatomically, the results of this study and previous studies all suggest that there might be functional interactions between the two regions. However, there is still no clear understanding of how the reduction of the auditory cortical-hippocampal connection is related with the occurrence of auditory hallucinations. Baojuan et al. (36) speculated that since there are long longitudinal fibers (inferior longitudinal fasciculus) linking the visual region and the hippocampus (55), there might be a similar "auditory cortical-hippocampal projection" participating in the processing of auditory information. We hypothesized that reduced auditory cortical-hippocampal connectivity may lead to aberrant memory retrieval, which is regulated by the hippocampus/parahippocampal gyrus, subsequently triggering memory pieces stored in subcortical regions, especially those related to language, thereby causing the appearance of unconscious auditory hallucinations.

TABLE 5 | Correlations between pairwise normalized ratios of GC values and PANSS P3 score.

Pairwise ROIs	R	*P*
STG.L - Broca.L	–0.419*	0.026
STG.L - Broca.R	–0.441*	0.019
STG.R - Broca.R	–0.586#	0.001
Broca.L -STG.R	0.523#	0.004
Broca.L - Hipp.L	0.382*	0.045
STG.R - Hipp.R	–0.334	0.082
Broca.R - Wernicke.R	0.147	0.456

*L, left; R, right; *P < 0.05; #P < 0.005.*
GC, granger causality; PANSS, Positive and Negative Syndrome Scale; Hipp, hippocampus; STG, superior temporal gyrus; Broca, Broca's area; Wernicke, Wernicke's area.

Limitations

There were some limitations associated with this study. First, the sample size was small, and the study only included patients from one hospital, which might limit the scope and statistical power of our findings. Second, ROI selection was mainly based on previous literature researches, which were frequently reported regions associated with auditory hallucinations. However, several other regions associated with auditory hallucination, but with no clear consensus, were not included, which might limit the exploration of alterations in effective connections. Third, GCA ignores the influence of neurohemodynamics, which may cause displacement distortion and therefore false causality. The dynamic causal model can quantify changes of the effective connection at the neuron level, which can make up for the defects of the Granger causality model but is directly affected by the accuracy of selected ROIs. Therefore, combining data-driven and model-driven analysis by using these two effective connection methods could provide more accurate results and is a promising avenue for future research.

CONCLUSION

In this effective connection study based on GCA, we found abnormal connections in specific directions involving the auditory cortex, auditory language formation regions, and hippocampus in schizophrenia with auditory hallucinations, which may explain the mechanism of auditory hallucination from insights of auditory processing, origin of internal speech and memory. These results also provide new imaging evidences in related neural mechanisms of auditory hallucinations from the direction of the information flow.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Shaanxi Provincial People's Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XiaZ and JY were responsible for the study concept and design. JG, DZ, and LW carried out the literature search and analyzed the data. JG wrote the first draft of the manuscript. YF, YW, and XinZ carried out the image acquisition. WW examined patients with psychopathological scales. JG, DZ, MT, and XL assisted with data analysis and interpretation of findings. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Polymorphisms in the Human Aquaporin 4 Gene Are Associated With Schizophrenia in the Southern Chinese Han Population: A Case–Control Study

Yung-Fu Wu[1,2], *Huey-Kang Sytwu*[3*] *and For-Wey Lung*[2,4*]

[1] Department of Psychiatry, Beitou Branch, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan, [2] Graduate Institute of Medical Science, National Defense Medical Center, Taipei, Taiwan, [3] Department of Microbiology and Immunology, National Defense Medical Center, Taipei, Taiwan, [4] Department of Psychiatry, Calo Psychiatric Center, Pingtung County, Taiwan

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***Correspondence:**
Huey-Kang Sytwu
sytwu@ndmctsgh.edu.tw
For-Wey Lung
forwey@seed.net.tw

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Background: In psychiatric illness, pathogenic role of neuroinflammation has been supported by multiple lines of evidence. Astrocytes contribute to the blood-brain barrier (BBB) with formation of the "glymphatic" drainage system of the central nervous system (CNS) through perivascular processes. Found primarily at the end-feet of astrocytes, the aquaporin 4 (AQP4) gene has been suspected to play putative roles in the development of psychiatric disorders as well as the clearance of the glymphatic system. However, there remain many uncertainties because of the limited research on AQP4. The present study is focused on the association between AQP4 gene polymorphisms and schizophrenia (SCZ) in the Southern Chinese Han population.

Methods: Two hundred ninety-two patients and 100 healthy controls were enrolled in this study. To study the relationship of AQP4 gene polymorphisms and SCZ, genetic information was drawn from a cohort of 100 healthy controls and 100 matched patients with SCZ of Southern Han Chinese descent. Comparisons of the allele and genotype distributions between control and case groups were made using the $\chi 2$ test. Two-group comparisons were made to assess the linkage equilibrium and haplotype.

Results: Three SNPs were found. In comparison to healthy controls, patients had higher T-allele frequencies at rs1058424 and G-allele frequencies at rs3763043 ($p = 0.043$ and $p = 0.045$, respectively). Furthermore, there is an association between the decreased risk of SCZ and the AA genotype at both rs1058424 ($p = 0.021$, OR = 2.04) and rs3763043 ($p = 0.018$, OR = 2.25) The TCG haplotype ($p = 0.036$) was associated with a potential risk of SCZ, while the ACA haplotype ($p = 0.0007$) was associated with a decreased risk of SCZ and retained statistical significance after Bonferroni correction ($p = 0.006$).

Conclusions: An etiological reference for SCZ is provided by the association between AQP4 gene polymorphisms and SCZ in Southern Han Chinese population.

Keywords: schizophrenia (SCZ), single-nucleotide polymorphisms (SNPs), aquaporin 4 (AQP4), haplotype, astrocyte

INTRODUCTION

Both environmental and genetic factors play an important role in schizophrenia (SCZ), which is a multifactorial common psychiatric disorder (1–3). Given the lack of diagnostic neuropathology and defined biological markers for SCZ, it is hard to monitor the disease progression and treatment response. Currently, there is an increasing recognition in the significance of SCZ genetic polymorphisms, given the findings of genome-wide association studies (4). The modest increase in relative risk in SCZ is due to the cause by multiple genes.

However, more and more researchers have now identified the illness of SCZ is an inflammatory disease of the central nervous system (CNS) characterized by relapsing attacks of neuronal dysfunction (5). For more than 20 years, research has focused on the interactions of neurons and neuroglial cells, such as astrocytes and microglia, which result in neuroinflammation (6). Furthermore, they found that many aspects of disrupted neuronal and synaptic function, such as disrupted glutamate homeostasis, impaired action of antipsychotics, development of antipsychotic resistance, and increased permeability to inflammatory molecules may be related to the complex nature of the dysfunction of the blood-brain barrier (BBB) (7, 8). Clearance of waste for vertebrate CNS is carried out by the glymphatic system (9). The pathway consists of a para-arterial influx route for cerebrospinal fluid (CSF) to enter the brain parenchyma by BBB transport, coupled to a clearance mechanism for the removal of interstitial fluid (ISF) and extracellular solutes from the interstitial compartments of the brain and spinal cord (10). Initial arterial pulsation and regulation of exchange of solutes between ISF and CSF also depends on brain extracellular space contraction and expansion. Astrocytic aquaporin 4 (AQP4) water channels facilitate the convective bulk flow of ISF, which carries out clearance of waste products, excess extracellular fluid, and soluble proteins (11). The most abundant protein found in ependymal cell lining and in astrocytes in the ventricles showing the highest expression on perivascular astrocytes end feet surrounding blood vessels in CNS is the AQP4 water-channel protein (12). The AQP4 protein encoded by the AQP4 gene, which is located on chromosome 18, consists of five exons and four introns and, through an alternative splicing mechanism of the AQP4 protein, has two isoforms (13, 14). Coding regions of AQP4 are highly conserved, but noncoding regions demonstrate high sequence variation (15, 16). Coding nucleotide variants of AQP4 may influence conformational changes in protein structure, and this may alter the function of AQP4 proteins. Nevertheless, noncoding regions of the AQP4 gene are also thought to be involved in transcriptional and post-transcriptional regulation because of their binding affinity for transcriptional factors. In particular, the 3′ untranslated region (UTR) may be a target for microRNAs (miRNAs), which affect gene expression patterns (17). Theoretically, the total protein content and the pathogenesis of SCZ may be affected by the single nucleotide polymorphisms (SNPs) in the 5′ or 3′ UTRs of the AQP4 gene.

To summarize, due to its importance in maintenance of BBB structure, permeability, and structure, AQP4 may influence the glymphatic system in SCZ. It seems that AQP4 polymorphisms may play an important role in disease incidence, progression, and treatment response. However, most of the current gene-level studies on SCZ have not focused on the AQP4 gene. In our published studies (5), we have identified that differences in the treatment response in SCZ may be associated with the impacts of AQP4 variation. Furthermore, we have noted an association between biomarkers of treatment response and 3′ UTRs of AQP4 SNPs (rs1058424, rs335929, and rs376043) in patients with SCZ (18). We used a graphic method to dichotomize the two groups using selected cutoff points. For prediction of a higher severity of negative symptoms of SCZ, a log value of S100 calcium-binding protein B (S100B) level >1.78 may be sufficient. In conclusion, negative symptoms, poor control of neuroinflammation, and increased serum levels of S100B are more likely in patients with TAA haplotype of the AQP4 polymorphism. However, one limitation of this approach may be that in some instances the study outcome is altered when it is used instead of the traditional analysis in comparison with healthy individuals.

In this study, we further investigated the contribution of the AQP4 gene in our patient cohort by carrying out a matched case–control study. We aimed to investigate the relationship between the AQP4 polymorphisms and the risk of SCZ among the Southern Chinese Han population.

METHODS

Study Participants

All patients were enrolled from the Department of Psychiatry of the Beitou Branch of Tri- Service General Hospital. Patients aged between 20 and 70 years who experienced SCZ between January 1, 2017 and December 31, 2019 were evaluated. The control group was drawn from the community. All the cases and controls were of Southern Han Chinese ethnicity. Written informed consent were collected from both healthy controls and patients in participation of this study, with approval from the independent ethics committee/institutional review board in Taiwan.

DNA Extraction

Peripheral blood samples were provided by participants, using ethylenediaminetetraacetic acid (ETDA) tubes. DNA was extracted using an AccuBiomed iColumn 12 purification system and AccuPure Cell/Blood DNA Mini Kit (AccuBiomed, New Taipei City, Taiwan). First, 20 µl proteinase K and 200 µl whole blood were added to a 2-ml sample tube. The sample was spun down at 6,000 rpm for 30 s, and the tube was placed in the above system. Finally, the DNA samples were eluted at 100 µl elution buffer and the concentration are quantitate using Nanodrop. (ThermoScientific, Wilmington, Delaware, USA).

Segment Selection and Primer Design

The entire sequence of the human AQP4 gene comprises the full-length human AQP4 gene plus 5 kb upstream and 2 kb downstream (21.3 kb in total). Data on the genetic variation of

the entire gene were obtained from the HapMap project (http://hapmap.ncbi.nlm.nih.gov/) for 45 unrelated Chinese Han individuals in Beijing (CHB). The real-time hybridization method was used to analyze and detect the rs1058424 (T/A), rs335929 (C/A), and rs3763043 (G/A) SNPs (Roche Light Cycler 480II, Switzerland). The primer probes are listed in **Table 1**.

Polymerase Chain Reaction (PCR) Amplification

The PCR mix was prepared using a KAPA HRM FAST qPCR Kit (KAPA Biosystems, Wilmington, Massachusetts, USA). The reaction mixture contained 20 ng DNA, 1 µl 10 µM Forward and Reverse Primer, 2 µl 25 mM $MgCl_2$, 10 µl 2× KAPA HRM FAST Master Mix, and PCR-grade water to a total of 20 µl. The PCR protocol began with pre-denaturation at 95°C for 120 s, followed by pre-denaturation at 95°C for 5 s and 60°C for 40 s, repeated for 45 cycles. After the PCR cycles, the melting-curve step was started at 95°C for 1 s and 35°C for 1 min. The melting curve was produced by increasing the temperature to 90°C and took a reading for every 0.14 s. Finally, the samples were cooled to 40°C for 1 min.

DNA Sequencing, SNP Selection, and Genotyping

Salting-out method was used to extract genomic DNA from peripheral blood leukocytes. Based on the HapMap data for Han Chinese in the Beijing population, SNPs were tagged across the entire region of the AQP4 gene, selected using the tagger algorithm (http://www.broadinstitute.org/mpg/tagger/) with a pairwise approach, an r2 cutoff of 0.8, and a minor allele frequency >0.05. A total of three tag SNPs in the 3′ UTR (rs1058424, rs335929, and rs3763043) in two distinct gene regions were retrieved. TaqMan allele-specific discrimination assays on an ABI PRISM_7700 Sequence Detection System and analyzed with SDS software (Applied Biosystems, Foster City, CA, USA) was used for tag SNP genotyping. The characteristics of genotyped AQP4 tag SNPs are listed in **Table 2**.

TABLE 1 | List of primers/probes.

Hyb qPCR	Primer	Sequence (5′→3′)
rs1058424	Primer-Forward	GCAAGTGTCACTGCTCATCA
rs1058424	Primer-Reverse	AGGTGCCCTTATGATTTGGGA
rs335929	Primer-Forward	TCCCACATTACCTTGGGCAT
rs335929	Primer-Reverse	CCTTATGCATAGACTACCTTGGC
rs3763043	Primer-Forward	ACCGTGTGTCAAGATTTGGT
rs3763043	Primer-Reverse	TGAATGTGCATGACTGTGACA

Statistical Analysis

Statistical analyses were performed using SPSS Software 22.0 (IBM, Armonk, NY, USA). The case and control groups were identified using propensity score matching methods.

Continuous variables were expressed as mean ± SD. Categorical variables were expressed as N (%). Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. Patients were genotyped for risk SNPs to investigate the difference between groups. Allele and genotype frequencies were found by calculation of the Hardy–Weinberg equilibrium (HWE) of the three SNPs. A goodness-of-fit $\chi 2$ test was used to detect the HWE, and the Pearson $\chi 2$ test was used to compare allele distributions. Haploview 4.2 software (Broad Institute, Cambridge, MA, USA) was used to visualize linkage disequilibrium (LD) structure and haplotype (19). Haplotype frequency comparisons under different modes of hereditary were conducted using R package haplo.stats (20). Statistical analysis of haplotypes with traits and covariates was performed when the linkage phase was ambiguous. The $\chi 2$ test was used to assess the associations between alleles, genotypes, haplotypes, and the risk of SCZ, respectively. The Bonferroni correction was used in multiple independent comparisons ($p < 0.00625$ was statistically significant) to control for type I error, and the p-value was divided by the total number of loci or haplotypes.

RESULTS

Patient Demographics and Clinical Features

We recruited 292 consecutive cases and 100 healthy controls. For the association study, the 100 cases were matched for age, sex, and education to 100 of the healthy controls. The demographic data for the cases and controls are listed in **Table 3**. There were 41 men (41.0%) and 59 women (59.0%) in the matched case group, and 48 men (48.0%) and 52 women (52.0%) in the control group. The mean age of the cases was 45.99 ± 23.26 years and that of the controls was 42.38 ± 13.11 years. The mean age at diagnosis of SCZ was 27.43 ± 9.31 years and the mean duration of illness was 18.56 ± 11.48 years.

Allele and Genotype Analysis

We detected three SNPs (rs1058424, rs3763043, and rs335929) through the analysis of sequencing results. Allele frequencies and genotype frequencies of the detected SNP loci are listed in **Table 4**. When compared with two groups, we found that the patients had higher frequencies of the T-allele at rs1058424 and the

TABLE 2 | Characteristics of genotyped AQP4 tag SNPs.

rs number	Chromosome position	Distance from gene start	Gene position	Function	HWpval	MAF
rs1058424	24435545	3543	3′ UTR	Regulatory	1.186×10^{-5}	A/T (0.428)
rs335929	24435587	3585	3′ UTR	Regulatory	0.549	A/C (0.492)
rs3763043	24435818	3816	3′ UTR	Regulatory	0.010	G/A (0.482)

*HWpval denotes p values for the Hardy–Weinberg equilibrium in the controls.
MAF, Minor allele frequency.*

TABLE 3 | Clinical and demographic information on the participants, and baseline statistical analysis of the groups.

Variables	Case group (*n* = 100)		Control group (*n* = 100)		*p* value
	Number	%	Number	%	
Male	41	41	48	48	0.319
Age distribution (years)					0.374
20–29	13	13	18	18	
30–39	20	20	29	29	
40–49	26	26	22	22	
50–59	25	25	18	18	
60–69	16	16	13	13	
Educational level (years)					0.710
< 6	2	2	2	2	
7–9	0	0	1	1	
10–12	27	27	23	23	
> 13	71	71	74	74	
	Mean	SD	Mean	SD	*p*-Value
Age	45.99	23.26	42.38	13.11	0.658
Age at diagnosis of SCZ (years)	27.43	9.31			
Duration of illness (years)	18.56	11.48			

SCZ, schizophrenia.

TABLE 4 | Genotype distribution and allele frequencies of AQP4 SNPs between groups.

AQP4 tag SNPs	Case group (*n* = 100)		Healthy group (*n* = 100)		χ^2	OR	95% CI	*p* value
	No.	%	No.	%				
rs1058424								0.041*
Genotype								
AT	40	40.0	27	27.0				
TT	28	28.0	24	24.0				
AT+TT	68	68.0	51	51.0				
AA	32	32.0	49	49.0	5.996	2.04	1.149–3.627	0.021*
Allele								
T	96	48.0	75	37.5	4.505	1.28	1.017–1.611	0.043*
A	104	52.0	125	62.5				
rs335929								0.306
Genotype								
AC	52	52.0	42	42.0				
AA	26	26.0	28	28.0				
AA+AC	78	78.0	70	70.0				
CC	22	22.0	30	30.0	1.663	1.52	0.803–2.875	0.259
Allele								
A	105	52.5	98	49.0	0.490	1.07	0.883–1.300	0.549
C	95	47.5	102	51.0				
rs3763043								0.036*
Genotype								
AG	46	46.0	34	34.0				
GG	34	34.0	30	30.0				
AG+GG	80	80.0	64	64.0				
AA	20	20.0	36	36.0	6.349	2.25	1.189–4.258	0.018*
Allele								
A	86	43.0	107	53.5	4.415	0.80	0.655–0.987	0.045*
G	114	57.0	93	46.5				

< 0.05, **< 0.01, *< 0.001.*

G-allele at rs3763043 than healthy controls had (p = 0.043 and p = 0.045, respectively). In addition, the AA genotype at both rs1058424 (P = 0.021, OR=2.04) and rs3763043 (P = 0.018, OR=2.25) was associated with lower frequencies and a lower risk of SCZ when compared with healthy controls.

Linkage Disequilibrium and Haplotypes

Each of the result of the LD and the relationship of haplotype distribution are showed in **Figure 1** and **Table 5**. Based on proximity of SNPs, prevalence of more than 1%, and LD, eight haplotypes were selected by authors. Global significance of the positive results were obtained using permutations (n = 100,000). The TCG haplotype (p = 0.036) was associated with a potential risk of SCZ, while the ACA haplotype (p = 0.0007) was associated with a decreased risk of SCZ and retained statistical significance after Bonferroni correction (p = 0.006).

DISCUSSION

Genetic factors play important roles in SCZ pathogenesis as indicated by epidemiological data (1). The genetic basis for SCZ may not be related to a single genetic variant but may be influenced by multiple genes acting synergistically with environmental factors to increase the likelihood of the disease developing. Genetic association studies offer a powerful approach for identifying the multiple and sometimes minor variables that modulate susceptibility to this common but complex disease. Despite the critical role of AQP4 in the pathogenesis of SCZ, polymorphisms in coding regions of the AQP4 gene are unlikely to confer a risk of SCZ. On the other hand, the noncoding regions of this gene showed considerable variation among various ethnic groups. We have noted that the 3′ UTR of the AQP4 gene locus presents a high degree of variation, with several polymorphic sites that may influence SCZ susceptibility in the Southern Chinese Han population (18). In this study, we confirmed the importance of these three SNPs in the 3′ UTR region and identified the possible risk and protective haplotypes in SCZ.

As we know, the CNS is the only organ of the body that lacks a traditional lymphatic system, and as a result, has developed unique adaptations for achieving fluid balance and interstitial waste removal. AQP4 channels, which are highly concentrated in astrocytes and ependymal cells lining in the ventricles, are the most common water channels in the CNS (21–23). Also, AQP4 is involved in BBB development, function, and integrity (24). Apart from its function in water homeostasis, many studies have shown possible inter-relations between AQP4 and the glymphatic system (25). Due to its particularly high expression at the BBB and blood CSF barrier, we try to emphasize that the genetic variants of AQP4 will modulate the bidirectional fluid exchange, the subsequent activation of microglia, the development of neuroinflammation, and the treatment response of therapeutics (5, 26).

AQP4 has been implicated in the pathophysiology of many neurological (23, 27–32) and psychiatric (33–35) diseases. In a previous study, AQP4 gene polymorphisms were examined in children with febrile seizures and revealed no association with the rs1058424 AT genotype or rs3763043 CT genotype SNPs.

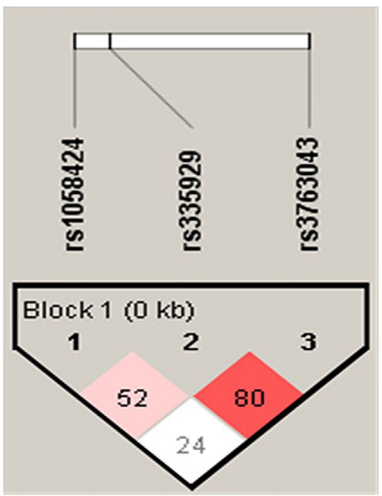

FIGURE 1 | Linkage disequilibrium (LD) in AQP4 markers (rs1058424, rs335929, and rs3763043) in SCZ and controls in a population.

Furthermore, the two selected SNPs have been researched in relation to other neurological disorders. Burfeind et al. (36) reported that rs3763043 was associated with a more rapid cognitive decline after diagnosis of Alzheimer's disease. Another study, involving patients with traumatic brain injury, revealed that the rs3763043 TT genotype was significantly more prevalent in those with poor outcomes (37). Heuser et al. (38) researched the AQP4 and Kir 4.1 genes in patients with temporal lobe epilepsy, and showed that rs1058424 was associated with the disease. Related studies have strongly pointed out the importance of water channels in the occurrence of neurological diseases, but the findings cannot be directly applied to psychiatric diseases that also originate from the brain.

Polymorphisms of the AQP4 gene have not been studied intensively in psychiatric diseases, possibly because of the highly conserved nature of the coding region. Studies in this area have been insufficient because AQP4 gene polymorphisms, especially in noncoding regions, vary among different populations. In the existing literature discussion, studies from Japan (39) and Taiwan (5, 18) have reported inconsistent results regarding the role of the AQP4 gene in SCZ. We found that Japanese research as early as 2005 was particularly focused on AQP4 SNPs (39). Although the results showed no direct association with rs3763043 in Japanese patients, it appears to be the earliest study of the correlation between SCZ and water channels. In an article published by our research team in 2012 (5), we hypothesized that the treatment response may be due to different genetic performance among individuals and the effect on changes in the function of water channels. Regarding the differences in the clinical findings related to drugs and clinical treatment effects, it seems that rs335929 may have an effect in influencing the dosage of antipsychotics. Both studies verified the importance of AQP4, but further discussion of the effect of AQP4 haplotypes was lacking. Our current study performed an analysis of eight haplotypes and showed the association of TAA haplotype and treatment response (18). We also noted that TCG haplotype was the most frequent haplotype while the ACA haplotype was the lowest frequent haplotype among 190 patients. In this case-control study, the TCG haplotype still had the highest frequencies in case group. However, the ACA haplotype was identified to be the most frequent haplotype in healthy control group. The significance of these two studies indicated that the 3′ UTRs of the AQP4 gene may be potentially associated with the incidence, progression and treatment response of SCZ in the Southern Chinese Han population.

Our study had some limitations. First, it was noted that patients with early onset of age and lower education levels were over-represented in the study. The majority of our 292 participants with SCZ had the characteristics of early onset of illness (age 25.30 vs. 27.43 years) and lower educational level (74.1% vs. 29.0% below 12 years) when compared with the 100 matched SCZ cases. Second, the current study had a small sample of healthy controls and a limited number of haplotypes (eight). Large numbers of cases and controls will be needed to increase the recombination of LD blocks and confirm our findings. Third, the Berkson bias, which is a kind of selection bias, may have existed, although the case group contained only patients with a diagnosis of SCZ. Most of the participants in this study had been hospitalized more than once and had received different medications for a long time before joining the study. Inclusion of patients with repeated hospitalizations was unavoidable. In addition, patients who could not cooperate with the treatment or for whom the treatment response was not good may have been transferred to another hospital and not enrolled in the study. Therefore, it was not possible to show the results for patients who have been diagnosed with SCZ for the first time and those who have not taken their medication regularly.

TABLE 5 | Predicted haplotypes from the AQP4 tag SNPs (rs1058424, rs335929, and rs376043) between 100 cases and 100 healthy controls.

Haplotype	Frequency	Case ratio	Control ratio	χ^2	*p* value	Adjusted *p* value[a]
TCG	0.185	45.2: 154.8	28.9: 171.1	4.418	0.036*	0.288
AAA	0.171	32.7: 167.3	35.5: 164.5	0.142	0.706	1.000
AAG	0.165	35.6: 164.4	30.3: 169.7	0.499	0.480	1.000
ACA	0.127	14.2: 185.8	36.6: 163.4	11.38	0.0007***	0.006**
TAA	0.115	25.0: 175.0	20.8: 179.2	0.437	0.508	1.000
ACG	0.110	21.6: 178.4	22.5: 177.5	0.023	0.880	1.000
TCA	0.070	14.1: 185.9	14.0: 186.0	0.000	0.987	1.000
TAG	0.057	11.7: 188.3	11.3: 188.7	0.007	0.935	1.000

< 0.05, **< 0.01, *< 0.001.*

[a]P values of no more than 0.00625 were considered statistically significant due to the Bonferroni correction.

To our knowledge, this is the first case-control study to report the associations between these three SNPs and SCZ in the Southern Chinese Han population. We conclude that patients had higher frequencies of the T-allele at rs1058424 and G-allele at rs3763043 than healthy controls had. Furthermore, the AA genotype at both rs1058424 and rs3763043 was associated with a decreased risk of SCZ. The TCG haplotype was associated with a potential risk of SCZ, while the ACA haplotype was associated with a decreased risk of SCZ and retained statistical significance after Bonferroni correction.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Tri-Service General Hospital, Taiwan. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Y-FW, with help of H-KS and F-WL, planned the present study's content and analysis, interpreted the data and wrote the paper. Y-FW, H-KS, and F-WL initiated and performed the whole survey, analyzed the data and helped to interpret the findings and to write the paper. All authors contributed to the article and approved the submitted version.

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Highly Recurrent Copy Number Variations in *GABRB2* Associated With Schizophrenia and Premenstrual Dysphoric Disorder

Ata Ullah[1†], Xi Long[1†], Wai-Kin Mat[1], Taobo Hu[1], Muhammad Ismail Khan[1], Li Hui[2], Xiangyang Zhang[3], Peng Sun[4], Mingzhou Gao[4], Jieqiong Wang[4], Haijun Wang[4], Xia Li[4], Wenjun Sun[4], Mingqi Qiao[4] and Hong Xue[1,4,5*]*

[1] Applied Genomics Center and State Key Laboratory of Molecular Neuroscience, Hong Kong University of Science and Technology, Division of Life Science, Hong Kong, Hong Kong, [2] Suzhou Guangji Hospital, The Affiliated Guangji Hospital of Soochow University, Suzhou, China, [3] Institute of Psychology, Chinese Academy of Sciences, Beijing, China, [4] School of Basic Medicine, Shandong University of Traditional Chinese Medicine, Jinan, China, [5] School of Basic Medicine and Clinical Pharmacy, China Pharmaceutical University, Nanjing, China

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United States

***Correspondence:**
Mingqi Qiao
qmingqi@163.com
Hong Xue
hxue@ust.hk

[†]These authors have contributed equally to this work

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Objective: Although single-nucleotide polymorphisms in *GABRB2*, the gene encoding for $GABA_A$ receptors β2 subunit, have been associated with schizophrenia (SCZ), it is unknown whether there is any association of copy number variations (CNVs) in this gene with either SCZ or premenstrual dysphoric disorder (PMDD).

Methods: In this study, the occurrences of the recurrent CNVs esv2730987 in Intron 6 and nsv1177513 in Exon 11 of *GABRB2* in Chinese and German SCZ, and Chinese PMDD patients were compared to controls of same ethnicity and gender by quantitative PCR (qPCR).

Results: The results demonstrated that copy-number-gains were enriched in both SCZ and PMDD patients with significant odds ratios (OR). For combined-gender SCZ patients versus controls, about two-fold increases were observed in both ethnic groups at both esv2730987 (OR = 2.15, p = 5.32E–4 in Chinese group; OR = 2.79, p = 8.84E–3 in German group) and nsv1177513 (OR = 3.29, p = 1.28E–11 in Chinese group; OR = 2.44, p = 6.17E–5 in German group). The most significant copy-number-gains were observed in Chinese females at nsv1177513 (OR = 3.41), and German females at esv2730987 (OR=3.96). Copy-number-gains were also enriched in Chinese PMDD patients versus controls at esv2730987 (OR = 10.53, p = 4.34E–26) and nsv1177513 (OR = 2.39, p = 3.19E–5).

Conclusion: These findings established for the first time the association of highly recurrent CNVs with SCZ and PMDD, suggesting the presence of an overlapping genetic basis with shared biomarkers for these two common psychiatric disorders.

Keywords: GABAA receptors, schizophrenia, premenstrual dysphoric disorder, copy number variations, genetic factors

INTRODUCTION

Schizophrenia (SCZ) is a major psychiatric disease with a significant genetic component (1, 2). Among the genes that have been related to SCZ, the single-nucleotide polymorphisms (SNPs) of the $GABA_A$ receptors β_2 subunit gene (*GABRB2*) introns 8 and 9 in chromosome 5 were found by us to be associated with SCZ in multiple populations (3, 4), and the association was confirmed in a genome-wide linkage scan (5). These SCZ-associated SNPs, located in the vicinity of the AluYi6AH-151 insertion on *GABRB2* (6, 7), influenced genotype-dependent expression, and alternative splicing of the *GABRB2* transcript giving rise to a reduced long-to-short isoform ratio of the β_2 subunit in SCZ brains (8, 9). The reduced ratio in turn rendered the β_2 subunit-containing $GABA_A$ receptors less susceptible to fatigue upon repeated stimulation, thereby retarding the turn-off of inhibitory activity of the neurons in the face of lowered energy status (9–12). As a result, some of the haplotypes in the region such as H26 and H73 have been identified as protective, while other haplotypes such as H19 and H81 have been identified as susceptibility-enhancing, toward the development of SCZ (7).

Besides the strong evidence from schizophrenic brains for an important role of *GABRB2* SNP in SCZ etiology, recently we have found that knock-out of either one or both copies of the *GABRB2* gene in mice also brought about a wide range of phenotypic alterations resembling the positive symptoms, negative symptoms and cognitive deficits of SCZ. Moreover, a range of theses SCZ-like phenotypic alterations in the knock-out mice could be ameliorated or reversed by the antipsychotic drug risperidone. These findings from the knock-out mice model were therefore in accord with those based on the SCZ-associated *GABRB2* SNPs and haplotypes in establishing a pivotal role of *GABRB2* in the origin of SCZ (13). In recent years, SCZ has been associated with a wide range of copy number variations (CNVs) outside of the *GABRB2* gene, pointing to the involvement of CNVs in some aspects of SCZ etiology (14). Accordingly, an objective of the present study was to determine whether any of the CNVs in *GABRB2* might be associated with SCZ.

Premenstrual dysphoric disorder (PMDD) is a severe form of premenstrual syndrome (PMS) that afflicts 5–10% of women in their reproductive years (15). Both animal and human studies were indicative of possible linkages of periods of neurosteroid fluctuation and alteration with the sensitivity of $GABA_A$ receptors to neurosteroids, leading to mood instability in PMDD (16, 17). Moreover, PMDD has been associated with two different groups of sex hormone-related genes, *viz.*, the estrogen receptor alpha gene *ESR1* (18), and the ESC/E(Z) genes that affect how sex hormones interact with other genes (19), but genetic association of $GABA_A$ receptors with PMDD has been largely lacking. Since the SCZ-associated SNPs in *GABRB2* have been associated with not only SCZ but also bipolar disorder (10), heroin addiction (20), and altruism (21), and some of the SCZ-associated CNVs have been associated with other mental disorders including autism and mental retardation (14), the present study was directed as well to the analysis of *GABRB2* CNVs in both SCZ and PMDD patients. The results obtained showed that the recurrent CNVs nsv1177513 in Exon 11 and esv2730987 in Intron 6 of *GABRB2* were associated with both SCZ and PMDD.

MATERIALS AND METHODS

Participants

For the SCZ study, genomic DNA was prepared using the phenol-chloroform method from the blood samples of 285 Chinese SCZ patients (160 male and 125 female) with a mean age of 39.7 ± 12.7 years, from Beijing Hui Long Guan Hospital, and 286 controls (161 male and 125 female) with a mean age of 24.4 ± 9.2 years from Hong Kong Red Cross; and 207 German SCZ patients (101 male and 106 female) with a mean age of 31.3 ± 9.3 years, and 210 controls (110 male and 100 female) with a mean age of 29.3 ± 9 years from hospitals affiliated with the University of Wurzburg. SCZ (ICD-11: F20.9) is a poorly understood mental disorder that presents hallucinations, delusions, and other cognitive issues. Typically, it has three phases—prodromal, acute, and recovery. Since the prodromal phase is mostly reported for subjects aged 20–35, and our mean sample size was 39.5 ± 12.7 years, a majority of the subjects would belong to the acute phase.

PMDD (ICD-11: F32.81) is characterized by cognitive-affective and physical symptoms in the week before mensis, and it has been designated as a disorder by American Psychiatric Association. For the PMDD study, genomic DNA was prepared using the phenol-chloroform method from the blood samples of 215 Chinese PMDD women with a mean age of 24.5 ± 4.1 years and 208 controls with a mean age of 21.4 ± 2.1 years from Shandong University of Traditional Chinese Medicine Hospital, Jinan, People's Republic of China. All samples were obtained with the written consent of subjects. The research was conducted with ethics approvals from the human participants' research panel of the Hong Kong University and Science and Technology. PMDD sample collection was approved by China ethics committee of registering clinical trials (ID: ChiECRCT-2013030).

Determination of Copy Number by Quantitative PCR

To monitor *GABRB2* CNVs in the peripheral white blood cell samples, two CNV regions with the highest recorded recurrence in the gene, *viz.*, nsv1177513 on Exon 11 (226 occurrences; chr5:160,715,688-160,717,804) and esv2730987 on Intron 6 (3 occurrences; chr5:160,796,703-160,797,020) were retrieved from the Database of Genomic Structural Variation (dbVar), along with the smaller CNV regions of esv2659732, esv3842929 and esv3077326 that overlapped with nsv1177513, and esv2667994 that overlapped with esv2730987 (see **Supplementary Table S1**). The copy number status at these regions in SCZ patients, PMDD women, and controls were determined by quantitative PCR (qPCR) using the LightCycler® 480 SYBR Green I Master kit on the Light Cycler 480 platform (Roche) with primers designed based on the sequences of nsv1177513 and esv2730987, respectively. *RNase P* and *ALB* were employed as reference

genes in qPCR, and all tests were run in triplicates. The qPCR reaction mix contained 5-µl Roche L480 SYBR Green (ROX Free), 0.2-µl 10 µM forward primer, 0.2-µl 10 µM reverse primer and 4.6-µl (4 ng/µl) DNA sample. The primer sequences for esv2730987 and nsv1177513 were designed using NCBI PrimerBlast (**Table 1**). The plates containing qPCR reaction mix in the wells were centrifuged and placed into LightCycler 480 qPCR. The PCR schedule consisted of 95°C for 5 min, 40 cycles of 95°C for 10 s, 60°C for 1 min, and 72°C for 30 s.

The threshold cycle number (Ct) was calculated for all the samples with LightCycler® 480 SW 1.5.1 software. Samples with a Ct value more than 30, and samples with a difference between any two of the replicate measurements greater than 0.5, were excluded from further analysis (24). The target gene expression level was normalized with respect to *RNase P* and *ALB* expression. The difference between the Ct value of the target and the average Ct values of *RNase P* and *ALB* was recorded as ΔCt. One known control sample was included on each plate to monitor the batch to batch variation, and to serve as the calibrator required by the ΔΔCt method (25). The normalized fold expression of the target gene was calculated as 2^(−ΔΔCt), and the exact copy number was defined as 2*2^(−ΔΔCt) (24). A copy number that fell between 1.5 and 2.5 was regarded as CN-neutral (N), and a copy number above or below these thresholds was considered to be a CN-gain (G) or CN-loss (L), respectively. The copy numbers of esv2730987 and nsv1177513 determined for the SCZ patients, PMDD patients and controls were indicated in **Supplementary Tables S2A–I**.

Statistical Analysis

All comparisons of CNV or CNV haplotype frequencies were conducted using Chi-square tests, and unadjusted *p* values were reported without multiple test correction. The 95% confidence interval (CI) of the odds ratio (OR) was estimated by

$$CI = e^{\ln(OR)\pm 1.96\times\sqrt{\frac{1}{x1}+\frac{1}{x2}+\frac{1}{y1}+\frac{1}{y2}}},$$

where *x*1, *x*2, *y*1, and *y*2 were the four figures employed to calculate the OR. The heritability of liability for SCZ was estimated by percentage of familial risk (or logRR genetic variance) attributable to CN-gain at the risk loci using INDI-V online tool at http://cnsgenomics.com/shiny/INDI-V/ with a baseline population risk of disease of 1.0% and a sibling recurrence risk of 8.8 (26). The logRR values were 2.53% and 9.30% for CN-gain at esv2730987 and nsv1177513, respectively. The combined contribution of the two loci was estimated by collapsing the CN-gains at the two loci into a single signal, and a sample with a CN-gain at either one of these two loci was counted as one CN-gain.

TABLE 1 | Primer sequences for measuring copy number status by qPCR.

Gene (CNV)	Primer	Ref.
GABRB2 (esv2730987)	Forward: 5′-CATAACAGGTTTGCTATATTTGCCA-3′ Reverse: 5′-GCCTTCACAAGTTAGATGCACA-3′	
GABRB2 (nsv1177513)	Forward: 5′-TTCTGTTCACTCCTTTCTGGTTT-3′ Reverse: 5′-TTGTTCCTTTCAACCAAAGACTCC-3′	
ALB	Forward: 5′-AATGCTGCACAGAATCCTTGGT-3′ Reverse: 5′-TCATCGACTTCCAGAGCTGAAA-3′	(22)
RNaseP	Forward: 5′-CTAACAGGGCTCTCCCTGAG-3′ Reverse: 5′-CAGCCATTGAACTCACTTCG-3′	(23)

RESULTS

CNVs in Schizophrenic Cohorts of Chinese and German Origins

GABRB2 is known to contain a considerable number of CNVs, comprising both CN-gains and CN-losses. Among them, nsv1177513 displays the highest CNV frequency in dbVar with 226 occurrences, while esv2730987 displays 3 occurrences (**Figure 1** and **Supplementary Tables S1A, B**). When combined male and female Chinese SCZ white blood cell DNA samples were analyzed by qPCR and compared to controls (**Table 2**), a two-fold increase of CN-gains was observed in the cases over controls, the frequency in esv2730987 was 24.21% in Chinese SCZ patients, exceeding that of 12.94% in controls with OR = 2.15 and p = 5.32E−4 (**Table 2**). For different genders, the frequency in esv2730987 was 26.88% in Chinese male SCZ patients, exceeding that of 16.15% in male controls with OR = 1.91 and p = 1.93E−2; and 20.80% in Chinese female SCZ patients, exceeding that of 8.80% in female controls with OR = 2.72 and p = 7.55E−3. Even more strikingly, the frequency in nsv1177513 was 54.39% in combined male and female Chinese SCZ patients, exceeding that of 26.57% in controls with OR = 3.29 and p = 1.28E−11. Its frequency was 53.13% in Chinese male SCZ patients, exceeding that of 26.09% in male controls with OR = 3.21 and p = 7.30E−7; and 56.00% in Chinese female SCZ patients, exceeding that of 27.20% in female controls (OR = 3.41, p = 3.85E−6).

For the German cohorts, the frequency of CN-gains in esv2730987 in the combined male and female SCZ samples was 11.11%, higher than the controls at 4.29% with OR = 2.79 and p = 8.84E−3. Its frequency in the male SCZ patients was 7.92%, which was not significantly higher than the male controls at 4.55% with only p = 3.08E−1 (OR = 1.81); whereas its frequency in the female SCZ patients was 14.15%, significantly higher than the female controls at 4.00% with OR = 3.96 and p = 1.19E−2. Moreover, the frequency of nsv1177513 was 37.20% for the combined male and female German SCZ patients, which was significantly higher than the controls at 19.52% with OR = 2.44 and p = 6.17E−5. Its frequency was 41.58% in the male SCZ patients, higher than the male controls at 23.64% with OR = 2.30 and p = 5.33E−3; its frequency was 33.02% in the female SCZ patients, higher than the female controls at 15.00% with OR = 2.79 and p = 2.57E−3. Therefore, for both the Chinese and German cohorts, female SCZ patients showed higher CN-gain occurrences at both esv2730987 and nsv1177513 compared to female controls. Overall, it was estimated that 9.36% of the variance in heritability of SCZ liability could be explained by carrying a CN-gain within either or both of the two highly recurrent CNVs in *GABRB2* (**Supplementary Table S3**).

Between the two ethnic populations, the frequency of CN-gain in esv2730987 was significantly higher in male Chinese

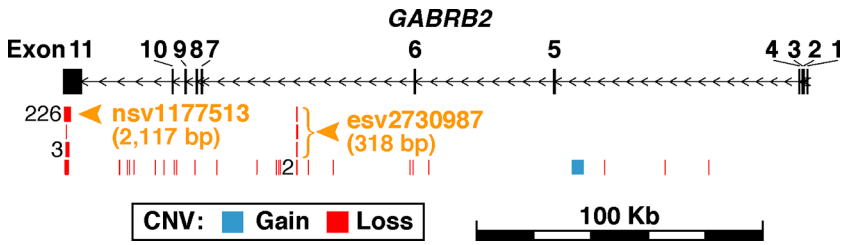

FIGURE 1 | Schematic representation of germline CNVs in *GABRB2*. The region shown spans 20-Kb upstream and downstream of *GABRB2* at chr5:160,715,436-160,975,130. Exons are represented by the black vertical rectangles, and introns by horizontal line segments between the exons. Each exon is numbered above its corresponding rectangle. Black arrows indicate direction of transcription. CNVs retrieved from the database of human genomic structural variation (dbVar; file date 2018-03-04) are shown in blue for copy-number-gains and red for copy-number-losses. CNVs with identical genomic coordinates are merged as one in the figure, with their number of occurrences labeled left to their positions. A total of 261 CNVs falling within *GABRB2* are mapped to 31 parental copy-number-regions in dbVar (see the full list of CNVs in **Supplementary Table S1A** and the summary statistics of their parental copy-number-regions in **Supplementary Table S1B**). CNVs are found to be highly recurrent in Exon 11 and Intron 6, where the parental copy-number-regions with the most CNV occurrences were selected for investigation in the present study. Orange arrowheads and bracket indicate the locations of the two investigated copy-number-regions, *viz.*, nsv1177513 at chr5:160,715,688-160,717,804 with identical CNVs from 226 samples, and esv2730987 at chr5:160,796,703-160,797,020 with three different CNVs from three samples.

TABLE 2 | Copy number variations in schizophrenia patients from two ethnic groups.

Ethnic Group	Gender[a]	Control (CNVs)[b]			SCZ (CNVs)[c]			*p*-Value[d]	OR (95% CI)[e]
		L	N	G (%)	L	N	G (%)		
esv2730987									
Chinese	M + F	7	242	37 (12.94)	1	215	69 (24.21)	5.32E–04	2.15 (1.39–3.33)
Chinese	M	6	129	26 (16.15)	0	117	43 (26.88)	1.93E–02	1.91 (1.11–3.30)
Chinese	F	1	113	11 (8.80)	1	98	26 (20.80)	7.55E–03	2.72 (1.28–5.79)
German	M + F	8	193	9 (4.29)	7	177	23 (11.11)	8.84E–03	2.79 (1.26–6.19)
German	M	4	101	5 (4.55)	6	87	8 (7.92)	3.08E–01	1.81 (0.57–5.71)
German	F	4	92	4 (4.00)	1	90	15 (14.15)	1.19E–02	3.96 (1.27–12.36)
nsv1177513									
Chinese	M + F	31	179	76 (26.57)	10	120	155 (54.39)	1.28E–11	3.29 (2.32–4.68)
Chinese	M	18	101	42 (26.09)	4	71	85 (53.13)	7.30E–07	3.21 (2.01–5.13)
Chinese	F	13	78	34 (27.20)	6	49	70 (56.00)	3.85E–06	3.41 (2.01–5.78)
German	M + F	20	149	41 (19.52)	21	109	77 (37.20)	6.17E–05	2.44 (1.57–3.80)
German	M	7	77	26 (23.64)	3	56	42 (41.58)	5.33E–03	2.3 (1.27–4.16)
German	F	13	72	15 (15.00)	18	53	35 (33.02)	2.57E–03	2.79 (1.41–5.53)

[a]*Gender: M = male, F = female, M + F = male + female.*
[b]*Control samples: mean age 24.4 ± 9.2 years in Chinese, and 29.3 ± 9 years in German; L = CN-loss, N = CN-neutral, G = CN-gain.*
[c]*SCZ samples: mean age 39.7 ± 12.7 years in Chinese, and 31.3 ± 9.3 years in German.*
[d]*p-Value (unadjusted for multiple testing) by Chi-square test between G and L+N of controls vs. G and L+N of SCZ patients.*
[e]*Odds ratio = [G/(L+N) in SCZ]/[G/(L+N) in Control]. 95% confidence interval is indicated in the parentheses.*

controls compared to male German controls with OR = 4.04 and $p = 5.91E{-}3$; whereas there was no significant difference between female Chinese and female German controls (**Supplementary Table S4A**). For SCZ-patients, CN-gains in both esv2730987 and nsv1177513 were significantly higher in the Chinese compared to the German cases for the male, female or combined male and female groups, with OR ranging from 1.59 to 4.27 and *p*-values from 2.06E–3 to 2.63E–44 (**Supplementary Table S4B**). No significant difference in CN-gains was observed between the genders in any of the ethnic groups (**Supplementary Table S4C**).

There were significant reductions in CN-losses in Chinese male SCZ patients compared to controls at both esv2730987 ($p = 4.01E{-}2$) and nsv1177513 ($p = 4.28E{-}3$). On the other hand, there was no case-control difference at either esv2730987 or nsv1177513 in the Chinese female group or the German male or female group (**Supplementary Table S4D**). CN-losses at nsv1177513 were fewer only in the male German SCZ group compared to the female German SCZ group ($p = 5.00E{-}3$, **Supplementary Table S4C**). Notably, there were fewer neutral copy numbers in the Chinese and German SCZ groups compared to the controls (**Table 2** and **Supplementary Table S4D**) in agreement with earlier reports (27).

CNVs in Premenstrual Dysphoric Disorder Cohort of Chinese Origin

When female DNA samples of women with PMDD were compared to those of female non-PMDD controls, the two groups showed a significant difference with respect to the

frequencies of CN-gains at both esv2730987 and nsv1177513. The 66.51% frequency in esv2730987 at PMDD patients exceeded that of 15.87% in controls (OR = 10.53, p = 4.34E−26), and the 43.72% frequency at nsv1177513 in PMDD patients likewise exceeded that of 24.52% in controls (OR = 2.39, p = 3.19E−5). While there was no significant change in CN-losses at either esv273098 or nsv1177513 between PMDD patients and controls, there were far fewer instances of neutral copy numbers at both esv273098 (p = 2.49E−33) and nsv1177513 (p = 6.08E−12) in PMDD patients compared to the controls (**Table 3** and **Supplementary Table S4E**).

CNV Haplotypes in SCZ and PMDD Cohorts

When the frequencies of the nine haplotypes based on the gain (G), neutral (N), or loss (L) at the esv2730987 and nsv1177513 loci were compared between the SCZ or PMDD patients and their controls, the frequency of the G-G haplotype (*viz.*, CN-gain at both esv2730987 and nsv1177513) showed the largest increase in both SCZ and PMDD patients relative to their controls, yielding ORs of 2.73 and 3.89 in the combined-gender groups of the Chinese and German SCZ cohorts, respectively, and OR of 5.81 in the PMDD cohort (**Table 4**). The OR between patients and controls for the G-G double-gain at these two CNV loci reached 6.78 in the German female SCZ group, exceeding that of 3.96 and 2.79 for the individual esv2730987 and nsv1177513 locus (**Table 2** and **Supplementary Table S4F**). However, in other case-control comparisons, the ORs for the double-gain haplotype was between that for the gain at esv2730987 and the nsv1177513 locus (**Supplementary Table S4F**). Another haplotype showing significant increase in SCZ patients relative to controls was the N-G haplotype in both the Chinese combined-gender group (OR = 2.36, p = 1.68E−5) and the German combined-gender group (OR = 1.87, p = 1.19E−2). The G-N haplotype also occurred more in the PMDD patients compared to controls reaching an OR of 5.57 (p = 5.26E−11). The N-N haplotype frequencies were all significantly reduced in patients compared to controls for both diseases throughout the different population or gender groupings (**Supplementary Table S4F**).

DISCUSSION

Previously, a range of rare, high-penetrant CNVs have been identified at various locations in the human genome that increased the risk of SCZ as well as other psychiatric diseases such as mental retardation and autism (28). Rare CNVs at 1q21.1, 2p16.3 (NRXN1), 3q29, 7q11.2, 15q13.3, distal 16p11.2, proximal 16p11.2, and 22q11.2 conferred significant risk of SCZ with ORs of 2-60, accounting for 0.85% of SCZ cases (29, 30). More generally, we have found that recurrent germline CNV profiles predict cancer susceptibility (31). Therefore, the present study was directly to recurrent rather than rare CNVs.

Highly Recurrent CNVs in *GABRB2* Associated With SCZ

The *GABRB2* gene, SNPs in which associated with SCZ in Chinese with an OR of 2.02 to 2.50 (3), harbored a number of recurrent germline CNVs including esv2730987 and nsv1177513, the latter of which has the highest frequency recorded in *GABRB2*. Results showed in **Table 2** indicated that the combined male-female frequencies for CN-gains in esv2730987 and nsv1177513 were both significantly higher in cases over controls, establishing the associations of highly recurrent CNVs with SCZ, in both Chinese and German. Based on the results from this study, it is estimated that 9.36% of the variance in SCZ liability can be explained by carrying a CN-gain within either or both of the two highly recurrent CNVs in *GABRB2* (**Supplementary Table S3**). In contrast, a previous study has reported that only about 2.5-3% of SCZ heritability can be explained collectively by a total of 32 SCZ-associated SNPs and CNVs (26).

The p-values were more significant for CN-gain increases in both nsv1177513 and esv2730987 in the Chinese relative to the German SCZ patients. With respect to genders, the CN-gains in both esv2730987 and nsv1177513 were significantly elevated in both female Chinese and female German patients, as well as the male Chinese patients. In contrast, the German male patients displayed significant elevation in CN-gains in nsv1177513 but not in esv2730987. This weaker correlation of CN-gains with SCZ in esv2730987 in the male German patients was also evident in the weaker correlation of the CN-gains in the nsv1177513 in the male German patients and in the esv2730987 in the female Chinese patients, but opposite for the stronger correlation observed in nsv1177513 in the male Chinese patients compared to the female ones.

Previously, with the rare high-penetrant CNVs that increased the risk of SCZ, autism and mental retardation, disease propensity was only associated with both CN-gains and CN-losses (28), in contrast to the association of CN-gains with SCZ

TABLE 3 | Copy number variations in PMDD female patients of Chinese origin.

CNV ID[a]	Control (CNVs)[b]			PMDD (CNVs)[c]			*p-Value*[d]	OR (95% CI)[e]
	L	N	G	L	N	G		
esv2730987	5	170	33 (15.87)	4	68	143 (66.51)	4.34E−26	10.53 (6.60–16.81)
nsv1177513	5	152	51 (24.52)	4	117	94 (43.72)	3.19E−05	2.39 (1.58–3.62)

[a]*CNV region in dbVar.*
[b]*Control samples: mean age 21.4 ± 2.1 years; L = CN-loss, N = CN-neutral, G = CN-gain.*
[c]*PMDD samples mean age 24.5 ± 4.1 years.*
[d]*p-Value (unadjusted for multiple testing) by Chi-square test between G and L+N of controls vs G and L+N of PMDD patients.*
[e]*Odds ratio = [G/(L+N) in PMDD]/[G/(L+N) in Control]. 95% confidence interval is indicated in the parentheses.*

TABLE 4 | Haplotypes of copy number variations in SCZ and PMDD cohorts.

Haplotype [a]	No. of Cases		No. of Controls		*p*-Value [b]	OR (95% CI) [c]
	With Haplotype (%)	Without Haplotype	With Haplotype (%)	Without Haplotype		
Chinese SCZ cohort (Male + Female)						
G-G	57 (20.00)	228	24 (8.39)	262	1.16E–04	2.73 (1.64–4.54)
G-N	12 (4.21)	273	13 (4.55)	273	1.00E+00	0.92 (0.41–2.06)
L-L	1 (0.35)	284	3 (1.05)	283	6.18E–01	0.33 (0.03–3.21)
L-N	0 (0.00)	285	4 (1.40)	282	1.33E–01	0.00 (0.00–NaN)
N-G	98 (34.39)	187	52 (18.18)	234	1.68E–05	2.36 (1.60–3.47)
N-L	9 (3.16)	276	28 (9.79)	258	2.30E–03	0.30 (0.14–0.65)
N-N	108 (37.89)	177	162 (56.64)	124	1.07E–05	0.47 (0.33–0.65)
German SCZ cohort (Male + Female)						
G-G	18 (8.70)	189	5 (2.39)	204	9.37E–03	3.89 (1.41–10.67)
G-L	1 (0.48)	206	0 (0.00)	209	9.96E–01	Inf (NaN–Inf)
G-N	4 (1.93)	203	4 (1.91)	205	1.00E+00	1.01 (0.25–4.09)
L-G	1 (0.48)	206	0 (0.00)	209	9.96E–01	Inf (NaN–Inf)
L-L	2 (0.97)	205	4 (1.91)	205	6.90E–01	0.50 (0.09–2.76)
L-N	4 (1.93)	203	4 (1.91)	205	1.00E+00	1.01 (0.25–4.09)
N-G	58 (28.02)	149	36 (17.22)	173	1.19E–02	1.87 (1.17–2.99)
N-L	18 (8.70)	189	16 (7.66)	193	8.35E–01	1.15 (0.57–2.32)
N-N	101 (48.79)	106	140 (66.99)	69	2.53E–04	0.47 (0.32–0.70)
Chinese PMDD cohort (Female)						
G-G	60 (27.91)	155	13 (6.25)	195	8.21E–09	5.81 (3.08–10.96)
G-L	3 (1.40)	212	0 (0.00)	208	2.58E–01	Inf (NaN–Inf)
G-N	80 (37.21)	135	20 (9.62)	188	5.26E–11	5.57 (3.25–9.54)
L-G	3 (1.40)	212	0 (0.00)	208	2.58E–01	Inf (NaN–Inf)
L-L	0 (0.00)	215	2 (0.96)	206	4.64E–01	0.00 (0.00–NaN)
L-N	1 (0.47)	214	3 (1.44)	205	5.92E–01	0.32 (0.03–3.09)
N-G	31 (14.42)	184	38 (18.27)	170	3.47E–01	0.75 (0.45–1.27)
N-L	1 (0.47)	214	3 (1.44)	205	5.92E–01	0.32 (0.03–3.09)
N-N	36 (16.74)	179	129 (62.02)	79	3.58E–21	0.12 (0.08–0.19)

[a]*Haplotype based on the gain (G), neutral (N), and loss (L) at the esv2730987 and nsv1177513 loci. The G, N or L before hyphen indicates CNV status at esv2730987 and that after hyphen the status at nsv1177513. Only seven of the nine possible haplotypes were found in the Chinese SCZ cohort.*

[b]*p-Value (unadjusted for multiple testing) by Chi-square test for numbers of samples with haplotype and without haplotype among cases vs those among controls.*

[c]*OR = (No. of cases with haplotype/No. of cases without haplotype)/(No. of controls with haplotype/No. of controls without haplotype). 95% confidence interval is indicated in the parentheses.*

in esv2730987 or nsv1177513. The nature of the perturbations occasioned by CNVs could be multifaceted and vary from one gene to another. Earlier, pairwise co-localizations of 42 genomic features have enabled a tripartite division of the human genomic sequence into the Genic, Proximal, and Distal sequence zones, which are distinguishable in terms of their constituent genomic variants (32). The DNA sequences in Proximal zones, where *GABRB2* locate, tolerate small genetic variants such as microsatellites but not long genetic variants such as large CNVs, possibly on account of excessive disruption of inter-gene distances involving the gene-regulatory elements by the long variants (32). The mechanisms of the disruption by CN-gains in either esv2730987 or nsv1177513 yet remain to be elucidated. Nevertheless, the strong association and high heritability of liability demonstrated in this study support that the two recurrent CNVs in *GABRB2* may serve as excellent biomarkers.

Association of Highly Recurrent CNVs With PMDD

Neuropsychiatric changes in PMDD have been observed and characterized extensively (19, 33, 34). Women with PMDD often present recurrent symptoms of anxiety and depression, which have been associated with genetic variations of *ESR1* (18). Studies have linked progesterone to modulation of the medial prefrontal cortex (mPFC) and testosterone to the orbitofrontal cortex (OFC), along with the implication of various cortical and subcortical regions in an attempt to explain the psychiatric vulnerability in women (35). In addition, other studies have shown that OFC function varies with changes in progesterone concentration throughout the menstrual cycle, suggesting a relationship between psychiatric effect of PMDD with the coupling of mPFC and OFC with the amygdala (34). Moreover, PMDD also has been linked to the impaired activation of the left

amygdala and dorsal anterior cingulate gyrus during emotional processing (36–38).

In another approach, it has been reported that the estrogen-sensitive epigenetic ESC/E(Z) complex in lymphoblastoid cell lines (LCLs) differed between women with PMDD and non-affected female controls at the RNA and protein levels (19), pointing to a linkage between periods of neurosteroid fluctuation and altered sensitivity of $GABA_A$ receptors to neurosteroids (16, 17, 39). The present finding that recurrent CN-gains at nsv1177513 and esv2730987 were enriched in PMDD subjects thus expanded further the spectrum of neuropsychiatric disorders associated with *GABRB2* beyond SCZ (3, 8), bipolar disorder (10), and heroin addiction (20), and provided the first known instance of PMDD association with a gene expressed in the central nervous system.

A striking parallel between PMDD and SCZ pertaining to their associations with the recurrent CNVs in *GABRB2* was that the double CN-neutral haplotype N-N at the two CNV sites was protective against both diseases. Whereas the double CN-gain haplotype G-G was risk-conferring to both diseases. However, disease-association was stronger at esv2730987 in PMDD, but stronger at nsv1177513 in SCZ. More significant associations were observed with PMDD compared to SCZ with respect to both genotypes (**Tables 2** and **3**) and haplotypes (**Table 4**).

Recurrent CNVs as Markers in Association Studies

The recurrent CNVs represent useful markers for the search of genetic components attributable to complex disorders. The association of recurrent CNVs with complex disorders have been revealed by their large ORs reaching up to 3.96 in the SCZ cohorts and 10.53 in the PMDD cohorts in the present study, as well as by the previous finding of useful recurrent CNVs for cancer predisposition (31). Different from the frequently used common SNPs and rare structural variations in association studies, the recurrent CNVs represent the intermediate form of genetic variants. On the one hand, they are more robust than SNPs regarding the confounding effects brought about by complex genetic forces like recombination and natural selection. On the other hand, due to their high recurrency, they could readily explain more of the disease variations in the population than rare structural variations do. Therefore, in-depth investigation of recurrent CNVs is needed for future association studies of complex diseases and traits.

In addition to the recurrent CNVs in *GABRB2*, SCZ has also been associated with SNPs in the same gene, underlining the central role of this gene in the SCZ etiology as well as the genetic instability of the gene. Such instability would enhance the occurrences of genetic variants, in both common, e.g., SNPs and rare, e.g., CNVs forms. Given the functional significance of *GABRB2* in the central nervous system, sequence and structural alterations could lead to functional perturbations of the gene, and thereby associations with psychiatric disorders. On the other hand, the associations of PMDD also with the same recurrent CN-gains in *GABRB2* helped to explain the symptomology of PMDD involving not only pain but also emotional disturbance, and pointed to possible overlap between the etiological mechanism for SCZ and PMDD.

Although, close association between *GABRB2* and SCZ has been confirmed by meta-analyses of candidate genes for SCZ (40, 41), genome-wide association studies (GWASs) have not reported to date any significant association between genetic variants in *GABRB2* and SCZ (42, 43). The GWAS results might seem inconsistent with the positive signals from the genome-wide linkage and candidate gene studies, as well as the SCZ-like phenotypic alterations displayed by the *GABRB2* knock-out mice (13). However, a fine-resolution linkage disequilibrium analysis of a 3,551-bp segment of *GABRB2* revealed active recombination together with intense positive selection on the derived alleles in this region (7). Such co-occurrence of recombination and positive selection could blur the local SNP-based signals in GWAS, causing the genetic components attributable to complex traits and diseases difficult to recognize, whereas CNV-based signal could be more resistant to such confounding factors. Therefore, genes and genomic regions subject to both active recombination and selection would merit close examination and require the employment of markers, such as CNVs, that are more robust to the effects of recombination-selection co-occurrence to avoid any missing heritability pertaining to the development of complex diseases.

Closing Remarks

With the employment of recurrent CNV markers, the present study has established for the first time the genetic associations between CN-gains in *GABRB2* and two different psychiatric disorders, namely SCZ and PMDD in face of the effects of active recombination and natural selection on this gene. Most neuropsychiatric disorders have significant genetic components. Different neuropsychiatric disorders are often related to one another in their phenotypic characteristics, which could stem out of common genetic variations, or "shared genetics", exemplified by the genetic overlap between Alzheimer's disease and bipolar disorder with respect to the *MARK2* and *VAC14* genes (44), the genetic overlap between SCZ, bipolar disorder, and intelligence (45), and the shared genetic variants between SCZ and lung cancer (46). In the same view, the present finding of the same set of recurrent CN-gains in *GABRB2* associated two clinically very different psychiatric disorders delineate further the central role of this gene in neuropsychiatric disorders, and providing useful insights into overlapping genetic mechanisms underlying the comorbidities of different psychiatric disorders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found at https://www.ncbi.nlm.nih.gov/clinvar/, under the accession numbers: SCV001334130, SCV001334131, SCV001334132, SCV001334133, SCV001334134, SCV001334135, SCV001334136, SCV001334137.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Beijing Hui Long Guan Hospital Hong Kong Red Cross. The hospitals affiliated with the University of Wurzburg German Shandong University of Traditional Chinese Medicine Hospital, Jinan, People's Republic of China. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HX conceived and designed the experiments. AU, W-KM, XLo, and MK performed the experiments and analysis of the data. TH identified the two recurrent CNV regions from database and designed the primers. LH and XZ coordinated the collection of schizophrenia samples of Chinese origin. PS, MG, JW, HW, XLi, WS, and MQ coordinated the collection of PMDD and control cohorts, and AU, XLo, and HX wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.00572/full#supplementary-material

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A Comprehensive Analysis of the Effect of SIRT1 Variation on the Risk of Schizophrenia and Depressive Symptoms

Dandan Wang[1†], Wei Tang[2†], Junxiong Zhao[3], Weixing Fan[3], Yi Zhang[1] and Chen Zhang[1]*

[1] Shanghai Mental Health Center, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2] Department of Psychiatry, The Affiliated Kangning Hospital of Wenzhou Medical University, Wenzhou, China, [3] Department of Psychiatry, Jinhua Second Hospital, Jinhua, China

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***Correspondence:**
Chen Zhang
zhangchen645@gmail.com

[†] These authors have contributed equally to this work

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Depressive symptoms could be considered a mutual manifestation of major depressive disorder and schizophrenia. Rs3758391 is a functional locus of Sirtuin (SIRT1) involving depression etiology. In this study, we hypothesized that the SIRT1 SNP rs3758391 might be a hazard for schizophrenia pathogenesis, especially related to the appearance of depressive symptoms. We recruited 723 healthy controls and 715 schizophrenia patients, the occurrence of psychotic and depressive symptoms was evaluated by Calgary Depression Scale (CDSS) and PANSS. Meanwhile, qt-PCR was used to detect the mRNA levels of SIRT1 in peripheral blood of 197 olanzapine monotherapy schizophrenia patients. 45.6% of schizophrenia patients had depressive symptoms. In the patient group, mRNA levels of patients with depressive symptoms were significantly lower than those without depressive symptoms ($P < 0.01$). CDSS scores of schizophrenia patients with different rs3758391 genotypes were significantly different ($P < 0.01$). *Post hoc* comparisons indicated that the CDSS scores of rs3758391 C/C and C/T carriers were higher than those of T/T carriers (Ps < 0.01). In the occipital cortex, our eQTL analysis showed that there was a clear correlation between rs3758391 and the SIRT1 mRNA levels. Our preliminary findings provide suggestive evidence that SIRT1 makes schizophrenia patients more prone to depressive symptoms. This SNP might be a biomarker of depression in schizophrenia.

Keywords: schizophrenia, depression, SIRT1, CDSS, association, polymorphism

INTRODUCTION

Schizophrenia is a heterogeneous group of syndromes whose main symptoms mainly involve multiple dimensions, including cognitive dysfunction, negative symptoms, and positive symptoms. And major depressive disorder (MDD) is another common serious mental illness, mainly manifested by symptoms such as inability to concentrate, loss of interest, and low mood.

In clinical practice, depressive symptoms commonly accompany schizophrenia (Green et al., 2003). Depressive symptoms could be considered to be mutual manifestations of both schizophrenia and MDD (Chen et al., 2017). Studies have shown that about 50% of patients with schizophrenia have comorbid depression (Buckley et al., 2009). Although the pathophysiology of both of these mental disorders remains unknown, numerous family studies have highlighted the crucial role of genetic factors in the pathogenesis in MDD and schizophrenia (Zhang et al., 2016a). Notably, a good deal of genome-wide association research has uncovered overlapping genetic risk factors shared by patients with either disorder (Lee et al., 2013; Xiao et al., 2018).

Sirtuin (SIRT1) is an important nicotinamide-dependent protein deacetylase in the sirtuin protein family. The function of SIRT1 involves many aspects, and the functions that have been discovered so far mainly include regulating cell survival and apoptosis and inhibiting the stress-induced inflammatory response (Rahman and Islam, 2011). Additionally, SIRT1 also plays an important role in regulating biological rhythm and transducing dopaminergic signals (Kim et al., 2016). Subsequently, genetic studies in Eastern Asian populations (mainly Japanese and Chinese Han populations) indicated that the SIRT1 gene is associated with schizophrenia (Wang et al., 2015). Therefore, at least in Asian populations, SIRT1 variation is likely to contribute to the risk of schizophrenia.

Lately, a whole-genome sequencing experiment containing thousands of homogeneous Chinese samples identified one locus near SIRT1 that is significantly associated with MDD (CONVERGE consortium, 2015). Then our further study found that the expression of SIRT1 in the peripheral blood of MDD patients was significantly downregulated at the mRNA levels compared with that in the blood of healthy subjects (decreased by 37%; Luo and Zhang, 2016). Large-scale MDD expression data have further confirmed this finding (Jansen et al., 2016). Recently, in the Han Chinese population, we discovered a functional locus, rs3758391, in SIRT1 related to the etiology of MDD (Tang et al., 2018). The rs3758391 (T/C) is a gene promoter of the SIRT1 gene. And the critical functions of this SNP in the pathophysiology of human diseases are mainly manifested in the C variation might destroy the p53-binding sequence and affect the expression of SIRT1 (Naqvi et al., 2010; Hu et al., 2015). And our previous work also indicated that low plasma SIRT1 levels in schizophrenia patients are associated with depressive symptoms (Fang et al., 2019). Based on the above discoveries, we hypothesized that the SIRT1 SNP rs3758391 variation might be one of the causes of schizophrenia, especially its depressive symptoms.

In this research, we used public databases to explore the differences in SIRT1 expression in the brains of schizophrenic patients and healthy controls. Later, we attempted to identify the association of SIRT1 mRNA and the rs3758391 polymorphism with susceptibility to schizophrenia and associated depressive symptoms. Finally, in order to detect the effect of rs3758391 polymorphism on brain SIRT1 mRNA expression, we used an available database for eQTL analysis.

MATERIALS AND METHODS

Subjects

Seven hundred and fifteen schizophrenia patients were recruited from mental health institute in Eastern China (Shanghai Mental Health Center, Affiliated Kangning Hospital, Wenzhou Medical University, and Jinhua Second Hospital), the inclusion criteria are in accordance with our previous publications and are as follows (Zhang et al., 2011; Cai et al., 2015; Wang et al., 2016; Zhang et al., 2018): (1) illness course less than 5 years; (2) with a stable condition over 6 months; (3) junior high school education or above; (4) not treated with a mood stabilizer or antidepressant; and (5) total scores for PANSS under 60 Patients who with other psychiatric disorders, had a severe physical disease, with substance dependence or abuse, are pregnant or nursing women were all excluded. Moreover, mRNA samples were obtained from 198 patients who treated with olanzapine monotherapy. The 723 healthy subjects were included as controls, and the detailed recruitment requirements were based on our previous studies (Zhang et al., 2017).

The Institutional Review Boards of Jinhua Second Hospital and other related institutions reviewed and approved this research. We obtained the written informed consent of all participants and strictly followed the experimental guidelines in the Declaration of Helsinki.

Evaluation

The psychiatric symptoms and the severity of depression were evaluated by the PANSS and the Calgary Depression Scale (CDSS). Patients were defined as having significant depression if they had a CDSS score of 7 or higher (Peitl et al., 2017).

Analysis of Brain SIRT1 Expression

The genetics and expression data platform for schizophrenia research named SZDB database[1] were used to compare the SIRT1 expression between case and controls (Wu et al., 2017).

RNA Preparation and Quantitative Real-Time Polymerase Chain Reaction

The RNA preparation and quantitative real-time polymerase chain reaction (qRT-PCR) were performed as our previous work (Luo and Zhang, 2016).

Genotyping

The SNP rs3758391 was genotyped as our previous work has described (Zhang et al., 2014).

PGC Data Analysis

The Psychiatric Genomics Consortium (PGC[2]) database was used to find out the association of rs3758391 polymorphism in schizophrenia. The more detailed description of this database sees our previous article (Zhang et al., 2016a).

[1] http://www.szdb.org/

[2] http://www.broadinstitute.org/mpg/ricopili/

Brain eQTL Analysis

The brain eQTL database[3] (Ramasamy et al., 2014) were utilized for eQTL analysis of the rs3758391 polymorphism in the brain.

Statistical Analysis

We used the SHEsis software to compare the genotype and allele distribution in the case-control study. The Hardy-Weinberg equilibrium (HWE) was calculated by Haploview 4.2.

The ANCOVA was used to compare the CDSS scores between groups with different genotypes. When comparing the difference in SIRT1 mRNA expression between groups, we used ANCOVA to further controlled the potential covariates including age, gender, duration of olanzapine treatment, and olanzapine daily dosage. In addition, Pearson's correlation analysis was conducted to explore the correlation between the levels of SIRT1 mRNA expression and CDSS scores in the patients. SPSS 17.0 was used for statistical analyses. All tests were two-tailed, with significance set to $p < 0.05$.

RESULTS

We extracted the data for SIRT1 mRNA expression in the brain from the SZDB database. But we failed to find any significant differences in SIRT1 mRNA expression in the striatum, prefrontal cortex, or hippocampus of the case and control groups (**Figure 1**). At the molecular level, the distribution of rs3758391 genotypes in healthy controls and schizophrenia patients all established HWE. **Table 1** presented comparisons of the allele frequency and genotype of the rs3758391 SNP in schizophrenia and healthy controls but found no difference. Next, we also found no significant association between the rs3758391 polymorphism and schizophrenia in the PGC database (**Supplementary Figure S1**).

After evaluating 715 schizophrenia patients, according to the CDSS, 326 patients (45.6%) were determined to have depressive symptoms. In the allele frequency and genotype comparison of the rs3758391 polymorphism between schizophrenia with and without depression, we observed no significant correlation between this SNP and depression in schizophrenic patients (P = 0.022). But we found that the T allele frequency of rs3758391 in schizophrenia patients with depression is significantly lower than that of schizophrenia patients without depression (OR = 1.43, 95% CI: 1.09–1.88, and P = 0.009). In addition, we measured the peripheral mRNA expression of SIRT1 in 89 schizophrenia patients with depression and 108 without depression. The primary clinical information of these two cohorts was well matched (**Supplementary Table S1**). **Figure 2** suggests that SIRT1 mRNA levels of schizophrenia patients with depression were significantly lower than those of schizophrenia without depression ($P < 0.01$). Consistent with our

[3]http://peana-od.inf.um.es:8080/UKBECv12/

Gene	Probe	Hippocampus			Prefrontal Cotex			Striatum		
		FoldChange	*P*-value	FDR	FoldChange	*P*-value	FDR	FoldChange	*P*-value	FDR
SIRT1	218878_s_at	1.12	0.031	0.149	1.18	0.003	0.154	0.98	0.689	0.855

FIGURE 1 | Differential expression of SIRT1 mRNA in brain between patients with schizophrenia and healthy controls. Each bar represents the average level of SIRT1 mRNA expression. Error bars represent the standard deviation of the mean value. Data was extracted from the SZDB database (http://www.szdb.org/).

TABLE 1 | Distribution of rs3758391 genotype and allele in schizophrenia and controls, and schizophrenia with or without depression.

SNP		Genotype, *N* (%)				Allele, *N* (%)			
rs3758391	N	C/C	C/T	T/T	P	C	T	P	OR (95%CI)
Schizophrenia	715	25 (3.5)	207 (29.0)	483 (67.6)	0.18	257 (18.0)	1173 (82.0)	0.11	1.17 (0.96–1.42)
Controls	723	15 (2.1)	198 (27.4)	510 (70.5)		228 (15.8)	1218 (84.2)		
Schizophrenia									
With depression	326	13 (4.0)	110 (33.7)	203 (62.3)	0.022	136 (20.9)	516 (79.1)	0.009	1.43 (1.09–1.88)
Without depression	389	12 (3.1)	97 (24.9)	280 (72.0)		121 (15.6)	657 (84.4)		

previously published results (Fang et al., 2019), the present study also suggested that the CDSS score was negatively correlated with SIRT1 mRNA levels ($r = -0.328$, $P < 0.01$).

To further explore the relationship between the rs3758391 polymorphism and depressive symptoms in patients with schizophrenia, we compared the CDSS scores of different genotypes of the rs3758391 polymorphism and found a significant difference ($P < 0.01$; **Figure 3**). *Post hoc* comparison showed that the CDSS score of rs3758391 C/C and C/T carriers were higher than that of T/T carriers (Ps < 0.01). Then, our eQTL analysis suggested a significant association between rs3758391 and SIRT1 mRNA expression in the occipital cortex (**Figure 4**), and SIRT1 mRNA expression levels in carriers with C/C genotypes were significantly lower than carriers with T/T genotype. However, we did not find this difference in the peripheral blood samples.

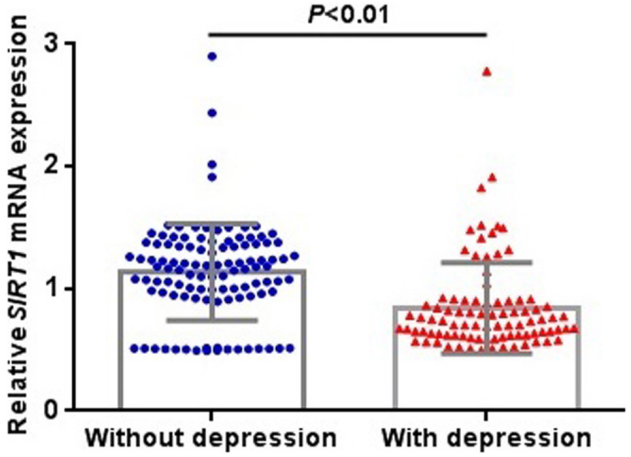

FIGURE 2 | Expression levels of SIRT1 mRNA in peripheral blood in schizophrenia patients with or without depression. SIRT1 mRNA was normalized to that of GAPDH.

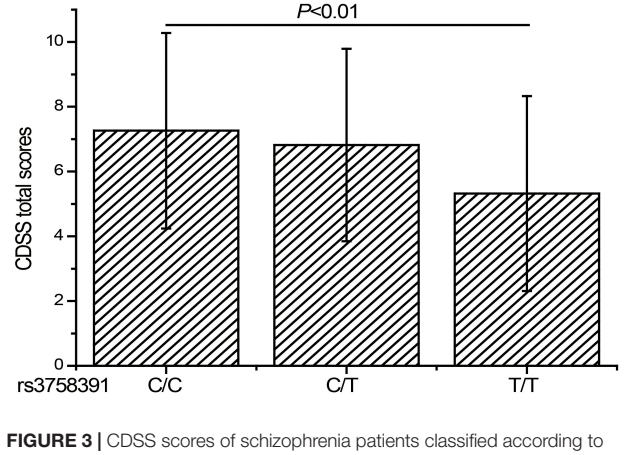

FIGURE 3 | CDSS scores of schizophrenia patients classified according to the genotypes of rs3758391 polymorphism. Each column represents the mean ± SD. C/C (n = 25), C/T (n = 207), and T/T (n = 483).

DISCUSSION

Sirtuin was identified as an MDD risk gene in a large-scale Chinese GWAS (CONVERGE consortium, 2015). Our recent meta-analysis indicated that among the Han Chinese population, the SIRT1 rs3758391 polymorphism confers susceptibility to MDD (Tang et al., 2018). Schizophrenia and MDD are distinct categorical diagnoses, but there is evidence of molecular genetic mechanisms overlap between the disorders (Schulze et al., 2014). Therefore, we aimed to identify the impact of SIRT1 rs3758391 polymorphism on the genetics and symptoms of schizophrenia in this research.

In this study, we found neither the association between SIRT1 mRNA expression in the brain and schizophrenia nor the significant effect of the rs3758391 polymorphism on susceptibility to schizophrenia in PGC GWAS. It suggests that the SIRT1 SNP rs3758391 probably does not confer an increased risk of schizophrenia. We further stratified the schizophrenia patients as either subject with or without depression based on the CDSS evaluation. Our study demonstrated that SIRT1 mRNA expression is significantly downregulated in schizophrenia with depression compared with that in those without depression, and patients with the C allele of rs3758391 have more severe symptoms of depression, which means the SNP rs3758391 might be the determinant of depressive symptoms in schizophrenia. To shed light on the impact of rs3758391 polymorphism on SIRT1 mRNA expression, an eQTL analysis was performed, which discovered that rs3758391 is significantly associated with SIRT1 mRNA expression in the occipital cortex. These consistent results indicated that genetic variation resulting from the rs3758391 polymorphism might lead to the dysregulation of SIRT1 mRNA expression and exert a significant influence on the occurrence and development of depressive symptoms in schizophrenia.

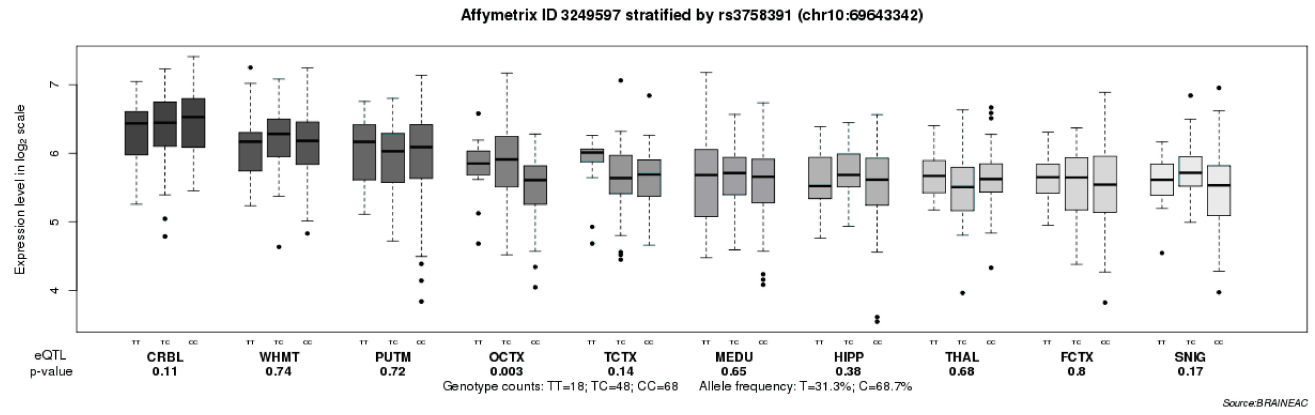

FIGURE 4 | Association of rs3758391 with SIRT1 mRNA expression levels in ten brain regions (Affymetrix ID 3249597; http://peana-od.inf.um.es:8080/UKBECv12/). SNIG, substantia nigra; PUTM, putamen (at the level of the anterior commissure); MEDU, the inferior olivary nucleus (sub-dissected from the medulla); THAL, thalamus (at the level of the lateral geniculate nucleus); OCTX, occipital cortex; HIPP, hippocampus; FCTX, frontal cortex; TCTX, temporal cortex; WHMT, intralobular white matter; and CRBL, cerebellar cortex.

Sirtuin is responsible for oxidative respiration and cellular survival and therefore is related to inflammation, glucose stasis, apoptosis, and aging (Kauppinen et al., 2013). Researchers demonstrated that chronic stress could increase the risk of depression-like behavior by reducing SIRT1 activity by establishing a mouse model of depression (Abe-Higuchi et al., 2016). However, SIRT activators can improve such phenotypes (Hurley et al., 2014). For humans, convergent genetic evidence has verified the important role of SIRT1 in the etiology of MDD (CONVERGE consortium, 2015; Luo and Zhang, 2016). Our recent work provided evidence that suggests that the low plasma SIRT1 concentration might cause depression in patients with schizophrenia (Fang et al., 2019), which is also consistent with the results shown in the present study. There is evidence showing that SIRT1 can promote the secretion of IL-6, which exerts a significant influence on the regulation of the inflammatory response (Tang et al., 2017). Interestingly, we found a significant increase in IL-6 mRNA levels in patients with MDD (Zhang et al., 2016b). In summary, we speculated that the effect of SIRT1 on the development of depressive symptoms in schizophrenia might be achieved by regulating the inflammatory response. Our previous study indicated that the pathophysiology of MDD might involve immune dysfunction (Zhang et al., 2016c). Thus, a question has naturally arisen regarding whether such a mechanism underlies the development of depressive symptoms in schizophrenia. Further investigations are required for clarification.

The SNP rs3758391 is a gene promoter located at the p53-binding site of the SIRT1 gene (Naqvi et al., 2010). This study indicated that rs3758391 is likely to affect the severity of depressive symptoms in schizophrenia. Our eQTL results showed that rs3758391 is closely related to the mRNA expression of SIRT1 in the occipital cortex and that rs3758391 C/C carriers have significantly lower SIRT1mRNA expression in the occipital cortex than T/T carriers. Emerging evidence suggested that the pathogenesis of MDD is related to the dysfunction of the occipital cortex mediated by the complement factor H (CFH), an important inflammatory molecule (Maciag et al., 2010; Zhang et al., 2016c). Thus, we assumed that inflammation might be the basis of the mechanism of the occipital cortex involved in the depressive symptoms in schizophrenia; however, this conclusion requires further investigations. The HapMap project has documented that the overall average heterozygosity frequency of the rs3758391 polymorphism is 50%. In the NCBI database, the C allele of rs3758391 is found at a frequency of 15.1% in HCB and a frequency of 73.0% in CEU. This implies that rs3758391 might be an ethnically-specific polymorphism. Thus, our analysis should be replicated in Caucasian populations.

Despite the promising implications of our results, there are several major limitations should be noted. First and foremost, we analyzed the effect of only one SNP in SIRT1 on schizophrenia. This means that further analysis of more SIRT1 gene variants is needed to verify our results. Second, the patients included in the group were all treated with antipsychotics and were stable for more than 6 months. As is well-known, antipsychotic treatment is likely to bias the symptomatology (Zhang et al., 2017). Finally, due to the inherent characteristics of cross-sectional studies, we failed to determine whether SIRT1 levels have changed before the onset of depressive symptoms in schizophrenia. In summary, our research was only exploratory and preliminary.

To the best of our knowledge, this study explored the relationship between SIRT1 rs3758391 polymorphism and schizophrenia for the first time. In this study, we performed a comprehensive investigation to detect the potential link between rs3758391 and schizophrenia. Our preliminary findings provide suggestive evidence that SIRT1 confers susceptibility to depressive symptoms in schizophrenia. The SNP rs3758391 functionally affects the severity of depressive symptoms in schizophrenic patients. This SNP probably served as one of the biomarkers of schizophrenia depression. Our work is a commendable attempt to promote the diagnosis and treatment

of different subtypes of schizophrenia, which provides useful information that improves the understanding of the genetic mechanism of depressive symptoms in schizophrenia. These findings should be further validated by more extensive sample studies in the broader population.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Boards of Jinhua Second Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CZ and DW contributed to the overall design of the study. JZ and WF wrote the protocol for the genotyping. JZ, DW, WF, WT, and YZ got involved sample collection. JZ and CZ undertook the statistical analysis and interpretation of data. CZ and DW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00832/full#supplementary-material

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Hyper-Activated Brain Resting-State Network and Mismatch Negativity Deficit in Schizophrenia With Auditory Verbal Hallucination Revealed by an Event-Related Potential Evidence

Qiaoling Sun[1†], Yehua Fang[2†], Xuemei Peng[3], Yongyan Shi[1], Jinhong Chen[4], Lifeng Wang[5] and Liwen Tan[1]*

[1] Department of Psychiatry, Mental Health Institute of the Second Xiangya Hospital, Central South University, Changsha, China, [2] Department of Clinical Psychology, Zhuzhou Central Hospital, Zhuzhou, China, [3] Department Psychology, Xiangtan Central Hospital, Xiangtan, China, [4] Department of Sleeping Disorders & Neurosis, Brain Hospital of Hunan Province, Changsha, China, [5] Department of Clinical Psychology, The Third Xiangya Hospital of Central South University, Changsha, China

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***Correspondence:**
Liwen Tan
tanliwen@csu.edu.cn

[†]These authors have contributed equally to this work

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Schizophrenia is a holergasia with unclear mechanism and high heterogeneity. Auditory verbal hallucination (AVH) study might help in understanding schizophrenia from the perspective of individual symptoms. This study aimed to investigate the activities of the resting-state networks (RSN) in the electroencephalogram (EEG) and mismatch negativity (MMN) in task-related state of schizophrenia patients with AVH. We recruited 30 schizophrenia patients without any medication for more than 4 weeks (15 AVH patients and 15 Non-AVH patients) and 15 healthy controls. We recorded the EEG data of the participants in the resting-state for 7 min and the event-related potential (ERP) data under an auditory oddball paradigm. In the resting-state EEG network, AVH patients exhibited a higher clustering coefficient than Non-AVH patients and healthy controls on delta and beta bands and a shorter characteristic path length than Non-AVH patients and healthy controls on all frequency bands. For ERP data, AVH patients showed a lower MMN amplitude than healthy controls ($p = 0.017$) and Non-AVH patients ($p = 0.033$). What's more, MMN amplitude was positively correlated with clustering coefficient, and negatively correlated with characteristic path length on delta, theta, beta and gamma band in AVH patients. Our results indicate that AVH patients showed a hyper-activity in resting-state and may have impaired higher-order auditory expectations in the task-related state than healthy controls and Non-AVH patients. And it seems reasonable to conclude that the formation of AVH may occupy certain brain resources and compete for brain resources with external auditory stimuli.

Keywords: schizophrenia, auditory verbal hallucination (AVH), event-related potential, mismatch negativity (MMN), resting-state network (RSN)

INTRODUCTION

Schizophrenia, which typically starts between late adolescence and early adulthood, is characterised by incompatibility among thought, behaviour, emotion and the surrounding environment (1, 2). The disease has an unclear cause, long course, tendency to relapse, and poor treatment effect, which bring a serious burden to society (3). Schizophrenia has always been regarded as a whole in research; however, this approach is inaccurate owing to the heterogeneity of the disorder (4). Schizophrenia from the perspective of individual symptoms may be easily understood (5). One of the most prevalent symptoms of schizophrenia is hallucination (6), which refers to an illusory perception (7) that occurs in the absence of external stimulus and with a clear consciousness state. Auditory verbal hallucination (AVH) involves the perception of speech in the absence of external sensory stimulation, occurs with a 50%–80% probability (8) and is the most common hallucination of schizophrenia. The study of independent AVH may be conducive to understanding schizophrenia (9).

In order to explore how AVH occurs spontaneously from the brain's intrinsic activity. Amount works had been done by studying the brain in its so-called "resting state," which refers to the intrinsic patterns of brain activity that are observable in the absence of an external task (10). Many early studies have found increased resting activity in the upper or middle temporal lobe in the presence of AVH (11). Dierks compared activation level of resting-state in the same schizophrenia patients group on and offset AVH and found that the activation level of auditory cortex in schizophrenia patients on AVH was increased (12). Allen et al. observed that the secondary auditory cortex was particularly active when AVH occurred (13). In a case study involving a patient with chronic AVH, elevated activity of the right medial temporal and left superior temporal gyri without external speech was observed (14). The bilateral temporal cortex is overactive when AVH occurs in schizophrenia (15).

The mismatch negativity (MMN) is an electrophysiological response that is elicited when a sequence of identical auditory stimuli is infrequently interrupted by a stimulus that deviates from the standard stimulus along one or more dimensions, which appears to represent the automatic change detection process that occurs when an acoustic event violates expectations maintained by the active auditory trace (16, 17). Some studies showed that the decreased amplitude and prolonged latency of MMN are related to the positive symptoms of schizophrenia (18), and MMN abnormality in schizophrenia patients is mainly manifested in the decrease of amplitude (19). MMN in the fronto-temporal lobe is atypical in schizophrenia patients with AVH (20).

Spontaneous potential is a kind of electroencephalogram (EEG) recorded without stimulation. Brain network analysis is a data processing method and brain network topology can be established from EEG data (21). When stimulation is introduced, the event-related potential (ERP), an EEG recorded during the advanced cognitive processing of an object (attention, memory or judgment), is obtained (22). ERP with an excellent temporal resolution is an ideal tool for studying the cognitive function of patients with psychiatric problems and has an extremely sensitive response to cognitive processing. The latency of ERP component refers to the time interval between the stimulus point and ERP component, reflecting the speed at which the brain processes stimuli, whereas the amplitude reflects the brain's ability to respond to stimuli and resources (such as the number of neurons initiated) devoted to it.

Amount of works had been done on resting-state or task-related state activities in schizophrenia patients with AVH. Various articles from functional magnetic resonance imaging have posited a specific link between RSN [such as default mode network (23, 24), central executive network (25), auditory, and language regions (26–28)] and AH. Few EEG/magnetoencephalography studies have examined the resting state in relation to AVH (29) and found that the occurrence of AVH has been associated with increased beta oscillations in left frontoparietal regions (30) and increased gamma-theta in frontotemporal areas (31).But inadequate AVH severity and the effects of antipsychotic drugs have not been considered in these studies (32), and it is still unclear how this hyper-activated resting-state can affect the activity of the task-related state.

In this present study, we recruited drug-free schizophrenia patients with or without AVH and age-matched healthy controls. We recorded the EEG data in the resting-state and their corresponding ERP data during the auditory oddball paradigm. We aimed to examine the activities of the resting EEG brain networks and MMN in task-related state of schizophrenia patients with AVH and explore whether there were some relations between these two states.

MATERIALS AND METHODS

Participants

Patients were recruited from the psychiatric outpatient department of the Second Xiangya Hospital of Central South University from June to December 2014. The inclusion criteria of the patients were as follows: (a) aged between 18 to 60 years, (b) met ICD-10 criteria for schizophrenia, (c) drug-free or has washed out more than 4 weeks and (d) normal hearing and right-handed. The exclusion criteria were as follows: (a) history of head injury resulting in loss of consciousness, (b) diseases of physical illness or neurological disorders, (c) comorbid with other mental disorders and with other forms of hallucinations, (d) alcohol or drug abuse history, and (e) received electroconvulsive therapy treatment in the past year. Patients meeting the diagnostic criteria for schizophrenia, according to ICD-10, were assessed by two senior clinical psychiatrists by using the Positive and Negative Symptom Scale (33).

We divided the schizophrenia patients into AVH patients and Non-AVH patients. The AVH patients consisted of 15 patients with a current history of AVH, as evidenced by a score ≥ 3 ("mild or greater hallucinatory experiences") on the hallucination item of the PANSS positive symptom scale and the Non-AVH patients consisted of 15 patients with no lifetime history of AVH. Healthy controls (n = 15) were recruited from the local community by advertisement. The study was approved by the

local ethics committee, and written informed consent was obtained from each participant.

Auditory Oddball Task

The program was performed using the E-prime 2.0 software. Briefly, the subjects received auditory stimulus sequences consisting of 540 standard stimuli and 60 deviant stimuli delivered randomly. MMN can be elicited by auditory oddball paradigm and is the negative waveform peaking between 100 and 250 ms after stimulation without subjective effort or subjective interference (16, 17). The processing of auditory stimuli is complex, especially the encoding of duration. If AVH and shortages of auditory resources are found, the brain is most likely to make mistakes in the encoding of duration. Therefore, this study chose duration deviation to induce MMN (34). The probabilities of hearing standard and deviant stimuli were 90% and 10%, respectively. The inter-stimulus interval was 500 ms. The stimulus was delivered binaurally through Sennheiser headphones. The subjects were instructed to watch a neutral exposition to ignore the stimuli. The auditory stimulus had a pure tone and was applied in different durations (standard: 100 ms, deviant: 50 ms). The frequency of the pure-tone stimuli was 1,000 Hz, and the loudness was 70 dB (35).

Data Recording

EEG data were recorded with an electrode cap with Ag/AgCl electrodes at 64 scalp sites according to the modified 10–20 system of electrode placement. Vertical electro-oscillogram was recorded from one electrode fixed below the right eye. The reference electrode was at FCz. All the electrode impedances were maintained below 5 kΩ. Electrical activity was recorded by using the Brain Vision Recorder software (Germany, Brain Products) with an amplifier band pass of 0.1 and 1,000 Hz and digitised at 500 Hz. The first block of the experiment was to record the resting-state EEG data for 7 min when the participants were asked to sit quietly with their eyes closed. After the first block, the participants had a break for 1–2 min. In the second block, the ERP data were recorded when the subjects received the auditory oddball task.

Analyses of the Resting-State EEG Data and ERP Data

We estimated Coherence (*Coh*), which is the linear relationship at a specific frequency between the two signals *x(t)* and *y(t)* on the basis of their cross-spectrum (36). In brief, *Coh* is expressed as follows,

$$Coh_{xy}(f) = \frac{|S_{xy}(f)|^2}{S_{xx}(f)S_{yy}(f)}$$

$S_{xy}(f)$ indicates the cross-spectrum of $x(t)$ and $y(t)$ at the frequency f; $S_{xx}(f)$ and $S_{yy}(f)$ denote the autospectrum from the fast Fourier transformation on $x(t)$ and $y(t)$, respectively.

Network analysis was performed using electrodes as the nodes. The coherence between electrode pairs was used to measure the interactions between two regions. After the weighted network was calculated, a threshold (0.200) that can guarantee the connection of network was used to binarize the network. Based on the binarised network, the characteristics of the network can be quantitatively denoted by the network measurements, including clustering coefficient (C) and characteristic path length (L) (21). The weighted matrix obtained from *Coh* is referred to as w, and w_{ij} represents the connectivity between nodes i and j. Node number is denoted by N, and the set of all nodes in the network is denoted by Ω. For a given threshold T, the adjacent matrix w is binarized as,

$$w_{ij} = \begin{cases} 1, w_{ij} \geq T \\ 0, w_{ij} < T \end{cases}$$

The threshold is 0.200. k_i is the degree of the node i that was defined as,

$$k_i = \sum_{j \in \Omega} w_{ij}$$

t_i is the number of triangles that can be formed between node i and its neighboring nodes, and was defined as,

$$t_i \frac{1}{2} \sum_{j,h \in \Omega} w_{ij} w_{ih} w_{jh}$$

d_{ij} is the shortest path between node i and node j. The characteristic path length L for a graph can then be defined as,

$$L = \frac{1}{N} \sum_{i \in \Omega} L_i = \frac{1}{N} \sum_{i \in \Omega} \frac{\Sigma_{j \in \Omega, j \neq i} d_{ij}}{N - 1}$$

The clustering coefficient can be calculated as,

$$C = \frac{1}{N} \sum_{i \in \Omega} \frac{2t_i}{k_i(k_i - 1)}$$

Five frequency bands including delta (1–4 Hz), theta (4–8 Hz), alpha (8–13 Hz), beta (13–30 Hz) and gamma (30–60 Hz), were selected for analysis.

MMN was acquired at Fz electrode, because MMN was predominantly distributed in the frontal–central area. The offline data of the second block was processed by using the Brain Vision Analyser 2.0 system (Brain Products GmbH, Germany). EEG data were referenced to the average of mastoids (TP9 and TP10). EEG signals were bandpass filtered using a 0.5–30 Hz (50 Hz notch). Eye movements and eye blinks were removed using an independent component analysis (ICA). Artefact rejection procedures were applied to all epochs (−200 ms pre-stimulus to 450 ms post-stimulus), with a baseline correction from −200 ms to 0 ms pre-stimulus. The latency and amplitude of MMN were measured. Latency refers to the time from the beginning of stimulation to the maximum negative peak between 100 and 250 ms, and its amplitude is the vertical distance from the baseline to the maximum peak.

STATISTICAL ANALYSIS

Statistical analyses were carried out by using the Statistics Product and Service Solutions (SPSS18.0) software package.

Cochran & Cox Approximate t-test was used in the analysis of PANSS-P3 score between the two patient groups. For the resting EEG data, repeated-measures ANOVA with group (AVH patients, Non-AVH patients, healthy controls) as between-subject variable, and frequency band (delta, theta, alpha, beta and gamma) as within-subject variable. For the ERP data, one-way ANOVA was performed. All post-hoc analyses used the Bonferroni adjustment. Statistical analyses were adjusted for variance nonsphericity using the Greenhouse-Geisser correction (37). Statistical significance was considered at $p < 0.05$. Spearman correlation analysis was applied to explore the relationship between MMN amplitude and properties of resting EEG data in AVH patients.

RESULTS

Demographic Results

The demographic information for all participants is presented in **Table 1**. The schizophrenia patients and healthy controls were matched with respect to age, gender and education level. The two patient groups had no significant difference in PANSS positive symptom, negative symptom and general psychopathology. The difference in the PANSS-P3 was statistically significant between AVH patients and Non-AVH patients, and AVH patients had a higher PANSS-P3 score ($t' = 15.08$, $p < 0.001$).

Resting EEG

For clustering coefficient, a significant interaction of group × frequency band ($F = 2.320$, $p = 0.037$) was also observed. And further simple effect analyses found that on delta and beta bands AVH patients had a higher clustering coefficient than Non-AVH patients (delta band: $p = 0.042$; beta band: $p = 0.038$) and healthy controls (delta band: $p = 0.001$; beta band: $p = 0.002$), on theta and gamma bands AVH patients showed a higher clustering coefficient than healthy controls (theta band: $p = 0.023$; gamma band: $p = 0.019$), and on alpha band, no significant difference was found among three groups. See in **Figure 1**.

For characteristic path length, main group was found ($F = 7.754$, $p = 0.002$) and post-hoc test showed that AVH patients had shorter characteristic path length than Non-AVH patients ($p = 0.034$) and healthy controls ($p = 0.002$). No interaction of group × frequency band was observed.

MMN

Grand averages of MMN is presented in **Figure 2**. For the MMN latency, no significant differences were observed (**Table 2**). For MMN amplitude, a significant effect was found. Post hoc test revealed statistical differences between AVH patients and healthy controls ($p = 0.017$), and the MMN amplitude of AVH patients was smaller than the healthy controls. Post hoc test also revealed statistical differences between AVH patients and Non-AVH patients ($p = 0.033$), and the MMN amplitude of AVH patients was smaller than Non-AVH patients. No statistical difference was found between healthy controls and Non-AVH patients.

The brain topographies at the Fz electrode are shown in **Figure 3**. It reflects the EEG changes from 100–248 ms after stimulation. The negative wave appeared gradually at approximately 138 ms and was increased with time. The amplitude reached the maximum during 176–212 ms and then decreased gradually. The differences between groups were obvious from 176–212 ms. The amplitude of the negative wave in AVH patients was lower than that in healthy controls and Non-AVH patients.

Correlation Analysis in AVH Patients

Spearman correlation analysis between MMN amplitude and properties of resting EEG data in AVH patients is presented in **Table 3**. MMN amplitude was positively correlated with clustering coefficient and negatively correlated with characteristic path length in the delta, theta, beta and gamma band. Because MMN is a negative ERP component, positive loadings indicate smaller amplitudes while negative loadings indicate larger amplitude.

DISCUSSION

Due to the complexity and variety of schizophrenia symptoms, as well as the interaction between symptoms, auditory-hallucination-related research is very difficult to implement. The pathogenesis of AVH in schizophrenia has not been fully understood, and no complete neurocognitive theory can

TABLE 1 | Demographic information for participant and trait questionnaires.

Item	AVH patients ($n = 15$)	Non-AVH patients ($n = 15$)	healthy controls ($n = 15$)	Statistic values	p
Age (year)	29.07 ± 5.67	27.64 ± 5.55	29.00 ± 5.16	$H = 0.437$	0.804
Gender (male/female)	10/5	11/4	10/5	$X^2 = 0.207$	0.902
Education level (year)	13.47 ± 2.29	13.64 ± 3.53	15.69 ± 1.82	$H = 0.161$	0.923
Illness duration (month)	8.23 ± 7.93	11.09 ± 5.45	–	$Z = -1.345$	0.164
PANSS positive symptom	15.80 ± 2.83	13.73 ± 2.90	–	$t = 1.824$	0.081
PANSS negative symptom	14.47 ± 7.65	15.09 ± 7.49	–	$t = -0.207$	0.837
PANSS general psychopathology	32.07 ± 6.78	30.45 ± 7.16	–	$t = 0.585$	0.564
PANSS-P3	5.33 ± 1.11	1.18 ± 0.40	–	$t' = 15.083$	0.000

H, kruskal-wallis H test of multiple samples; X^2, Pearson's chi-squared test; t, independent t-test; t', Cochran & Cox Approximate t test; W, Wilcoxon rank sum test; Values are presented as mean ± SE. Where the data were unavailable or no data, a hyphen "-" was used.

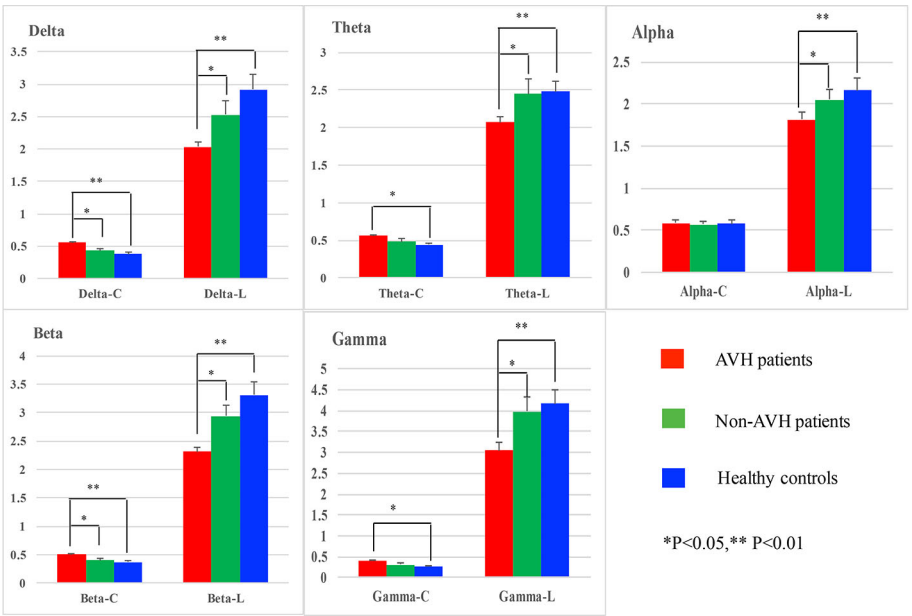

FIGURE 1 | Network properties on delta, theta, alpha, beta, and gamma bands for auditory verbal hallucination (AVH) patients, non-AVH patients, and healthy controls.

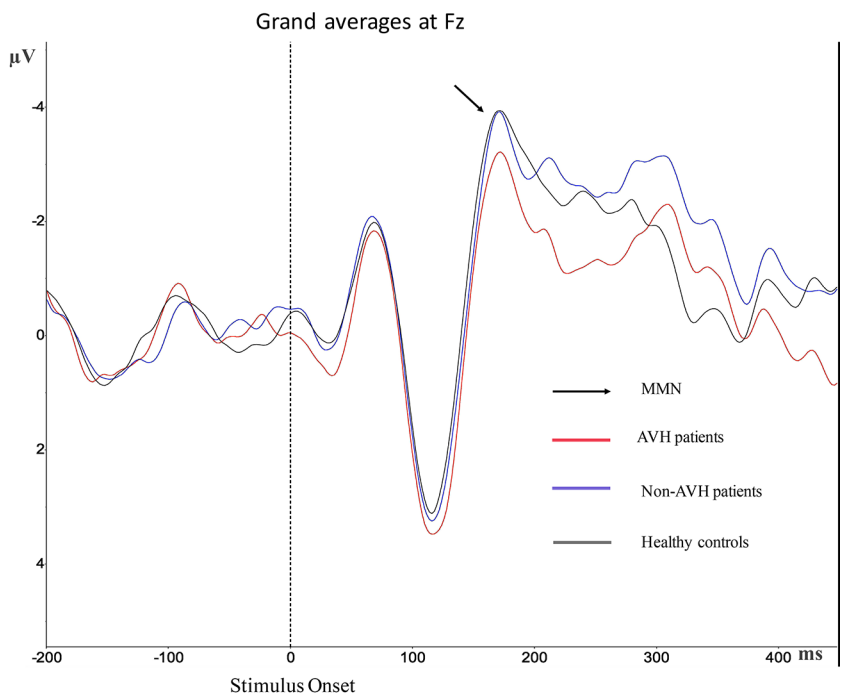

FIGURE 2 | Grand average mismatch negativity (MMN) waveforms for auditory verbal hallucination (AVH) patients, non-AVH patients, and healthy controls at Fz.

explain it yet (38–40). In this study, we explored the EEG changes from the resting-state to the task-related sate in schizophrenia patients with AVH by using the ERP method. We processed the resting EEG data by brain network analysis and compared the MMN induced by auditory oddball paradigm among AVH patients, Non-AVH patients, and healthy controls. In the resting-state, we found AVH patients had a higher clustering coefficient on delta and beta band and a

TABLE 2 | ANOVA results for the mismatch negativity (MMN) latency and amplitude.

	AVH patients	Non-AVH patients	healthy controls	F	*p*
latency	179.20 ± 12.667	177.36 ± 14.762	177.23 ± 16.361	0.063	0.939
amplitude	2.93 ± 1.684	4.48 ± 1.290	4.56 ± 1.305	5.514	0.008

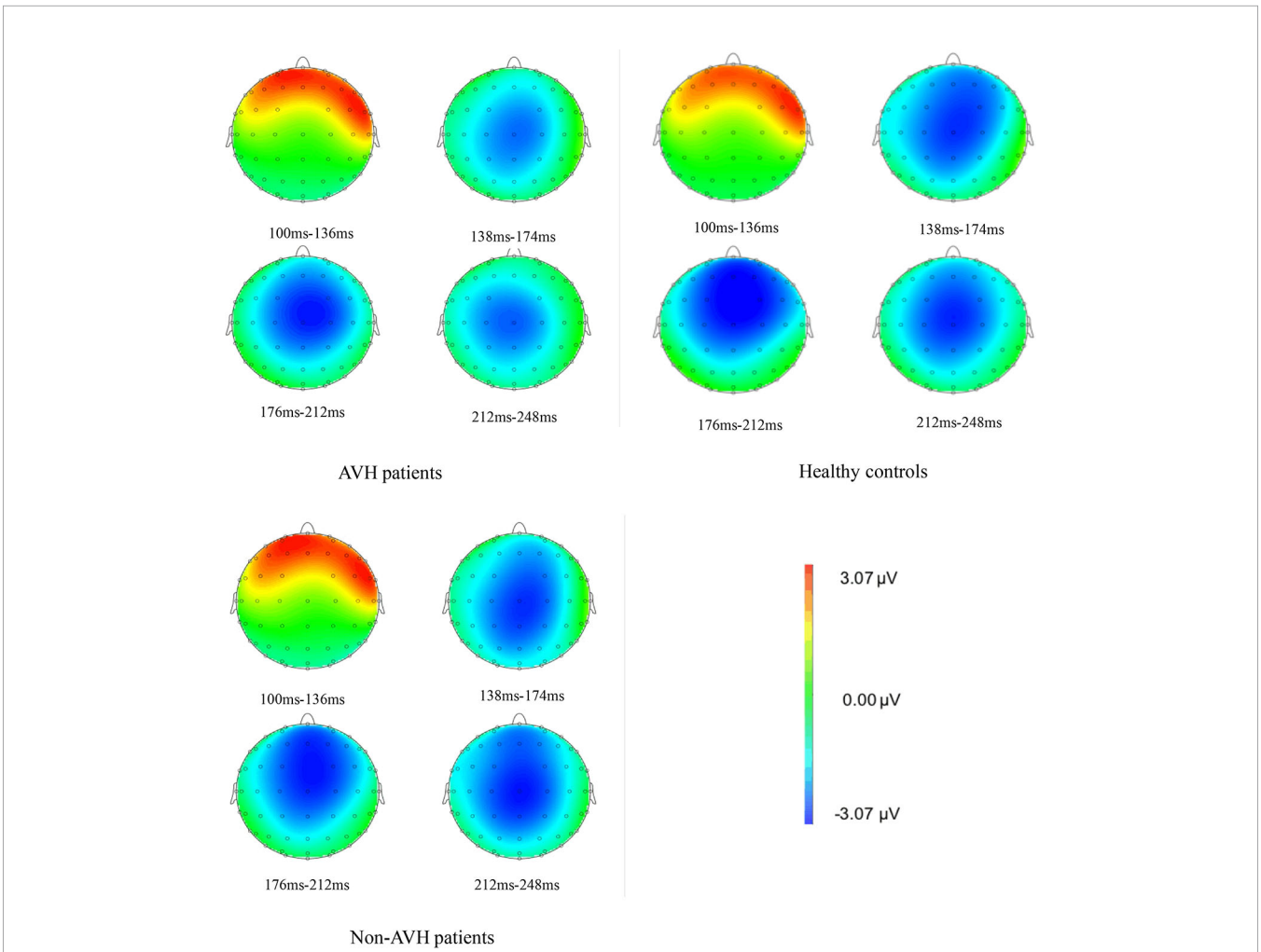

FIGURE 3 | Brain topographies at Fz electrode of an interval 100–115 ms for auditory verbal hallucination (AVH) patients, non-AVH patients, and healthy controls.

TABLE 3 | Spearman correlation analysis between mismatch negativity (MMN) amplitude and properties of resting network in auditory verbal hallucination (AVH) patients.

	clustering coefficient					characteristic path length				
	delta	theta	alpha	beta	gamma	delta	theta	alpha	beta	gamma
MMN	.604**	.582**	.192	.571**	.365*	-.574**	-.570**	-.294	-.605**	-.325*

*Spearman correlation, $p < 0.05$; **Spearman correlation, $p < 0.01$.

shorter characteristic path length on all bands than Non-AVH patients and healthy controls. In the task-related state, AVH patients showed smaller MMN amplitude than Non-AVH patients and healthy controls. What's more, MMN amplitude was positively correlated with the clustering coefficient, and negatively correlated with characteristic path length.

The clustering coefficient evaluates the number of connections between neighbors, which provides a measure of the local

structure of the network (local segregation) and an indicator of the efficiency of information transfer (41, 42). The characteristic path length evaluates the number of steps from one node to another for all possible pairs of nodes and it is usually interpreted as a metric of information integration across the overall network, which refers to the capacity of the network to become interconnected and exchange information (43, 44). The larger baseline clustering coefficient in AVH patients may reflect higher and more segregated cortical activity and the shorter characteristic path length in AVH patients may reflect more active information exchange in the resting-state brain network.

Our results are similar to previous findings. At rest, Lee et al. (30) reported greater amplitude of beta oscillations in schizophrenia patients with treatment-refractory AVH compared those without AVH, with group differences localizing to left frontoparietal regions implicated in speech and language processing. A meta-analysis demonstrated that experiencing AVH is associated with increased activity in fronto-temporal areas involved in speech generation and perception (11). In schizophrenia patients, the bilateral temporal cortex was over-activated during AVH (15). Repetitive transcranial magnetic stimulation could reduce intractable AVH by reducing the degree of activation of the left superior temporal gyrus (45). So, AVH patients may have an activated brain RSN.

In addition to schizophrenia, AVH in non-schizophrenia is also related to the activation of brain regions (46). In healthy individuals, high hallucination-prone participants reported high false alarms (i.e., reported a voice when it was not), while the temporal cortex showed high activation during these false alarms (47). To exclude the effects of delusions, negative symptoms, and antipsychotics, Remko van Lutterveld and coworkers (48) collected non-psychotic individuals with AVH to study AVHs by using resting-state fMRI. They found that in comparison with non-hallucinating controls, nonpsychotic individuals with AVH exhibit increased function in the temporal cortices (48). Therefore, AVH may be related to the activation of brain regions, and the activated brain RSN might be a neurobiological basis for auditory hallucination in schizophrenia.

In comparison with healthy controls and Non-AVH patients, AVH patients showed smaller MMN amplitude. These results are consistent with those of previous studies. AVH patients generally exhibited a lower duration MMN compared with healthy controls, particularly at F3 and Fz (49). And as measures of AVH increase, there is a corresponding decrease in MMN amplitude (50). Schizophrenia patients with clear, persistent AVH exhibited reduced MMN amplitude to duration than healthy controls and Non-AVH patients, while Non-AVH patients were not significantly different than healthy controls (4). AVH may contribute to MMN deficits in schizophrenia patients (49), and the MMN amplitude was correlated to the state and trait measures of AVH (19, 50), which further confirmed that AVH might affect the formation of MMN. MMN reflects a failure in higher-order auditory expectations (16, 17). So, AVH patients may have impaired higher-order auditory expectations than healthy controls and Non-AVH patients.

MMN was predominantly distributed in the frontal–central area, so in this study, we chose Fz electrode to extract MMN. The EEG topographies show that negative waves reached their maximum from 176–212 ms after stimulation and the largest negative wave appeared in the frontal–central area. This finding was in line with previous magnetoencephalography findings (51) and agree with the characteristic that MMN enhanced the processing in the central and frontal regions (52).

We found that AVH patients showed higher activity level in the resting-state brain network, and when external auditory stimuli occurred, AVH patients showed smaller MMN amplitude. What's more, correlation analysis found that MMN amplitude positively correlated with clustering coefficient, and negatively correlated with characteristic path length characteristic path length in the delta, theta, beta, and gamma band. So, there may be some relation between anomalies in the two states. We speculated that the formation of AVH might occupy certain brain resources, which leads to an increased brain activation level and compete for the limited brain resources with external auditory stimuli (51, 53). Which then leads to relatively inadequate brain resources in the process of external auditory stimuli and MMN deficits. The activation of the auditory cortex in schizophrenia patients with AVH was reduced when receiving external speech (54), which might be caused by the competition between AVH and normal external speech for resources within the temporal cortex. Hubl et al. found that AVH lowered the N100 amplitude and changed the topography presumably due to a reduced left temporal responsivity (55), which indicates a competition between AVH and the normal stimuli for physiological resources in the primary auditory cortex, and that the abnormal activation of the primary auditory cortex may be a constituent of AVH (55).

This study has some limitations which need to be considered when interpreting the results. The biggest is the relatively small sample size, which limitied our statistical power to detect the smaller between-group differences and reduces the reliability of results. In this study, the values of clustering coefficient, characteristic path length and MMN amplitude in Non-AVH patients were between those in AVH patients and healthy controls. Unfortunately, no statistical difference was found between healthy controls and Non-AVH patients, which was inconsistent with previous studies (17, 56) and may be partly due to the small sample size. Increasing the sample size in future studies would provide opportunities to identify additional differences. In addition, patients collected in this study were untreated patients with serious auditory verbal hallucination. But we did not assess whether AVH actually occurred when the EEG data were recorded. This topic is the first step for us to study the specific symptoms of schizophrenia. In the future, we will further confirm our results by changing stimulus materials, enlarging the sample size, comparing the situation before and after medication, and collecting refractory AVH patients for a better understanding of the mechanism of AVH and schizophrenia.

CONCLUSION

In summary, our results indicate that AVH patients showed a hyper-activity in resting-state and may have an impaired higher-order auditory expectations in the task-related state than healthy

controls and Non-AVH patients. And it seems reasonable to conclude that the formation of AVH may occupy certain brain resources and compete for brain resources with external auditory stimuli.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Biomedical Ethics Board of the Second Xiangya Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LT designed the study and wrote the protocol. YF managed the analyses, and QS wrote the first draft of the manuscript. XP, YS, JC, and LW contributed to conducting the study. All authors contributed to the article and approved the submitted version.

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NRG1, *PIP4K2A*, and *HTR2C* as Potential Candidate Biomarker Genes for Several Clinical Subphenotypes of Depression and Bipolar Disorder

Anastasia Levchenko[1], Natalia M. Vyalova[2], Timur Nurgaliev[3], Ivan V. Pozhidaev[2], German G. Simutkin[2], Nikolay A. Bokhan[2,4,5] and Svetlana A. Ivanova[2,5,6]*

[1] Theodosius Dobzhansky Center for Genome Bioinformatics, Saint Petersburg State University, Saint Petersburg, Russia, [2] Tomsk National Research Medical Center, Mental Health Research Institute, Russian Academy of Sciences, Tomsk, Russia, [3] Institute of Translational Biomedicine, Saint Petersburg State University, Saint Petersburg, Russia, [4] National Research Tomsk State University, Tomsk, Russia, [5] Siberian State Medical University, Tomsk, Russia, [6] National Research Tomsk Polytechnic University, Tomsk, Russia

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Saint Petersburg State University, Russia

***Correspondence:**
Anastasia Levchenko
a.levchenko@spbu.ru;
anastasia.levchenko@mail.mcgill.ca

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GSK3B, BDNF, NGF, NRG1, HTR2C, and PIP4K2A play important roles in molecular mechanisms of psychiatric disorders. GSK3B occupies a central position in these molecular mechanisms and is also modulated by psychotropic drugs. BDNF regulates a number of key aspects in neurodevelopment and synaptic plasticity. NGF exerts a trophic action and is implicated in cerebral alterations associated with psychiatric disorders. NRG1 is active in neural development, synaptic plasticity, and neurotransmission. HTR2C is another important psychopharmacological target. PIP4K2A catalyzes the phosphorylation of PI5P to form PIP2, the latter being implicated in various aspects of neuronal signal transduction. In the present study, the six genes were sequenced in a cohort of 19 patients with bipolar affective disorder, 41 patients with recurrent depressive disorder, and 55 patients with depressive episode. The study revealed a number of genetic variants associated with antidepressant treatment response, time to recurrence of episodes, and depression severity. Namely, alleles of rs35641374 and rs10508649 (*NRG1* and *PIP4K2A*) may be prognostic biomarkers of time to recurrence of depressive and manic/mixed episodes among patients with bipolar affective disorder. Alleles of NC_000008.11:g.32614509_32614510del, rs61731109, and rs10508649 (also *NRG1* and *PIP4K2A*) seem to be predictive biomarkers of response to pharmacological antidepressant treatment on the 28th day assessed by the HDRS-17 or CGI-I scale. In particular, the allele G of rs10508649 (*PIP4K2A*) may increase resistance to antidepressant treatment and be at the same time protective against recurrent manic/mixed episodes. These results support previous data indicating a biological link between resistance to antidepressant treatment and mania.

Bioinformatic functional annotation of associated variants revealed possible impact for transcriptional regulation of *PIP4K2A*. In addition, the allele A of rs2248440 (*HTR2C*) may be a prognostic biomarker of depression severity. This allele decreases expression of the neighboring immune system gene *IL13RA2* in the putamen according to the GTEx portal. The variant rs2248440 is near rs6318 (previously associated with depression and effects of psychotropic drugs) that is an eQTL for the same gene and tissue. Finally, the study points to several protein interactions relevant in the pathogenesis of mood disorders. Functional studies using cellular or animal models are warranted to support these results.

Keywords: neuregulin 1, serotonin 2C receptor, phosphatidylinositol-5-phosphate 4-kinase type 2 alpha, depressive episode, bipolar affective disorder, treatment response, severity, time to recurrence

INTRODUCTION

GSK3B (Beaulieu, 2012), *BDNF* (Lu et al., 2014), *NGF* (Cirulli and Alleva, 2009), *NRG1* (Mei and Nave, 2014), *HTR2C* (Chagraoui et al., 2016; Palacios et al., 2017), and *PIP4K2A* (Wilcox and Hinchliffe, 2008) are genes that showed some importance in the study of the molecular etiology of psychiatric disorders (**Table 1**). Specifically, *GSK3B* that codes for glycogen synthase kinase 3β plays a central role in the pathogenesis of psychiatric disorders (Beaulieu, 2012; Emamian, 2012). This gene is directly or indirectly inhibited by antidepressants, lithium, and antipsychotics (Beaulieu et al., 2009; Freyberg et al., 2010). It is furthermore associated with response to antidepressant medication in patients with depressive disorders (Tsai et al., 2008; Levchenko et al., 2018) and lithium treatment in patients with bipolar disorder (Benedetti et al., 2005; Lin et al., 2013; Iwahashi et al., 2014; Benedetti et al., 2015).

BDNF codes for Brain-Derived Neurotrophic Factor, active in neurodevelopment and synaptic plasticity (Begni et al., 2017; Numakawa et al., 2018); the gene has been extensively studied in the context of psychiatric disorders (Numakawa et al., 2018). In patients with depression, BDNF levels are decreased and methylation levels in the gene's promoter are increased, indicating attenuated gene expression (Kishi et al., 2017; Schroter et al., 2019). Variants in this gene are associated with severity of depression in drug-naive depressed patients (Losenkov et al., 2020) and with drug response in patients who were taking antidepressant medication for the first time (Ochi et al., 2019).

NGF codes for nerve growth factor that exerts a trophic action on the cholinergic neurons of the basal forebrain nuclei and is implicated in cerebral alterations associated with psychiatric disorders (Bersani et al., 2000). Levels of this neurotrophin are decreased in bipolar disorder (Barbosa et al., 2011) and schizophrenia (Qin et al., 2017).

NRG1, coding for neuregulin, is implicated in neural development, including myelination, synaptic plasticity, and neurotransmission (Mei and Nave, 2014). It is associated with schizophrenia, bipolar disorder, and depression (Mei and Nave, 2014; Dang et al., 2016; Wen et al., 2016; Chen et al., 2017).

HTR2C codes for the serotonin 2C receptor that is an extremely important target of drugs used to treat a number of psychiatric disorders (Di Giovanni and De Deurwaerdere, 2016; Palacios et al., 2017), including depression (Chagraoui et al., 2016). In addition, this gene is associated with severity of depression and response to pharmacological antidepressant treatment (Brummett et al., 2014; Vyalova et al., 2017).

PIP4K2A codes for phosphatidylinositol-5-phosphate 4-kinase type 2α, a kinase that catalyzes the phosphorylation of phosphatidylinositol-5-phosphate (PI5P) to form phosphatidylinositol-5,4-bisphosphate (PIP2) (Wilcox and Hinchliffe, 2008). The lipid PIP2 is implicated in various aspects of neuronal signal transduction (Di Paolo et al., 2004; Fedorenko et al., 2008; Fedorenko et al., 2009; Seebohm et al., 2014). Genetic studies of *PIP4K2A* showed an association with bipolar disorder and schizophrenia (Stopkova et al., 2003; Schwab et al., 2006), as well as with response to pharmacological antidepressant treatment (Vyalova et al., 2017). In addition, the mechanisms underlying lithium's therapeutic efficacy in the chronic treatment of bipolar disorder include differential expression of *PIP4K2A* (Seelan et al., 2008).

GSK3B, *BDNF*, *NGF*, *NRG1*, *HTR2C*, and *PIP4K2A* are functionally connected. GSK3B plays a central role in the AKT/GSK3 molecular pathway (Beaulieu, 2012). The binding of either BDNF, NGF, or NRG1 to their respective receptor tyrosine kinases (TrkB, encoded by *NTRK2*, TrkA, encoded by *NTRK1*, and ERBB4, encoded by the gene with the same name) leads to inhibition of GSK3B via activation of phosphoinositide 3-kinase (PI3K), 3-phosphoinositide-dependent protein kinase-1 (PDPK1), and AKT1 (Beaulieu, 2012; Kim et al., 2014; Mei and Nave, 2014; Numakawa et al., 2018). PI3K is a heterodimer made of a catalytic p110 subunit and an adapter regulatory p85 subunit; in the brain, the former is encoded by *PIK3CA* and *PIK3CB*, while the latter is encoded by *PIK3R1* and *PIK3R2* (Dwivedi et al., 2008). Binding of serotonin to HTR2C leads to the opposite effect: GSK3B activation (Beaulieu, 2012). A link between PIP4K2A and GSK3B involves activation of M-channels by PIP2 and GSK3B (**Figure 1**; Delmas and Brown, 2005; Carter, 2007; Fedorenko et al., 2008; Wildburger and Laezza, 2012; Jiang et al., 2015). These channels, formed in the brain by tetramers of subunits potassium voltage-gated channel subfamily Q members 2, 3, and 5 (KCNQ2, KCNQ3, and KCNQ5), play a critical role in the regulation of neuronal excitability

TABLE 1 | Previous reports of association between the six sequenced genes and psychiatric phenotypes, including response to mediation.

Genes	Variants	Associated phenotypes	References
GSK3B	rs6438552	Brain structural changes in major depressive disorder (MDD)	Inkster et al., 2009
	rs6438552 rs334558	Age of onset of bipolar disorder (BD) in female patients Response to lithium treatment	Lin et al., 2013
	Haplotype containing rs334558 and rs3755557	Response to lithium treatment	Iwahashi et al., 2014
	rs334558	Response to lithium augmentation	Adli et al., 2007
	rs334558	Gray matter volumes in the right frontal lobe of patients with BD	Benedetti et al., 2015
	rs334558	Remission in patients with depressive disorders	Levchenko et al., 2018
	rs334558, rs13321783, rs2319398	Response to antidepressant therapy	Tsai et al., 2008
	rs12630592	Severity of mania (in both acute and stabilized periods) and depression in stabilized periods	Bureau et al., 2017
	Haplotype containing rs334555, rs119258668, and rs11927974	Age of onset of MDD	Saus et al., 2010
	Interaction of rs6782799, rs6265 (*BDNF*), and negative life events	MDD	Yang et al., 2010
BDNF	rs6265	MDD	Tsai et al., 2003; Schroter et al., 2020
	Interaction of rs6265 and stressful life events	MDD	Hosang et al., 2014
	rs6265	Recurrent MDD	Xiao et al., 2011; Lee Y. et al., 2013
	rs6265	Severity of depression	Losenkov et al., 2020
	rs6265	Response to antidepressant therapy and remission in MDD	Choi et al., 2006; Alexopoulos et al., 2010; Chi et al., 2010; Zou et al., 2010; Taylor et al., 2011; Murphy et al., 2013; Wang et al., 2014; Colle et al., 2015; Xin et al., 2020
	rs712442	Response to antidepressant therapy	Ochi et al., 2019
NGF	Haplotype containing rs2254527, rs6678788, and rs12760036	Remission rate in MDD	Yeh et al., 2015
	Interaction with *BDNF*, among 590 other polygenes	Suicide attempt	Sokolowski et al., 2016
NRG1	rs4733272 (together with other chromosome 8-associated SNPs)	Schizophrenia, BD and MDD	Chen et al., 2017
	rs4236710 and rs4512342; haplotype containing rs4512342 and rs6982890	Schizophrenia	Wen et al., 2016
	rs2919375 Haplotype containing rs4531002 and rs11989919	MDD MDD and BD	
	rs35753505 and rs7014762	BD	Mei and Nave, 2014
	rs6994992, rs2439272, rs62510682, rs10503929, and rs3924999	Prepulse inhibition (PPI, a measure of inhibitory sensorimotor gating)	Hong et al., 2008; Roussos et al., 2011
	rs6994992	Activity of frontal and temporal lobes, premorbid IQ, and positive symptoms in schizophrenia	Hall et al., 2006; Papiol et al., 2011
	rs3924999	Perceptual aberrations in schizotypal personality disorder	Lin et al., 2005
HTR2C	rs6318	Severity of depression	Brummett et al., 2014
	rs6318	Response to antidepressant therapy	Vyalova et al., 2017
	rs6318	Suicide attempt	Karanovic et al., 2015

(Continued)

TABLE 1 | Continued

Genes	Variants	Associated phenotypes	References
	rs6318	Dysregulated stress responding and risk for depression	Avery and Vrshek-Schallhorn, 2016
	rs6318	Stress-induced mesoaccumbal dopamine release	Mickey et al., 2012
	rs6318 and rs3813929	Feeding behavior and antipsychotics-induced weight gain and movement disorders	Drago and Serretti, 2009
	rs1414334	Metabolic syndrome in patients using antipsychotics	Mulder et al., 2009; Risselada et al., 2012
PIP4K2A	rs10828317	Tardive dyskinesia in schizophrenia patients	Fedorenko et al., 2014
	rs10828317	Schizophrenia	Schwab et al., 2006; Bakker et al., 2007; Fedorenko et al., 2013
	rs10828317 and rs10430590	CGI-S total score at day 28 of antidepressant therapy	Vyalova et al., 2017
	rs11013052	Schizophrenia	Saggers-Gray et al., 2008
	rs8341	Schizophrenia	He et al., 2007
	Various intronic deletions and insertions 29 bp from the exon 9–intron 9 junction	BD	Stopkova et al., 2003

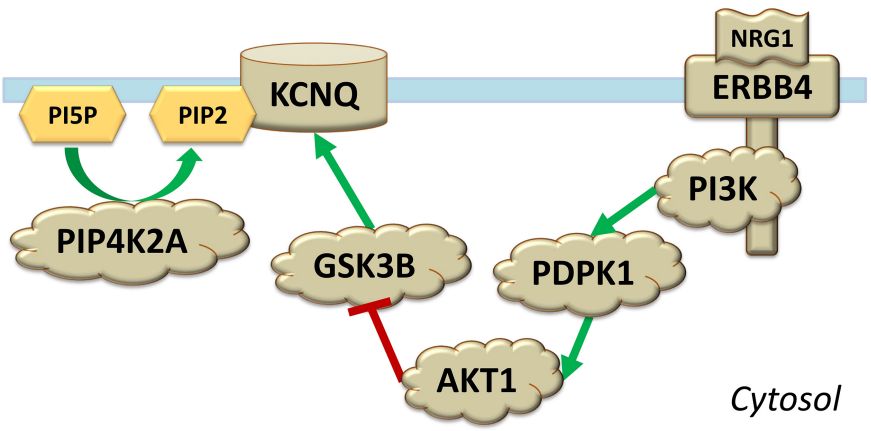

FIGURE 1 | Shown are molecular interactions involving protein products of *PIP4K2A* and *NRG1* associated with time to recurrence of an episode in bipolar disorder and antidepressant treatment response. Phospholipids phosphatidylinositol-5-phosphate (PI5P) and phosphatidylinositol-5,4-bisphosphate (PIP2) are located on the intracellular leaflet of the plasma membrane, while the channel KCNQ and the receptor ERBB4 are transmembrane. The binding of NRG1 to the receptor tyrosine kinase ERBB4 results in subsequent activation of phosphoinositide 3-kinase (PI3K), 3-phosphoinositide-dependent protein kinase-1 (PDPK1), and AKT1; the latter kinase inhibits GSK3B.

(Greene and Hoshi, 2017) and are potential treatment targets for manic symptoms (Grunnet et al., 2014).

In the present study, we sequenced exons and flanking intronic regions of *GSK3B, BDNF*, *NGF*, *NRG1*, *HTR2C*, and *PIP4K2A*, using the ion semiconductor next-generation sequencing technology. A statistical evaluation revealed that several alleles of *NRG1*, *HTR2C*, and *PIP4K2A* are associated with a number of clinical subphenotypes of depression and bipolar disorder, namely, time to recurrence of a depressive episode and of manic or mixed episode, response to pharmacological antidepressant treatment on the 28th day assessed by the Hamilton Depression Rating Scale—17 items (HDRS-17) or the Clinical Global Impression – Improvement (CGI-I) scale, and severity of depression. A bioinformatic assessment of the functional impact of these alleles suggests possible molecular mechanisms in the etiology of these clinical subphenotypes.

MATERIALS AND METHODS

Study Subjects

The genetic study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki 1975, revised in Fortaleza, Brazil, 2013) for experiments involving humans. After approval of the study protocol by the Local Bioethics Committee of the Mental Health Research Institute in Tomsk, Russia, 115 patients were recruited from an in-patient facility of the same institute. As a negative control, 34

individuals without psychiatric disorders were also recruited into the study. Only subjects with European ancestry were considered. All participants gave written informed consent after a proper explanation of the prospective study.

In particular, we included the following numbers of patients with mood disorders, diagnosed using the criteria of the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10): 19 patients with bipolar affective disorder (ICD-10: F31), 55 patients with depressive episode (ICD-10: F32), and 41 patients with recurrent depressive disorder (ICD-10: F33). A summary of clinical features for this cohort is shown in **Table 2**. Bipolar I and II disorders among patients with bipolar affective disorder were established using the Diagnostic and Statistical Manual of Mental Disorders, 4th edition, criteria. Severity of illness was assessed using the Clinical Global Impression—Severity scale (CGI-S) (Guy, 1976). Manic and mixed episodes were considered in the association study under a single category, because some patients only had depressive and mixed episodes. The 115 patients taken together constituted the cross-disorder group.

The duration of antidepressant treatment was not less than 28 days. During their follow-up in the clinic, patients were given several different groups of antidepressants, including tricyclic antidepressants, selective serotonin reuptake inhibitors, serotonin–norepinephrine reuptake inhibitors, noradrenergic and specific serotonergic antidepressants, and agomelatine (an agonist at melatonin receptors and an antagonist at serotonin 2C receptors). All antidepressants were used in recommended average therapeutic doses. For definition of response and remission, the HDRS-17 (Hamilton, 1960) and CGI scale (Guy, 1976) was used. Evaluation was made on the 28th day of treatment. Responders were identified if the HDRS-17 scores were reduced by 50% or if the CGI-I scores were ≤ 2. Remitters were identified if the HDRS-17 scores ≤ 7 or if the CGI-S scores were ≤ 2.

TABLE 2 | Summary of clinical features of the cohort.

Bipolar affective disorder		**nb of individuals/mean and standard deviation**
Type	Bipolar I disorder	9
	Bipolar II disorder	10
Disease severity	Mild	7
	Moderate	7
	Severe	5
	Age of onset	30.42 ± 12.46
	Disease duration	10.32 ± 7.23
Total number of episodes	Depressive episodes	4.26 ± 3.33
	Manic and mixed episodes	7.42 ± 13.22
Gender	Male	8
	Female	11
Depression		**nb of individuals/mean and standard deviation**
Diagnosis	Recurrent depressive disorder	41
	Depressive episode	55
Syndrome	Depressive	45
	Anxious-depressive	42
	Asteno-depressive	9
Disease severity	Mild	0
	Moderate	86
	Severe	10
	Age of onset	45.84 ± 11.37
	Disease duration	4.59 ± 7.19
	Total number of episodes	2.29 ± 2.09
Gender	Male	16
	Female	80
HDRS-17	Response*	93
	No response	3
	Remission*	64
	No remission	32
CGI-I	Response*	91
	No response	5
CGI-S	Remission*	67
	No remission	29
	–	**nb of individuals/mean and standard deviation**
	Controls	34
	Age	29.44 ± 8.14
Gender	Male	11
	Female	23

**Response to/remission following pharmacological antidepressant treatment.*

Targeted DNA Sequencing

Evacuated blood collection tubes "Vacutainer" (Becton Dickinson, Franklin Lakes, NJ, United States) with EDTA as the anticoagulant were used. Extraction of DNA from whole venous blood was performed using the phenol–chloroform method. Concentration and purity of DNA were measured using the NanoDrop 8000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States).

Targeted next-generation sequencing was performed using the Ion Torrent semiconductor technology (Thermo Fisher Scientific, Waltham, MA, United States) at the Center "Medical Genomics" of the Tomsk National Research Medical Center. A custom DNA panel of 86 amplicons, covering 57 targets, was designed using the Ion AmpliSeq Designer[1]. The targets comprised exons and flanking intronic regions of at least 50 base pairs (bp) of *GSK3B*, *BDNF*, *NRG1*, *NGF*, *HTR2C*, and *PIP4K2A* in the human genome assembly GRCh37/hg19. The Ion AmpliSeq DNA Library kit 2.0 and the Ion Xpress Barcode Adapters 1–16 and 17–32 kits were used to prepare amplicon libraries based on the custom Ion AmpliSeq panels. Library normalization was performed using the Ion Library Equalizer kit. The Ion PGM Template OT2 200 Kit was used to prepare the template on the Ion OneTouch 2 System. Sequencing was done with the mean coverage of 473 × on the Ion Torrent Personal Genome Machine (PGM) System, using the Ion PGM Sequencing 200 kit v2 and Ion 316 Chips v2.

[1]https://www.ampliseq.com

Sequencing Analysis Workflow

The unmapped BAM files were converted to the FASTQ format using Picard tools[2]. Quality control (QC) of raw sequencing data and trimming of low-quality bases and adapters was done using FastQC[3] and Trimmomatic (Bolger et al., 2014), respectively. The following Trimmomatic options were used: SLIDINGWINDOW: 4:20; LEADING: 15; TRAILING: 15; MINLEN: 36. QC of trimmed reads was also done using FastQC. Reads were aligned to the human genome assembly GRCh38.p12/hg38 using the default options of BWA-MEM[4]. SAMtools (Li et al., 2009) flagstat indicated that 99.84% of reads were aligned. SAMtools view with options -F 4 and –Sb, SAMtools sort, and SAMtools index were run to obtain indexed BAM files.

Discovery of single-nucleotide variants (SNV) and short indels was done using GATK (version 4.1.2.0) (McKenna et al., 2010)[5]. Genomic coordinates were lifted from the hg19 to hg38 assembly using the Lift Genome Annotations tool[6]. GATK HaplotypeCaller option stand-call-conf 20 was used. Variants were annotated with rsIDs listed in the Database of Single Nucleotide Polymorphisms build 153 (dbSNP)[7], the Exome Aggregation Consortium (ExAC)[8], and the Genome Aggregation Database (gnomAD)[9].

In order to assure the extraction of reliable sequencing results, we used hard filtering parameters (DePristo et al., 2011; Van der Auwera et al., 2013)[10]. First, the following GATK VariantsFiltration cutoff was applied: QUAL < 100.0. Variants, which were mostly indels, called with this filter in ten or more samples, but not listed in the three databases, were considered ion semiconductor technology artifacts (Laehnemann et al., 2016) and removed. Next, the following GATK VariantsFiltration cutoffs were applied to the remaining variants: QD < 2.0, FS > 200.0, MQ < 40.0, MQRankSum < -12.5, ReadPosRankSum < -8. Males were considered as homozygotes for variants on chromosome X. PLINK 2.0[11] was used to make the output files .bed, .bim, and .fam for the upcoming association study.

Functional Annotation of Variants

Functional annotation of all discovered variants was done with ANNOVAR (Wang et al., 2010) that estimates the degree of deleteriousness of coding variants on protein function using 21 different prediction algorithms/conservation scores, SnpEff (Cingolani et al., 2012) (version 4.3) that was used to predict deleterious effects of both coding and non-coding variants (including effects of intronic variants on consensus sequences for splicing), HumanSplicingFinder (Desmet et al., 2009) that was used to predict impact of exonic variants on splicing, the GeneCards database (Stelzer et al., 2016) that lists GeneHancer (Fishilevich et al., 2017) regulatory elements for genes (for the purposes of this study, we considered only Elite GeneHancer elements), and the Genotype-Tissue Expression (GTEx) portal[12] that lists expression quantitative trait loci (eQTLs) in various tissues. The GTEx Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. The data used for the analyses described in this manuscript were obtained from the GTEx Portal on 10/16/2019.

Association Study

Tests under a number of statistical models were run to evaluate the association between SNVs or indels, discovered during sequencing, and clinical subphenotypes. For analysis of quantitative data (time to recurrence of an episode, age of onset) linear regression was used, whereas for binary data (response to treatment, remission following treatment) the statistical model was binomial logistic regression. These two types of tests were carried out using a series of applications that run in the R environment: snpStats (Sole et al., 2006), SNPRelate[13], dplyr[14], GenABEL[15], ggplot2[16], manhattanly[17], GWASTools[18], and GENESIS[19]. Multiple comparisons were dealt with using the Bonferroni correction and by controlling the false discovery rate (FDR). The type I error rate in this case was set to 5% (i.e., Bonferroni- and/or FDR-corrected p-values were considered significant when ≤ 0.05).

To evaluate the association between genetic variants and multinomial data (three disorders vs. controls, bipolar I and II disorders vs. controls, syndromes among patients with depression), multinomial logistic regression was deployed, while for ordinal data (severity of disease) the model was ordinal logistic regression. These last two types of statistical tests were carried out using Trinculo, a program run in the C++ environment that evaluates likelihood ratios within Bayesian and frequentist frameworks (Jostins and McVean, 2016). The type I error rate was set to 1% (i.e., likelihood ratio p-values were considered significant when ≤ 0.01).

RESULTS

Sequencing Analysis

Sequencing analysis that included the indicated parameters of hard filtering resulted in 149 variant call format (VCF) files with 49 different variants, of which eight are novel, i.e., not listed in any of the three databases – dbSNP, ExAC, or gnomAD. Among

[2]https://broadinstitute.github.io/picard/
[3]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
[4]http://bio-bwa.sourceforge.net/
[5]https://software.broadinstitute.org/gatk/documentation/tooldocs/4.1.2.0/
[6]http://genome.ucsc.edu/cgi-bin/hgLiftOver
[7]https://www.ncbi.nlm.nih.gov/snp/
[8]http://exac.broadinstitute.org/
[9]https://gnomad.broadinstitute.org/
[10]https://software.broadinstitute.org/gatk/
[11]https://www.cog-genomics.org/plink/2.0/
[12]https://gtexportal.org
[13]https://www.rdocumentation.org/packages/SNPRelate
[14]https://www.rdocumentation.org/packages/dplyr
[15]https://www.rdocumentation.org/packages/GenABEL
[16]https://www.rdocumentation.org/packages/ggplot2
[17]https://www.rdocumentation.org/packages/manhattanly
[18]https://www.rdocumentation.org/packages/GWASTools
[19]https://www.rdocumentation.org/packages/GENESIS

the novel variants, four are 2–81 bp deletions, one is a 1 bp duplication, and three are 1 bp substitutions (**Table 3**). The novel variants (all in a heterozygous state, except for the 81 bp deletion present on the X chromosome in a male) were each present in one to three patients with depression, indicating minor allele frequency from 0.7 to 2% in the population under study.

Predicted Biological Impact of Discovered Variants

The results of functional annotation with SnpEff did not indicate high impact of either variant. Likewise, neither variant according to ANNOVAR is considered damaging/deleterious by all 21 prediction algorithms/conservation scores. HumanSplicingFinder indicated a number of new or disrupted exonic splicing enhancers (ESE) and exonic splicing silencers (ESS) (we show results only for novel and associated variants in section "Further Analysis of Biological Function of Associated and Novel Variants"). According to the list of Elite GeneHancer regulatory elements, both rs66866077 and rs79679324 (polymorphic in the cohort under study, but not associated with either clinical subphenotype) are found within the promoter GH11J027696 for *BDNF*. GeneHancer results for the variant rs10508649, associated with clinical subphenotypes, are described in section "Variants in *PIP4K2A*." The GTEx database indicated that rs1053454 and rs2230469 (polymorphic in the cohort under study, but not associated with either clinical subphenotype) are expression quantitative trait loci (eQTLs) for *PIP4K2A* in various parts of the brain cortex and a number of subcortical nuclei. GTEx results for rs2248440 and rs6318 are described in section "**Variant in *HTR2C*.**"

Several Alleles Are Associated With Clinical Subphenotypes

Statistical tests indicated eight associations between alleles and clinical subphenotypes (**Table 4**). Two variants in *NRG1*, NC_000008.11:g.32614509_32614510del, and rs35641374, are associated with a number of subphenotypes related to depressive symptoms. Namely, the allele "del" of NC_000008.11:g.32614509_32614510del is associated with absence of response to antidepressant medication on the 28th day assessed by the HDRS-17 among patients with depression. Longer intervals between depressive episodes in the bipolar and cross-disorder groups are associated with the allele C of rs35641374. The same is for longer intervals between episodes of any type (i.e., depressive, manic or mixed) in the bipolar disorder group.

The allele A (in reverse complement) of rs61731109, found in *PIP4K2A*, is associated with absence of response to antidepressant medication on the 28th day assessed by HDRS-17 among patients with depression. This association passed FDR, but not Bonferroni correction for multiple comparisons. The allele G (in reverse complement) of rs10508649, located in the same gene, is associated with absence of response to antidepressant medication on the 28th day assessed by the CGI-I scale among patients with depression and with longer intervals between manic or mixed episodes among patients with bipolar disorder.

There was no association with response to antidepressant medication for different groups of antidepressants.

Finally, the allele A of rs2248440 (it is a reference allele that is in fact minor, ranging in frequency from 0 to 25% in all eight mentioned populations in dbSNP build 153), located in *HTR2C*, is associated with higher severity of depression among patients with depression.

Further Analysis of Biological Function of Associated and Novel Variants

Functional Annotation of Associated Variants

Variants in NRG1

The variant NC_000008.11:g.32614509_32614510del, associated with response to antidepressant therapy assessed by the HDRS-17 is intronic, with no obvious biological function that could be predicted with bioinformatic tools used (**Table 4**). Although rs35641374, associated with time to recurrence of depression among bipolar and cross-disorder patients and with time to recurrence of episodes of any type among bipolar disorder patients, is a missense variant, it does not seem to affect protein function (both amino acids in the Val > Leu substitution have uncharged hydrophobic side chain).

Variants in PIP4K2A

The allele A (in reverse complement) of the synonymous variant rs61731109, associated with response to antidepressant therapy assessed by the HDRS-17, may affect splicing of introns flanking the constitutive exon 8 (ENST00000376573.9 and ENST00000545335.5) or 6 (ENST00000323883.11) of the *PIP4K2A*'s transcripts, by creating an exonic splicing silencer, as predicted by HumanSplicingFinder. The allele G (in reverse compliment) of another synonymous variant rs10508649, associated with response to antidepressant therapy assessed by the CGI-I scale, and with time to recurrence of a manic or mixed episode, may also affect splicing of introns flanking the constitutive exon 5 (ENST00000376573.9 and ENST00000545335.5) or 2 (ENST00000323883.11) of the *PIP4K2A*'s transcripts, by erasing an exonic splicing enhancer motif, as predicted by HumanSplicingFinder. Furthermore, this variant is found within the GeneHancer's elite enhancer GH10J022572 that regulates the expression of *PIP4K2A*.

Variant in HTR2C

The allele A of the intronic variant rs2248440, associated with higher severity of depression, decreases the expression of the neighboring gene *IL13RA2* in the putamen, according to GTEx. The variant rs6318 also regulates the expression level of *IL13RA2* in the putamen; it is only polymorphic in the cohort under study but was previously associated with a number of phenotypes relevant to the pathogenesis of depression and to effects of psychotropic drugs (Drago and Serretti, 2009; Mickey et al., 2012; Brummett et al., 2014; Karanovic et al., 2015; Avery and Vrshek-Schallhorn, 2016; Chagraoui et al., 2016; Vyalova et al., 2017). The two variants are located 4325 bp apart and are in linkage disequilibrium (LD), according to LDlink[20]. *IL13RA2* is expressed

[20]https://ldlink.nci.nih.gov/

TABLE 3 | Novel variants discovered by sequencing.

Chr	Pos (hg38)	Ref	Alt	Variant	Gene	Strand	Possible function	nb of chr	Carriers
3	119947358	A	G	NC_000003.12:g.119947358A > G	*GSK3B*	R	Intronic, no obvious function	3	d31, d138, d140
8	32614509-32614510	TT	del	NC_000008.11:g.32614509_32614510del	*NRG1*	F		2	d95, d129
	32756363	A	dup	NC_000008.11:g.32756363dup				1	d61
	32763214	T	G	NC_000008.11:g.32763214T > G				2	d59, d128
10	22539905-22539911	GAGAGAG	del	NC_000010.11:g.22539905_22539911del	*PIP4K2A*	R		1	d104
	22539924-22539937	AGAGAGAGGGAGAG	del	NC_000010.11:g.22539924_22539937del				2	d18, d87
11	27658302	T	C	NC_000011.10:g.27658302T > C	*BDNF*	R	Missense in all transcripts, Asp > Gly (acidic to neutral non-polar); deleterious/damaging (†ANNOVAR); allele G (reverse compliment) may affect splicing of the last intron of all *BDNF*'s transcripts, by erasing an exonic splicing enhancer motif (†HumanSplicingFinder)	1	d99
X	114906768-114906848	CAAGCTTTGATGTTACTGCACGGCCACACCGAGGAACCGCCTGGACTAAGTCTGGATTTCCTGAAGTGCTGCAAGAGGAAT	del	NC_000023.11:g.114906768_114906848del	*HTR2C*	F	Inframe deletion of 27 aa or frameshift deletion of 27 aa, resulting in 1 aa inserted; deleterious (†PROVEAN); may affect splicing of the last intron of all *HTR2C*'s transcripts, by erasing multiple exonic splicing enhancer motifs (†HumanSplicingFinder)	1	d128

Chr, chromosome; pos, position in the human genome; ref, reference allele; alt, alternate allele; †*used algorithms.*

TABLE 4 | Genetic variants, associated with clinical subphenotypes.

Chr	Pos (hg38)	Ref	Alt	Variant	Trait	Cohort	Model	Association	*p*-value	Gene	Strand	Variant biological function
8	32614509-32614510	TT	del	NC_000008.11: g.32614509_32614510del	Drug treatment response	Depression	Binomial logistic regression	Absence of response on the 28th day assessed by HDRS-17 is associated with allele "del"	3.22E-04	*NRG1*	F	Intronic, no obvious function
	32648114	G	C	rs35641374	Time to recurrence of a depressive episode	Cross disorder	Linear regression	Longer intervals between depressive episodes are associated with allele C	3.14E-06			Missense in five transcripts, Val > Leu, likely benign for protein function
					Time to recurrence of a depressive episode	Bipolar		Longer intervals between depressive episodes are associated with allele C	4.37E-07			
					Time to recurrence of an episode	Bipolar		Longer intervals between episodes of any type are associated with allele C	3.43E-04			
10	22541913	C	T	rs61731109	Drug treatment response	Depression	Binomial logistic regression	absence of response on the 28th day assessed by HDRS-17 is associated with allele T *(passed FDR, but not Bonferroni correction for multiple comparisons)*	1.11E-03	*PIP4K2A*	R	Synonymous in all transcripts; allele A (reverse complement) may affect splicing (†HumanSplicingFinder)
	22573353	T	C	rs10508649	Drug treatment response	Depression	Binomial logistic regression	Absence of response on the 28th day assessed by CGI-I scale is associated with allele C	9.43E-04			Synonymous in all transcripts; allele G (reverse compliment) may affect splicing (†HumanSplicingFinder); found within enhancer GH10J022572 that regulates expression of *PIP4K2A* (†GeneHancer)
					Time to recurrence of a manic or mixed episode	Bipolar	Linear regression	Longer intervals between manic or mixed episodes are associated with allele C	3.09E-04			
X	114727001	A*	G	rs2248440	Severity	Depression	Ordinal logistic regression	Higher severity of depression is associated with allele A	3.00E-03	*HTR2C*	F	Intronic; allele A decreases expression of *IL13RA2* in putamen (†GTEx)

*Chr, chromosome; pos, position in the human genome; ref, reference allele; alt, alternate allele; †used algorithms; * minor allele.*

in the brain, codes for the interleukin 13 receptor α2 subunit, and is found almost 94 kilobases (kb) downstream from *HTR2C*.

Functional Annotation of Novel Variants

The intronic variants NC_000003.12:g.119947358A > G, NC_000008.11:g.32614509_32614510del (associated with absence of response to antidepressant medication), NC_000008.11:g.32756363dup, NC_000008.11:g.32763214T > G, NC_000010.11:g.22539905_22539911del, and NC_000010.11:g.22539924_22539937del, found in genes *GSK3B*, *NRG1*, and *PIP4K2A*, bear no obvious biological function as reported by the bioinformatic tools used.

The missense variant NC_000011.10:g.27658302T > C in *BDNF* creates the substitution Asp > Gly, i.e., a change from an acidic to uncharged hydrophobic amino acid. This change is deemed deleterious/damaging for the protein function by PROVEAN, PolyPhen-2, LRT, MutationTaster, and FATHMM-MKL algorithms run in ANNOVAR. The implicated allele G (in reverse compliment) may also affect splicing of the last intron of all *BDNF* transcripts, by erasing an exonic splicing enhancer motif, as predicted by HumanSplicingFinder. This variant is present in a heterozygous state in one patient with depressive episode of moderate severity and is not associated with clinical subphenotypes of mood disorders.

Finally, the 81 bp deletion in the last exon of all *HTR2C* transcripts, NC_000023.11:g.114906768_114906848del, creates an in-frame deletion of 27 amino acids (ENST00000276198.5 and ENST00000371951.5) or frameshift deletion of 27 amino acids, resulting in 1 aa inserted (ENST00000371950.3). The variant is deleterious for ENST00000276198.5 and ENST00000371951.5 according to PROVEAN (Choi and Chan, 2015). Moreover, it may affect splicing of the last intron of all *HTR2C* transcripts, by erasing multiple exonic splicing enhancer motifs, as deemed by HumanSplicingFinder. This deletion was present on the X chromosome in one male patient with recurrent depressive disorder of moderate severity. No association with clinical subphenotypes was established for that variant.

DISCUSSION

Possible Genetic Biomarkers of Clinical Subphenotypes of Depression and Bipolar Disorder

Prognostic Biomarkers of Time to Recurrence of Mania and Depression in Bipolar Disorder

Time to recurrence of a depressive episode and time to recurrence of a manic or mixed episode among patients with bipolar disorder may be indicated by alleles of two different variants: rs35641374 in *NRG1* and rs10508649 in *PIP4K2A*, respectively (**Table 4**). The association between rs35641374 and time to recurrence of an episode of any type in the bipolar disorder group seems to be driven by the association between this genetic marker and time to recurrence of depression (the p-value increases from 4.37×10^{-7} for the latter association to 3.43×10^{-4}). Likewise, the association signal at the same marker in the cross-disorder group may in fact be driven by the same association in the bipolar disorder group (the *p*-value increases from 4.37×10^{-7} for the latter association to 3.14×10^{-6}). In other words, rs35641374 may be only associated with time to recurrence of a depressive episode among patients with bipolar disorder.

These results indicate that the allele C of rs35641374 located in *NRG1* and the allele G (reverse compliment) of rs10508649 located in *PIP4K2A* may be protective against recurrent depression and recurrent manic or mixed episodes, respectively. Although rs35641374 seems to be benign to the biological function of mRNA and protein, rs10508649 is found within the GeneHancer's elite enhancer GH10J022572 that regulates the expression of *PIP4K2A*. Furthermore, the allele G of rs10508649 may affect mRNA splicing, which indicates that this variant may be functional for the *PIP4K2A* gene expression on two levels.

This conclusion suggests that *PIP4K2A* plays a role in the pathogenesis of manic or mixed symptoms. Previous data indicate that this gene is associated with bipolar disorder and schizophrenia (Stopkova et al., 2003; Schwab et al., 2006), both disorders sharing a number of clinical and molecular features (International Schizophrenia Consortium et al., 2009; Bipolar Disorder and Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2018).

Implication of *NRG1* in the pathogenesis of depressive symptoms among patients with bipolar disorder is also supported by previous studies, linking this gene to bipolar disorder and depression (Mei and Nave, 2014; Dang et al., 2016; Wen et al., 2016; Chen et al., 2017).

Predictive Biomarkers of Response to Antidepressant Medication

Absence of response on the 28th day assessed by the HDRS-17 or CGI-I scale among patients with depression might be predicted by alleles of the variant NC_000008.11:g.32614509_32614510del in *NRG1* and of the two variants in *PIP4K2A*: rs61731109 and rs10508649. The indicated novel intronic variant should perhaps be listed under the dbSNP entry rs750640301 that differs from it only by the number of deleted nucleotides (one T instead of two) at the same genomic location. Although NC_000008.11:g.32614509_32614510del bears no obvious biological function, both rs61731109 and rs10508649 seem to affect splicing and, in case of rs10508649, transcriptional regulation of *PIP4K2A*.

Cross-Disorder Aspects of Mania and Antidepressant Therapeutic Response

It is worthy of note that the alleles of *NRG1* and *PIP4K2A* may be simultaneous predictors of time to recurrence of manic and depressive episodes among patients with bipolar disorder and of absence of drug treatment response among patients with depression (**Table 4**). In particular, the allele G (reverse compliment) of rs10508649 in *PIP4K2A* may increase resistance to antidepressant treatment and be at the same time protective against recurrent manic or mixed episodes. These data suggest interesting avenues for the study of the pathogenesis of mania and its possible connections

with the pathogenesis of treatment-resistant depression (McIntyre et al., 2014). It is known that antidepressant drugs are not suitable in treatment of bipolar disorder, because they do not provide the desired response; moreover, evidence from clinical practice suggests that antidepressants may precipitate manic symptoms (Hirschfeld, 2014). It has even been suggested that switching to mania is a consequence of increased antidepressant efficacy (Lee H. J. et al., 2013), which would support the results implicating rs10508649. Both treatment-resistant depression and mania were also suggested to have one common underlying biological mechanism: circadian rhythms (Lee H. J. et al., 2013). Although *NRG1* and *PIP4K2A* are not regarded as biological clock genes, they both take part in signaling networks where *GSK3B* (Carter, 2007; Beaulieu, 2012), a gene implicated in circadian rhythms (Harada et al., 2005), plays a prominent role. An additional clue in this puzzle is that lithium, which acts upon this pathway and inhibits GSK3B (Freland and Beaulieu, 2012), modulates circadian rhythms in patients with bipolar disorder (Lee H. J. et al., 2013). Further research is necessary to continue uncovering the complex interplay between molecular networks involving GSK3B, circadian rhythms, manic symptoms, and antidepressant resistance.

Prognostic Biomarker of Depression Severity

Depression severity in patients with a diagnosis of depression may be determined by alleles of the variant rs2248440 found in the intron following the first coding exon of *HTR2C*. This variant is in LD with the missense *HTR2C* variant rs6318, previously associated with mood disorders and response to antidepressant medication (Chagraoui et al., 2016; Vyalova et al., 2017). An interesting finding is that both variants are eQTLs in the putamen for the gene of a subunit of the interleukin 13 (IL-13) receptor (*IL13RA2*). This might indicate either that the serotonin 2C receptor is not a genetic factor influencing on severity of depressive symptoms or that both receptors act in concert in influencing on this clinical subphenotype. Knowing that the involvement of the serotonin 2C receptor in the pathogenesis of depression is supported by a considerable amount of scientific data (Chagraoui et al., 2016; Palacios et al., 2017) and that the pathogenesis of depression is associated with the immune system abnormalities (Felger and Lotrich, 2013), the latter scenario seems to be more plausible. Although *IL13RA2* is known to be implicated mainly in cancer (Sengupta et al., 2014), its ligand IL-13 regulates inflammation (Mao et al., 2019), so this gene could also play a part in increased inflammation seen in patients with depression. More research is needed to clarify these data.

Estimation of Sample Size

Estimation of sample size is possible, but not trivial, because it necessitates prior knowledge of relative risks and frequencies of genotypes associated with the disease. These parameters are unavailable in the case of genetic variants that were not studied previously. It is also worthy of note that application of algorithms that can calculate sample size for genetic association studies is limited in the case of complex study designs. For example, Genetic Power Calculator[21] can be used only for discrete traits. Application of this calculator, assuming that disease prevalence = 0.05, disease allele frequency = 0.05, and genotype relative risk = 2, indicates a sample size of 676 needed to achieve power of 80% for an allelic test at $\alpha = 0.05$. However, our study design also includes quantitative traits. The instrument Power for Genetic Association Analyses[22] (Menashe et al., 2008) does not include allelic tests. Nevertheless, assuming the dominant mode of inheritance, this statistical instrument indicates approximately the same sample size under the same parameters. General sample size calculators (such as[23]) indicate a sample size of 385 at $\alpha = 0.05$, assuming that the actual population is very large (more than 1 million).

Power refers to the number of patients required to avoid a type II error (false-negative results) in a comparative study. Sample size estimation indicates that power of the present study is low. Nevertheless, the study showed a number of statistically significant results, probably due to detailed phenotyping that enabled using a smaller subset of patients (Yehia and Eng, 2019).

Protein Networks

Interactions Suggested by This Study

NRG1 and PIP4K2A could act in concert via their connections with GSK3B (**Figure 1**; Carter, 2007; Beaulieu, 2012; Mei and Nave, 2014; Jiang et al., 2015; Greene and Hoshi, 2017). ERBB4 and its ligand NRG1 play an important role in neurodevelopment, neurotransmission, and synaptic plasticity, and this receptor is present on GABAergic, glutamatergic and dopaminergic neurons (Mei and Nave, 2014). One of its functions is to promote the inhibitory GABA release. Thus, together with KCNQ/M-channels activated by PIP2 (Greene and Hoshi, 2017), ERBB4 takes part in regulation of neuronal excitability, an aspect that could be important in the pathogenesis of bipolar disorder (Berridge, 2014).

Serotonin acts upon the brain and peripheral immune system components, while both types of these components regulate the serotonin neurotransmission (Robson et al., 2017; Wu et al., 2019). This connection could be the key to the observed immune system abnormalities strongly associated with depression (Felger and Lotrich, 2013) and suggests a possibility of an interaction between HTR2C and IL13RA2. The immune system could therefore be a biological factor that modulates the severity of depression (Felger and Lotrich, 2013).

On the other hand, knowing that serotonin leads to inhibition of KCNQ/M-channels (Colino and Halliwell, 1987; Stephens et al., 2018) and inflammation leads to production of reactive oxygen species resulting in increased neuronal excitability (Berridge, 2014),

[21] http://zzz.bwh.harvard.edu/gpc/

[22] https://dceg.cancer.gov/tools/design/pga

[23] https://www.surveymonkey.com/mp/sample-size-calculator/

NRG1, PIP4K2A, HTR2C, and IL13RA2 may have further interconnected roles in the pathophysiology of depressive and manic episodes and the response to antidepressant medication.

Interactions Predicted by the String Database Relevant to This Study

Generalized protein networks may be visualized with String V.11 (Szklarczyk et al., 2019). In fact, the protein products of the genes described in this study – *GSK3B*, *BDNF*, *NRG1*, *NGF*, *HTR2C*, *PIP4K2A, IL13RA2*, *IL13*, *ERBB4*, *PIK3CA*, *PIK3CB*, *PDPK1*, *AKT1*, the three M-channel subunits *KCNQ2*, *KCNQ3*, and *KCNQ5*, and neurotrophic receptors *NTRK1* and *NTRK2* – are deemed to be functionally connected (**Supplementary Figure S1A**). Adding top 20 predicted functional partners to this network within the first shell of interactions (a direct connection with the input proteins) reveals a larger network (**Supplementary Figure S1B**).

It is important to note that predicted interactions should be interpreted with caution: bioinformatic instruments that predict interaction networks (such as String) cannot extract *all* connections relevant in psychiatric disorders, not they can draw protein networks relevant *only* in psychiatric disorders. For example, despite evidence in literature indicating regulation of KCNQ2 by GSK3B (Carter, 2007; Wildburger and Laezza, 2012; Jiang et al., 2015; **Figure 1**), no such interaction was predicted by String (**Supplementary Figures S1A,B**).

Despite this, predicted interactions may be used to pinpoint interesting functional candidates that may become subjects of future studies. For instance, String indicates a functional connection between HTR2C and IL13RA2 through the interleukin 4 receptor subunit α (IL-4R). A heterodimer formed by this subunit and the interleukin 13 receptor subunit α1 binds both IL-13 and IL-4 (Karo-Atar et al., 2018). IL-4 is known to be implicated in psychiatric disorders, in particular, in the pathogenesis of depression (Wachholz et al., 2017). One of IL-4's roles is regulation of the serotonin transporter (Mossner et al., 2001). *PPP2CA* codes for the protein phosphatase 2 catalytic subunit α of protein phosphatase 2A (PP2A), an important component of AKT/GSK3 signaling implicated in a number of psychiatric disorders, including depression and bipolar disorder (Beaulieu, 2012), as well as response to psychotropic drugs, including antidepressants and lithium (Beaulieu et al., 2009). Protein products of *MTOR* and *TSC2* are implicated via their connections with AKT1 in regulation of synaptic plasticity and memory (Emamian, 2012); these are impaired in depression, possibly as a result of a reduction of hippocampal volumes (MacQueen and Frodl, 2011). As mentioned earlier, *PIK3R1* and *PIK3R2* code for regulatory subunits p85α and p85β, while *PIK3CA* codes for the catalytic subunit p110α of PI3K; expression levels of these subunits were significantly altered in suicide completers (Dwivedi et al., 2008). Suicide is a psychiatric phenotype associated with depression (Mullins et al., 2019), so pathogenic mechanisms may be shared between the two phenotypes. Finally, β-catenin encoded by *CTNNB1* is implicated in a number of molecular pathways relevant in psychiatric disorders (Freyberg et al., 2010; Wisniewska, 2013). *CTNNB1* also contains damaging genetic variants in several neurodevelopmental disorders, including schizophrenia (Levchenko et al., 2015), while some antipsychotic drugs result in increased levels of β-catenin in the brain (Freyberg et al., 2010). Bipolar disorder and schizophrenia share pathogenic mechanisms (Gordovez and McMahon, 2020), so β-catenin might be relevant in the pathogenesis of bipolar disorder as well (Guo et al., 2019).

Study Limitations

The main limitation of this genetic study is a modest number of patients, which is reflected in reduced statistical power. This might have been the reason several interesting (from the point of view of predicted biological function) genetic candidates failed to show an association with clinical subphenotypes. Among these variants are rs66866077 and rs79679324 (that may modify the *BDNF* promoter activity), as well as rs1053454 and rs2230469 (that determine the *PIP4K2A* level of expression in various parts of the brain). The same may be noted about two novel, apparently deleterious variants NC_000011.10:g.27658302T > C (*BDNF*) and NC_000023.11:g.114906768_114906848del (*HTR2C*). Larger cohorts are necessary in order to investigate significance of these putative functional variants.

Another limitation is that any statistics-based study only points out avenues for further research, so in order to prove biological relevance of candidate genetic biomarkers, described in this paper, functional studies deploying cellular or animal models are warranted.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are available at dbSNP: https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ss.cgi?subsnp_id=ss2137544101, https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ss.cgi?subsnp_id=ss3986007706, https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ss.cgi?subsnp_id=ss3986007707, https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ss.cgi?subsnp_id=ss3986007708, https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ss.cgi?subsnp_id=ss3986007709, https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ss.cgi?subsnp_id=ss3986007710, https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ss.cgi?subsnp_id=ss3986007711, and https://www.ncbi.nlm.nih.gov/dbvar/studies/nstd180.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Local Bioethics Committee of the Mental Health Research Institute in Tomsk, Russia. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NV, NB, and SI contributed to the conception and design of the study. GS was the psychiatrist who recruited and clinically assessed the patients. SI recruited the controls. GS and NV built the clinical database. NV and IP performed the sequencing. TN performed the sequencing analysis, association study, and a part of functional annotation. AL supervised the work of TN, performed the remaining part of functional annotation, integrated the study results, and wrote the manuscript. All authors contributed to the manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00936/full#supplementary-material

FIGURE S1 | Shown are molecular interactions as predicted by String. **(A)** A network limited only to input proteins. In evidence mode in String, an edge is drawn with differently colored lines that represent the existence of various types of evidence used in predicting the associations. Blue line – cooccurrence evidence, purple line – experimental evidence, yellow line – textmining evidence (i.e., the two proteins are co-mentioned in PubMed abstracts), light blue line – database evidence, and black line – coexpression evidence. **(B)** The same network expanded with additional top 20 predicted functional partners within the first shell of interactions (a direct connection with the input proteins). In confidence mode in String the thickness of the line indicate the degree of confidence prediction of the interaction.

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Solute Carrier Family 1 (*SLC1A1*) Contributes to Susceptibility and Psychopathology Symptoms of Schizophrenia in the Han Chinese Population

Wenqiang Li[1,2†], *Xi Su*[1,2†], *Tengfei Chen*[1,2], *Zhen Li*[1,2], *Yongfeng Yang*[1,2], *Luwen Zhang*[1,2], *Qing Liu*[1,2], *Minglong Shao*[1,2], *Yan Zhang*[1,2], *Minli Ding*[1], *Yanli Lu*[1], *Hongyan Yu*[1], *Xiaoduo Fan*[3], *Meng Song*[1,2*] and *Luxian Lv*[1,2*]

[1] Department of Mental Health, The Second Affiliated Hospital of Xinxiang Medical University, Henan Mental Hospital, Xinxiang, China, [2] Henan Key Lab of Biological Psychiatry, International Joint Research Laboratory for Psychiatry and Neuroscience of Henan, Xinxiang Medical University, Xinxiang, China, [3] Department of Psychiatry, University of Massachusetts Medical School/UMass Memorial Medical Center, Worcester, MA, United States

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Shanghai Jiao Tong University, China

***Correspondence:**
Meng Song
song.meng.201@163.com
Luxian Lv
lvx928@126.com

[†]*These authors have contributed equally to this work*

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Objective: Schizophrenia (SZ) is a common and complex psychiatric disorder that has a significant genetic component. The glutamate hypothesis describes one possible pathogenesis of SZ. The solute carrier family 1 gene (*SLC1A1*) is one of several genes thought to play a critical role in regulating the glutamatergic system and is strongly implicated in the pathophysiology of SZ. In this study, we identify polymorphisms of the *SLC1A1* gene that may confer susceptibility to SZ in the Han Chinese population.

Methods: We genotyped 36 single-nucleotide polymorphisms (SNPs) using Illumina GoldenGate assays on a BeadStation 500G Genotyping System in 528 paranoid SZ patients and 528 healthy controls. Psychopathology was rated by the Positive and Negative Symptom Scale.

Results: Significant associations were found in genotype and allele frequencies for SNPs rs10815017 (p = 0.002, 0.030, respectively) and rs2026828 (p = 0.020, 0.005, respectively) between SZ and healthy controls. There were significant associations in genotype frequency at rs6476875 (p = 0.020) and rs7024664 (p = 0.021) and allele frequency at rs3780412 (p = 0.026) and rs10974573 (p = 0.047) between SZ and healthy controls. Meanwhile, significant differences were found in genotype frequency at rs10815017 (p = 0.015), rs2026828 (p = 0.011), and rs3780411 (p = 0.040) in males, and rs7021569 in females (p = 0.020) between cases and controls when subdivided by gender. Also, significant differences were found in allele frequency at rs2026828 (p = 0.003), and rs7021569 (p = 0.045) in males, and rs10974619 in females (p = 0.044). However, those associations disappeared after Bonferroni's correction (p's > 0.05). Significant associations were found in the frequencies of four haplotypes (AA, CA, AGA, and GG) between SZ and healthy controls (χ^2 = 3.974, 7.433, 4.699, 4.526, p = 0.046, 0.006, 0.030, 0.033, respectively). There were significant associations between

rs7032326 genotypes and PANSS total, positive symptoms, negative symptoms, and general psychopathology in SZ (p = 0.002, 0.011, 0.028, 0.008, respectively).

Conclusion: The present study provides further evidence that *SLC1A1* may be not a susceptibility gene for SZ. However, the genetic variations of *SLC1A1* may affect psychopathology symptoms.

Keywords: schizophrenia, *SLC1A1*, single-nucleotide polymorphisms, psychopathology symptoms, association

INTRODUCTION

Schizophrenia (SZ) is a complex disease with multiple susceptibility genes (1). The pathogenesis of SZ is unknown, and the glutamate hypothesis is one possible suggestion (2). Previously, our studies have revealed susceptibility genes (e.g., SCL1A6) in the glutamate pathway (3). This suggests that research on the glutamate pathway can provide important evidence for the pathogenesis of SZ. At present, a large number of genome-wide association study (GWAS) have revealed that SZ is a complex disease involving multiple genes (4–8). However, there are no consistent results for the important genetic susceptibility genes of SZ. Therefore, identifying SZ susceptibility genes from numerous candidates is an ongoing challenge.

Glutamate is a key primary excitatory neurotransmitter that plays a critical role in synaptic plasticity, neuronal toxicity, neuronal development, and signal transduction in the brain (9), and glutamatergic dysfunction could be involved in the pathogenesis of SZ (2, 10). The glutamatergic dysfunction hypothesis, supported by previous and our recent studies that involved glutamate receptor genes, such as GRIN2A (11), GRIN2B (12), and NRG1 (13, 14), and genes related to glutamatergic transmission, e.g., SCL1A6 (3) and SLC1A3 (15). Thus, further exploration of the genes of the glutamate pathway is important for the research of susceptibility genes for SZ.

The solute carrier family 1 gene (*SLC1A1*), a member of the neuronal high-affinity glutamate transporter family, is located at 9p24.2 and codes for the excitatory amino acid transporter (EAAT) 3 (16), and is expressed throughout the central nervous system, especially in the forebrain (17). Previous studies have reported that *SLC1A1* is associated with risk of SZ (16, 18–20). Expression changes of *SLC1A1* transcripts in SZ have strongly implicated it in SZ pathophysiology (16). Moreover, a 5-generation Palauan family study revealed that an *SLC1A1* mutation and co-segregation correlated with the pathophysiology of SZ (20). Recent GWAS also suggested this gene as an SZ susceptibility gene (21). Some studies have reported that *SLC1A1* SNP rs2228622 (15) and rs7022369 (19) were susceptibility markers involved in the pathogenesis of SZ. Meanwhile, rs16921385 of *SLC1A1* was found to be associated with treatment response to risperidone in SZ (22). Those studies provide more evidence that variation of *SLC1A1* may play a critical role in SZ pathogenesis. However, the findings were inconsistent and scarce (15, 19, 23). Therefore, we further explored the association between *SLC1A1* and SZ in the Chinese Han population.

MATERIALS AND METHODS

Subjects

SZ patients were recruited as inpatients of the Second Affiliated Hospital of Xinxiang Medical University (China) from March of 2005 to December of 2008. The diagnostic criteria of SZ were according to the Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition (DSM-IV). Psychopathology symptoms were measured by Positive and Negative Symptom Scale (PANSS) (24). As in our previous studies (3, 12), family history (FH) was used to explore genetic susceptibility. Inclusion and exclusion criteria of SZ patients and healthy controls were in line with our previous studies (12, 25). Inclusion of SZ: 1) patients with paranoid SZ according to DSM-IV; 2) PANSS ≥ 60; 3) male vs. female = 1:1; 4) Age range from 18 to 55 years old; 5) Han Chinese population. Inclusion of healthy controls: 1) Han Chinese population; 2) male vs. female = 1:1; 3) Age range from 18 to 55 years old. Both SZ and healthy controls were born and lived in the north of Henan Province (China), and unrelated individuals of Chinese Han population. Individuals with other psychiatric disorders, substance dependence, organic brain disease, and severe medical complications were excluded. In the sample collection, the clinical raters had rich experience in administering psychopathological tests and ensure the inter-rater consistency of diagnoses and test results through the training of every 6 months. This study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Xinxiang Medical University (China). All subjects were informed and signed a written informed consent form.

SNP Selection

In this study, we used the FASTSNP online service (26) to perform functional analysis, and all 36 SNPs covering the genomic region chr9: 4477575 - 4576808. Meanwhile, those SNPs were a minor allele frequency ≥0.05 and highly ranked risk in the Chinese Beijing population at the HapMap database.

eQTL Analysis

Further, explore significant SNPs affect the expression level of SLC1A1 gene in brain tissues according to public eQTL databases (BrainSeq Phase 1: http://eqtl.brainseq.org/phase1/eqtl/; BrainSeq Phase 2: http://eqtl.brainseq.org/phase2/eqtl/; Brain xQTL: http://mostafavilab.stat.ubc.ca/xQTLServe/snp_query/).

Genotyping

Peripheral blood samples were collected from SZ and healthy controls by using evacuated tubes containing EDTA anticoagulant. RelaxGene Blood DNA System (Tiangen Biotech, Beijing, China) was used to extract genomic DNA from white blood cells. The method of genotyping was described in our previous studies that use the Illumina GoldenGate assays on a BeadStation 500G Genotyping System (Illumina, San Diego, CA, USA) (3, 12, 25).

Statistical Analyses

Statistical analyses were in line with and described in our previous studies (3, 12, 25). G*Power software was used to calculate power (http://www.gpower.hhu.de/). The Haploview V4.1 program was used to assess alleles, genotypes, and haplotype frequency (27). One-way analysis of variance (ANOVA) tests were used for association analyses between PANSS scores and different genotype (SPSS version 25.0, SPSS, Inc. Chicago, IL, USA). Statistical significance was set as $p < 0.05$. Bonferroni correction was used to adjust for multiple testing.

RESULTS

A total of 1,056 subjects, 528 SZ and 528 healthy controls, were included in this study. There were no significant differences in age or gender between cases and healthy controls ($p = 0.095$, 1.000, respectively) **(Table 1)**.

The genotypes of 36 SNPs were detected in 1056 samples, with a genotyping success rate of 99.79%. There were significant associations in genotype frequency and allele frequency at rs10815017 ($p = 0.002$, 0.030; respectively) and rs2026828 ($p = 0.020$, 0.005, respectively) between cases and healthy controls. Additionally, there were significant associations in genotype frequency at rs6476875 and rs7024664 between cases and healthy controls ($p = 0.020$, 0.021, respectively), and significant associations in allele frequency at rs3780412 and rs10974573 between cases and healthy controls ($p = 0.026$, 0.047, respectively). Meanwhile, there was an association trend in genotype frequency at rs7021569 and rs4742007 between SZ and health controls ($p = 0.05$ for both). Those associations disappeared after Bonferroni's correction (p's > 0.05). There were no significant associations in genotype frequency or allele frequency at the other 28 SNPs between the two groups **(Table 2)**.

TABLE 1 | Demographics of the schizophrenia patients and healthy controls.

Variables	SZ	HC	p
N	528	528	
Age (years)	27.32 ± 8.03	27.73 ± 8.01	0.95
Age of Onset (years)	23.47 ± 8.26	NA	
Duration of Illness (years)	6.18 ± 5.91	NA	
Gender (male/female)			1.00
Male	264	264	
Female	264	264	
Family history			
Yes	82	0	
No	446	528	

When the subjects were divided by sex, we found significant differences in genotype frequency at rs10815017 ($p = 0.015$), rs2026828 ($p = 0.011$), and rs3780411 ($p = 0.040$) in males, and rs7021569 in females ($p = 0.020$) between cases and controls. Also, significant differences were found in allele frequency at rs2026828 and rs7021569 in males ($p = 0.003$, 0.045, respectively), and rs10974619 in females ($p = 0.044$) **(Supplementary Table 1)**.

We also observed significant differences in genotype frequency at rs10814991 ($p = 0.015$) and rs1471786 ($p = 0.046$) between patients with and without family histories (FH) of SZ. A difference trend was found in genotype frequency at rs3780411 between FH (+) and FH (−) in SZ but was not significant **(Supplementary Table 2)**.

As shown in **Figure 1**, 9 LD blocks and 32 haplotypes were formed from 36 SNPs. There were significant associations in the frequencies of four haplotypes (AA, CA, AGA, and GG) between SZ and healthy control ($\chi^2 = 3.974$, 7.433, 4.699, 4.526, $p = 0.046$, 0.006, 0.030, 0.033, respectively) **(Table 3).**

Further, we make association analysis in six significant SNPs of the *SLC1A1* gene in the PGC samples, including European and East Asian population (**Supplementary Table 3**). We found significant associations at rs10974573 in European population ($p = 0.008$, OR=1.031). Meanwhile, there no significant associations at six SNPs affect the expression level of SLC1A1 gene in main brain tissues, including frontal cortex and hippocampus (p's > 0.05, **Supplementary Table 4).**

There were significant associations between rs7032326 genotypes and PANSS total, positive symptoms, negative symptoms, or general psychopathology in SZ ($p = 0.002$, 0.011, 0.028, 0.008, respectively). There were significant associations between rs7860087 genotypes and PANSS total, negative symptoms, or general psychopathology in SZ ($p = 0.011$, 0.015, 0.041, respectively). Genotypes of rs2039291 and rs4742007 were associated with positive symptoms ($p = 0.029$, 0.039, respectively). Genotypes of rs301430 were associated with negative symptoms ($p = 0.031$) **(Table 4).**

DISCUSSION

We investigated *SLC1A1* mutations associated with the pathogenesis and psychopathology symptoms of SZ in a Han Chinese population. None significant differences were found in genotype and allele frequencies of rs10815017 and rs2026828 between SZ patients and healthy controls after Bonferroni's correction. Therefore, our study suggests that *SLC1A1* may be not a susceptibility gene for SZ. However, we found that rs7032326 genotypes were associated with psychopathology symptoms of SZ.

Previous studies reported the decreased EAAT3 (encoded product of *SLC1A1*) transcript expression (28), and increased expression of transcripts encoding EAAT1 and EAAT2 (29) in SZ. These findings suggested that the glutamate receptors and related molecules were abnormally expressed in glutamatergic synapses in SZ. In addition, expression of *SLC1A1* was decreased following chronic antipsychotic treatment in animal models.

TABLE 2 | Genotype and allele frequencies of 36 SNPs in the *SLC1A1* gene in SZ patients and healthy controls.

SNP#	dbSNP ID	Allele (D/d)[a]	SZ								HCs								P value	
			N[b]	HWE (p)	Genotype			Allele		MAF	Nb	HWE (p)	Genotype			Allele		MAF	Genotype	Allele
					DD	Dd	dd	D	d				DD	Dd	dd	D	D			
1	rs10815017	G/A	528	0.021	391	119	18	901	155	0.147	528	0.111	348	168	12	864	192	0.182	**0.002 (0.08)***	**0.030 (1.00)***
2	rs2026828	A/G	528	0.240	204	238	86	646	410	0.388	528	0.716	163	257	108	583	473	0.448	**0.020 (0.73)***	**0.005 (0.22)***
3	rs6476875	A/G	528	0.001	353	143	32	849	207	0.196	526	0.906	316	184	26	816	236	0.224	**0.020 (0.71)***	0.111
4	rs7024664	T/A	527	0.000	258	174	95	690	364	0.345	523	0.000	283	132	108	698	348	0.333	**0.021 (0.76)***	0.540
5	rs7021569	C/G	528	0.877	309	189	30	807	249	0.236	526	0.003	295	180	51	770	282	0.268	0.050	0.088
6	rs4742007	A/G	528	0.041	131	287	110	549	507	0.480	527	0.240	139	250	138	528	526	0.499	0.051	0.384
7	rs3780412	A/G	528	0.832	323	181	24	827	229	0.217	527	0.665	292	198	37	782	272	0.258	0.078	**0.026 (0.94)***
8	rs10974573	A/C	528	0.364	338	173	17	849	207	0.196	528	0.748	369	146	13	884	172	0.163	0.124	**0.047 (1.00)***
9	rs7860087	G/C	528	0.555	387	132	9	906	150	0.142	528	0.375	416	103	9	935	121	0.115	0.099	0.059
10	rs2039291	C/A	528	0.529	174	252	102	600	456	0.432	528	0.994	148	263	117	559	497	0.471	0.186	0.073
11	rs2228622	G/A	528	0.858	320	183	25	823	233	0.221	528	0.605	297	195	36	789	267	0.253	0.200	0.082
12	rs10739062	G/C	528	0.359	246	222	60	714	342	0.324	528	0.799	219	240	69	678	378	0.358	0.235	0.098
13	rs10974619	G/A	528	0.522	464	61	3	989	67	0.063	528	0.353	448	75	5	971	85	0.080	0.329	0.130
14	rs12682807	A/C	527	0.805	292	202	33	786	268	0.254	528	0.857	316	184	28	816	240	0.227	0.334	0.147
15	rs2072657	A/C	526	0.107	254	234	38	742	310	0.295	527	0.613	285	202	40	772	282	0.268	0.124	0.166
16	rs16921385	A/G	528	0.177	377	143	8	897	159	0.151	528	0.413	366	144	18	876	180	0.170	0.134	0.213
17	rs10491731	A/C	528	0.687	309	192	27	810	246	0.233	528	0.087	300	186	42	786	270	0.256	0.175	0.224
18	rs3780413	G/C	528	0.089	276	222	30	774	282	0.267	528	0.558	304	190	34	798	258	0.244	0.130	0.231
19	rs188537	C/A	528	0.628	396	124	8	916	140	0.133	528	0.194	410	114	4	934	122	0.116	0.368	0.235
20	rs10814995	A/C	528	0.369	265	212	51	742	314	0.297	528	0.676	242	234	52	718	338	0.320	0.343	0.258
21	rs3087879	G/C	528	0.219	422	97	9	941	115	0.109	526	0.697	405	112	9	922	130	0.124	0.491	0.293
22	rs301432	A/T	528	0.540	327	174	27	828	228	0.216	527	0.427	308	194	25	810	244	0.231	0.421	0.390
23	rs12378107	G/C	506	0.776	396	104	6	896	116	0.115	499	0.101	385	102	12	872	126	0.126	0.345	0.423
24	rs3780411	G/C	528	0.077	131	284	113	546	510	0.483	527	0.632	135	258	134	528	526	0.499	0.213	0.460
25	rs10814991	A/G	528	0.201	152	276	100	580	476	0.451	527	0.292	162	271	94	595	459	0.435	0.760	0.480
26	rs7021409	G/A	528	0.661	267	214	47	748	308	0.292	527	0.643	274	209	44	757	297	0.282	0.884	0.616
27	rs7032326	A/G	528	0.138	213	232	83	658	398	0.377	528	0.893	212	244	72	668	388	0.367	0.581	0.653
28	rs1471786	G/A	528	0.820	152	265	111	569	487	0.461	527	0.807	156	264	107	576	478	0.454	0.939	0.724
29	rs301430	G/A	528	0.211	213	255	60	681	375	0.355	528	0.584	218	238	72	674	382	0.362	0.420	0.751
30	rs6476879	C/A	528	0.284	212	254	62	678	378	0.358	527	0.312	216	251	60	683	371	0.352	0.957	0.775
31	rs10758624	G/A	528	0.247	347	167	14	861	195	0.185	528	0.086	353	150	25	856	200	0.189	0.131	0.780
32	rs10758632	C/G	528	0.437	197	244	87	638	418	0.396	527	0.267	195	241	91	631	423	0.401	0.943	0.797
33	rs3780415	A/G	528	0.671	373	140	15	886	170	0.161	527	0.527	376	136	15	888	166	0.157	0.966	0.827
34	rs7045401	A/C	528	0.277	196	261	71	653	403	0.382	528	0.889	203	250	75	656	400	0.379	0.791	0.893
35	rs301431	C/G	528	0.678	284	209	35	777	279	0.264	527	0.380	281	213	33	775	279	0.265	0.946	0.979
36	rs972519	G/C	528	0.547	452	72	4	976	80	0.076	528	0.547	452	72	4	976	80	0.076	1.000	1.000

SZ, schizophrenia; HCs, healthy controls.

[a]*Major and minor alleles are denoted by D and d, respectively.*

[b]*Number of samples with successful genotype.*

*****p value of Bonferroni correction.*

The bold value indicates a value less than 0.05.

Some studies have also reported that SNP rs2228622 and rs7022369 in the *SLC1A1* gene were susceptibility gene sites for SZ (15, 19). Moreover, the SNP rs16921385, located in an intron of *SLC1A1*, was found to be associated with risperidone treatment response (22, 30). Therefore, *SLC1A1* variation was associated with the pathogeny of SZ. However, there were little studies to explore this association (15, 19, 23) and the mechanism of variation in *SLC1A1* still unknown.

In the present study, there were no significant association with SZ at SNPs rs10815017, rs6476875, rs7024664, and rs10974573. These SNPs are novel gene site findings that have not been reported previously. Further, there had a consistent finding in East Asian population of PGC samples. However, significant association was found at rs10974573 in European population indicated that this SNP may be a susceptibility gene site for SZ. Our study also found that rs3087879, rs301430, rs972519, rs10814991, rs7032326, rs7860087, rs3780415, rs3780413, and rs2072657 were not the susceptibility gene sites for SZ, a result that is consistent with previous reports (15, 19, 23). Meanwhile, we found SNPs rs2026828 and rs3780412 were not susceptibility markers for SZ, which is consistent with previous studies (15, 23). Previous studies reported rs2228622 (15) and rs10814995 (19) of *SLC1A1* were susceptibility markers for SZ in a Japanese population, which is inconsistent with our present finding. However, our finding for rs2228622 is in line with research in the Chinese population (23).

In current, there are only three studied (15, 19, 23) reported SNPs of *SLC1A1* in SZ. Thus, our study provides further evidences for the susceptibility of *SLC1A1* in SZ. As is well known, SZ is highly heterogeneous at genetic and symptomatic levels. Haplotypes of AA, CA, AGA, and GG were also associated with SZ and provided further evidence for the susceptibility of

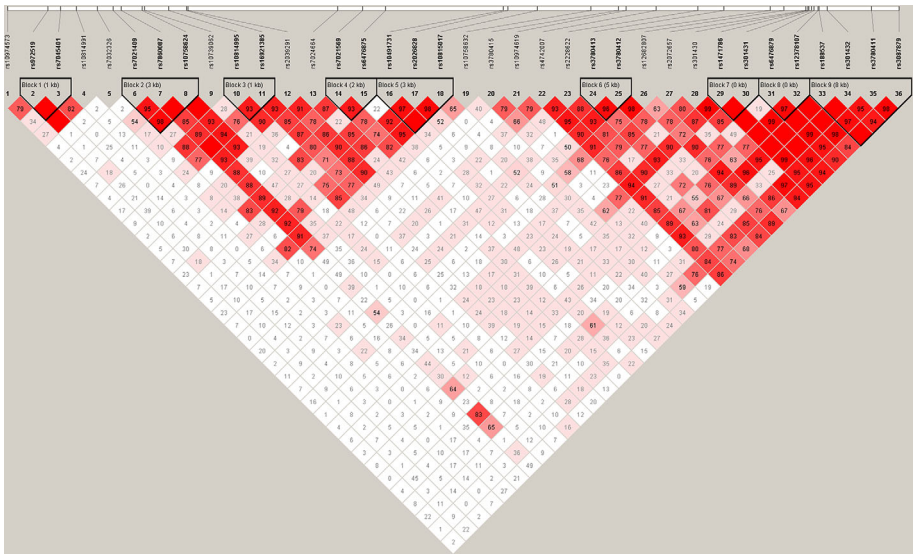

FIGURE 1 | Haplotype block structure of the *SLC1A1* gene in both SZ patients and HCs. The index association SNP is represented by a diamond. The colors of the remaining SNPs (circles) indicate LD with the index SNP based on pairwise r^2 values from our data.

TABLE 3 | Associated haplotype frequencies of 36 SNPs in the *SLC1A1* gene between SZ and healthy controls.

Block	Haplotype	Frequencies	SZ Frequencies	HC Frequencies	Chi Square	P Value
1	GA	0.544	0.545	0.543	0.017	0.896
	GC	0.380	0.379	0.382	0.018	0.893
	CA	0.076	0.076	0.076	0.001	1.000
2	GGG	0.523	0.525	0.521	0.029	0.864
	GGA	0.187	0.189	0.184	0.099	0.753
	AGG	0.163	0.171	0.154	1.217	0.270
	ACG	0.123	0.110	0.137	3.443	0.064
3	AA	0.534	0.512	0.556	3.974	**0.046**
	CA	0.306	0.317	0.294	1.361	0.243
	AG	0.157	0.168	0.147	1.681	0.195
4	CA	0.541	0.512	0.571	7.433	**0.006**
	GA	0.249	0.264	0.233	2.688	0.101
	CG	0.207	0.220	0.193	2.326	0.127
5	AAG	0.576	0.545	0.606	7.940	0.005
	CGG	0.240	0.251	0.229	1.405	0.236
	AGA	0.162	0.180	0.145	4.699	**0.030**
	AGG	0.016	0.017	0.014	0.276	0.600
6	GA	0.509	0.500	0.517	0.580	0.447
	CA	0.254	0.241	0.266	1.708	0.191
	GG	0.236	0.255	0.216	4.526	**0.033**
7	AC	0.457	0.453	0.461	0.134	0.714
	GC	0.278	0.282	0.275	0.147	0.702
	GG	0.264	0.265	0.264	0.001	0.981
8	CG	0.525	0.522	0.529	0.108	0.743
	AG	0.354	0.352	0.357	0.050	0.823
	CC	0.120	0.126	0.115	0.692	0.406
9	CAGG	0.502	0.495	0.510	0.477	0.490
	CAGG	0.502	0.495	0.510	0.477	0.490
	CTCG	0.217	0.227	0.207	1.185	0.276
	AACG	0.121	0.112	0.130	1.509	0.219
	CACC	0.112	0.120	0.104	1.241	0.265
	CACG	0.038	0.038	0.037	0.003	0.959

Block 1: rs972519- rs7045401; Block 2: rs7021409-rs7860087-rs10758624; Block 3: rs10814995-rs16921385;
Block 4: rs7021569-rs6476875; Block 5: rs10491731-rs2026828-rs1081507; Block 6: rs3780413-rs3780412;
Block 7: rs1471786-rs301431; Block 8: rs6476879-rs12378107; Block 9: rs188537-rs301432-rs3780411-rs3087879.
The bold value indicates a value less than 0.05.

TABLE 4 | Association analyses between SNPs and sub-scores of PANSS in SZ patients.

SNP	Genotype	N	PANSS Total		Positive Symptoms		Negative Symptoms		General Psychopathology	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD
rs7032326	AA	98	92.86	23.57*	26.17	6.46*	22.20	8.33*	44.48	13.34*
	AG	107	83.62	18.88	23.93	5.80	19.86	6.94	39.82	11.40
	GG	32	97.50	23.35	26.50	7.18	23.09	7.24	47.91	12.94
rs7860087	GG	188	87.74	20.15*	24.86	5.87	20.69	7.07*	42.20	11.96*
	CG	46	96.93	28.01	26.76	8.06	23.74	9.54	46.43	15.00
	CC	3	70.67	5.86	23.33	3.79	19.33	5.51	28.00	5.29
rs2039291	AA	49	90.82	22.71	26.71	6.47*	20.04	7.51	44.06	13.33
	AC	119	88.27	20.48	23.95	5.84	21.45	7.54	42.87	11.97
	CC	69	90.04	24.53	26.30	6.78	21.83	8.00	41.91	13.67
rs4742007	AA	69	86.28	21.11	23.75	6.35*	21.20	7.72	41.32	11.71
	AG	107	92.40	23.25	26.19	6.49	21.85	7.64	44.36	13.60
	GG	61	87.33	20.78	25.13	5.89	20.31	7.67	41.89	12.17
rs301430	AA	28	84.82	15.29	26.25	5.69	18.36	6.91*	40.21	7.98
	AG	106	92.20	24.02	25.33	6.66	22.52	7.76	44.35	14.09
	GG	103	87.56	21.42	24.80	6.22	20.77	7.55	42.00	12.21

*$p < 0.05$, compared with other genotypes; LSD tests, Bonferroni.

SLC1A1 in SZ. Compared with previous studies (15, 19, 23), our study has the following innovation and advantages: 1) SZ patient selection: only paranoid SZ patients were included and examined; 2) all subjects were living in the north Henan provinces of China and belonged to the same population group, this ensure the consistency of genetic background; 3) more SNP sites (36 SNPs) were tested than previous studies were including 8 SNPs (15), 19 SNPs (19), and 4 SNPs (23). Therefore, our studies were not only reduced the influence of phenotypic heterogeneity, improved the numbers of SNPs for examination.

SZ is characterized by positive symptoms, negative symptoms, disorganization of thoughts, behaviors, and cognitive deficits. Our previous studies observed the genetic basis for SZ psychopathology symptoms, such as *GRIN2B* was related to cognition deficit symptoms (12), and *CDNF2* was related to negative symptoms (25). However, there are few studies regarding the association between SZ psychopathology symptoms and *SLC1A1* (15, 19, 23). In this study, we found SNP rs7032326 was related to positive symptoms, negative symptoms, and general psychopathology. In addition, another four SNPs (rs7860087, rs2039291, rs4742007, and rs301430) were also related to clinical subtype symptoms. Although these SNPs were not the susceptibility markers for SZ, our finding also provided some evidence for the genetic basis for SZ psychopathology symptoms.

The present study also had some limitations. First, independent samples are needed to verify the finding. Second, the sample size that included PANSS scores was small and insufficient, and should be expanded to further explore the association between genotypes and psychopathology symptoms.

CONCLUSION

In conclusion, our study provides further evidence that *SLC1A1* may be not a susceptibility gene for SZ in Chinese Han population. These suggesting the variation of SLC1A1 may be not a genetic mechanism of SZ. However, the genetic variations of *SLC1A1* may affect psychopathology symptoms. Therefore, further studies need to explore other susceptibility genes of SZ.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.oebiotech.com/Article/slc1a1jyyj.html.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Second Affiliated Hospital of Xinxiang Medical University (China). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Author LL designed the study protocol. Authors TC, YY, YZ, MD, YL, and HY conducted sample selection and data management. Authors XS, ZL, LZ, QL, MLS, and XF undertook the genotyping identify and statistical analysis, and authors WL and MS wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.559210/full#supplementary-material

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Insulin Resistance and Oxidative Stress: In Relation to Cognitive Function and Psychopathology in Drug-Naïve, First-Episode Drug-Free Schizophrenia

Qi Tao[1,2,3,4†], *Yu Miao*[1,2,3†], *Huihui Li*[1,2,3], *Xiuxia Yuan*[1,2,3], *Xufeng Huang*[5], *Yunpeng Wang*[1,6], *Ole A. Andreassen*[1,7], *Xiaoduo Fan*[8], *Yongfeng Yang*[9,10,11*] *and Xueqin Song*[1,2,3*]

[1] Department of Psychiatry, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, China, [2] Biological Psychiatry International Joint Laboratory of Henan/Zhengzhou University, Zhengzhou, China, [3] Henan Psychiatric Transformation Research Key Laboratory/Zhengzhou University, Zhengzhou, China, [4] Academy of Medical Sciences/Zhengzhou University, Zhengzhou, China, [5] Illawarra Health and Medical Research Institute and University of Wollongong, Wollongong, NSW, Australia, [6] Lifespan Changes in Brain and Cognition (LCBC), Department of Psychology, University of Oslo, Oslo, Norway, [7] Norwegian Center for Mental Disorders Research (NORMENT), Institute of Clinical Medicine, University of Oslo, Oslo, Norway, [8] Psychotic Disorders Program, UMass Memorial Medical Center, University of Massachusetts Medical School, Worcester, MA, United States, [9] Henan Mental Hospital, The Second Affiliated Hospital of Xinxiang Medical University, Xinxiang, China, [10] Henan Key Lab of Biological Psychiatry of Xinxiang Medical University, Xinxiang, China, [11] International Joint Research Laboratory for Psychiatry and Neuroscience of Henan, Xinxiang, China

Objective: The present study aimed to examine whether insulin resistance and oxidative stress are associated with cognitive impairment in first-episode drug-free schizophrenia (SZ) patients.

Methods: Ninety first-episode SZ patients and 70 healthy controls were enrolled. Fasting insulin (FINS) and markers of oxidative stress [oxidized glutathione (GSSG), superoxide dismutase (SOD), nitric oxide (NO) and uric acid (UA) levels] were measured in serum before pharmacological treatment was initiated. Psychiatric symptoms and cognitive function were assessed with the Positive and Negative Syndrome Scale (PANSS) and MATRICS Consensus Cognitive Battery (MCCB), respectively. In addition, the homeostatic model assessment of insulin resistance (HOMA-IR) was also studied.

Results: HOMA-IR and serum levels of GSSG and NO were significantly higher in SZ patients than in healthy controls ($P < 0.001$), while the serum levels of SOD were significantly lower than in healthy controls ($P < 0.001$). HOMA-IR, GSSG and NO levels were significantly correlated to the total cognitive function scores of the patient group ($r = -0.345, -0.369, -0.444$, respectively, $P < 0.05$). But these factors were not co-related to the cognitive functions in the healthy control group. And, levels of SOD, UA were not associated with the total cognitive function scores in both the patient and the healthy control groups. NO was positively correlated with general pathological and the total score in the PANSS, and was negatively correlated with six cognitive domains ($r = -0.316$ to -0.553, $P < 0.05$).

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Edited by:
Xiancang Ma,
First Affiliated Hospital of Xi'an Jiaotong University, China

Reviewed by:
Manli Huang,
Zhejiang University, China
Philip D. Harvey,
University of Miami, United States

***Correspondence:**
Yongfeng Yang
yongfeng_200888@126.com
Xueqin Song
fccsongxq@zzu.edu.cn

† These authors have contributed equally to this work

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Conclusions: The levels of insulin resistance and oxidative stress are elevated, and correlated with the severity of cognitive impairment in drug-naïve, first-episode SZ patients. Treatment approaches targeting on reducing insulin resistance and oxidative stress may improve cognitive function in SZ patients.

Keywords: schizophrenia, insulin resistance, oxidative stress, cognitive impairment, psychopathology

INTRODUCTION

Schizophrenia (SZ) is a chronic severe mental illness with mainly unknown etiology, which incurs heavy burden on the persons affected, their families and the society (1). Cognitive impairment has been increasingly recognized as a core feature of SZ, and is associated with reduced social functioning, which is important for the prognosis of patients. N-methyl-D-aspartate (NMDA) receptor hypofunction has been implicated in pathophysiology of SZ (2). Previous studies have suggested that cognitive impairment is related to the hypofunction of the NMDA receptor, a type of ionic glutamate (Glu) receptor (3). Long-term potentiation (LTP) is induced through the NMDA receptor pathway to enhance learning and memory (4). When the NMDA receptor is over-activated, it causes excitatory toxicity to neural cells, leading to cell damage and death. Moreover, the proper function of the NMDA channel in the central nervous systems has been reported to be regulated by many other factors (5, 6).

Insulin resistance (IR) refers to the decreased efficiency of insulin in the promotion of glucose uptake and use. Insulin have effect on brain function, as it regulates the activity of NMDA and improves synaptic plasticity (7). When the biological efficacy of insulin decreases, the learning and memory function are reduced. Chen et al. believed that IR seems to play a role in cognitive impairment in SZ (8). Studies have found that increased IR may occur earlier in first-episode SZ patients than in healthy controls (9). Ringen (10) also proposed that IR has been associated with SZ.

Studies have found that the levels of oxidized products such as GSSG and NO are increased, and the levels of antioxidant products such as SOD are decreased in patients with schizophrenia (11), and such changes are associated with cognitive function and psychopathology (12). Studies have shown that that oxidative stress causes cognitive impairment by damaging neurons (13). The excitatory amino acid Glu and NMDA receptors are closely related to oxidative stress, which may cause cognitive impairment through the Glu-NMDAR-NO pathway (14). Glu can activate NMDA receptors (15), and produce excessive oxide and nitric oxide (NO), leading to neuronal excitotoxicity involved in the development of various neuropsychiatric diseases (16). Oxidative stress may be an intermediary mechanism of NMDA receptor dysfunction involved in the occurrence of schizophrenia. Boskovic suggested that oxidative stress may be involved in the occurrence and development of SZ (17). UA scavenges singlet oxygen and free radicals, and it can also trap peroxynitrite anions (free radicals in the ONOO –), thereby reduce the damage mediated by ONOO–. Thus, UA is an effective neuroprotective compounds (18). In addition, serum levels of UA can indicate the oxidative stress state of the body. Houlihan et al. (19) studied that UA at a high level may improve memory-related behaviors in cognitive function. However, there is no definitive conclusion on the relationship between UA and cognitive function.

Previous studies have reported an associations between IR, oxidative stress and the risk of type 2 diabetes (20, 21), but there has been little research on the associations between IR, oxidative stress and SZ. In addition, several lines of evidence showed that the antioxidant defense system may be disrupted in SZ patients, and excessive free radical production and oxidative stress damage response may be involved in the pathogenesis of SZ (22–24). Few studies have focused on the association between IR, oxidative stress and cognitive function in SZ patients.

The present study was to investigate if serum levels of biomarkers reflecting IR and oxidative stress are elevated in patients with drug-naïve, first episode SZ, and whether such biomarkers are associated with cognitive function in these patients.

MATERIALS AND METHODS

Participants

All subjects in this study were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University and provided written informed consent. Inpatients 18 and 45 years old diagnosed with first-episode SZ (disease duration <2 years) were recruited. The inclusion criteria for patients were: (1) diagnosis of first episode SZ based on the Diagnostic and Statistical Manual of Mental Disorders fourth version (DSM-IV) criteria and confirmed using the Structured Clinical Interview for DSM-IV (SCID) (25); (2) never treated with antipsychotic medications or other psychotropics; (3) the PANSS total score >60 points. Exclusion criteria included: (1) diagnose neurological or other mental illnesses, autoimmune diseases, diabetes and other organic diseases; (2) alcohol or substance abuse history; (3) pregnant or lactating women; (4) have taken any antibiotics, anti-inflammatory agents or probiotics in the past month; (5) major changes in living environment or diet in the past month. Recruitment of normal-weight healthy control subjects through advertisement, they had the same exclusion criteria as the patient group; Moreover, none of them had a history of any psychiatric diseases.

Assessments

The psychiatric symptoms of SZ were assessed in all enrolled patients using the Positive and Negative Syndrome Scale

(PANSS) (26). The PANSS was administered by a professionally trained and experienced psychiatrist. All subjects received a baseline cognitive evaluation using the MATRICS Consensus Cognitive Battery (MCCB) (27). It involves seven cognitive areas: (1) Speed of Processing Information; (2) Attention and Vigilance Awareness; (3) Working Memory; (4): Verbal Learning; (5) Visual Learning; (6) Reasoning and Problem Solving; (7) Social Cognition. The MCCB scoring program generates T-scores that are standardized and corrected for age and sex (27). The "cognitive composite" is the standardized sum of the seven domains. Training, data collection and data quality assurance were implemented or supervised by experienced psychologists in accordance with the guidelines outlined in the MCCB manual (27).

Biochemical Measurements

After all subjects were enrolled, 5 ml of venous blood was collected from the elbow under fasting condition (12 h fasting) by full-time laboratory personnel in the morning of the next day from 6:30 a.m. to 7:30 a.m. to avoid the influence of biological rhythm changes of the measured factors. Venous blood samples were collected from the elbows into EDTA anticoagulant tubes at 4°C for 10 min at 3,000 r/rain to separate the upper serum. FPG levels were measured by the glucose oxidase method and an automated analyzer (Roche Diagnostics, C8000, Germany), serum FINS levels were measured by radioimmunoassay (Elecsys 2010, Roche, Basel, Switzerland); GSSG was measured by a microenzyme method (A061-1,China); Serum SOD levels was measured using the kits and Roche automatic biochemical analyzer (Roche Diagnostics, C8000, Germany); NO levels was detected by one-step method (A013-2,China); The serum uric acid (UA)levels was measured by uricase-peroxidase method (Roche, C720, Switzerland). The current group of non-smokers had never smoked before. The homeostasis model of assessment of insulin resistance (HOMA-IR) was calculated using the following formula: HOMA-IR = FPG (mmol/L) × FINS (mu/L)/22.5 (28). Body mass index (BMI) = height/body mass ^{2} (kg/m ^{2}).

Statistical Analysis

SPSS 21.0 statistical software was used for all data analysis. All numeric variables data were expressed as mean ±SD. All categorical variables data were expressed as ratios and frequencies. Group comparison was performed using an unpaired *t*-test, and Chi-square test was used for testing independence among categorical variables. The correlations between IR, oxidative stress and cognitive function was computed by Pearson's correlation. two-sided $P < 0.05$ indicated that the difference was statistically significant.

RESULTS

Table 1 shows that there were no significant differences in age, education, gender, smoking status, and BMI between the SZ patients and healthy control groups ($P > 0.05$). In the seven domains of cognitive function, the scores of the SZ patient group were significantly lower than those of the healthy control group ($P < 0.05$, **Table 1**).

Table 2 shows that FPG levels in SZ patients showed an increased trend but fell short of statistical significance ($t = 1.448$, $P = 0.150$); However, FINS, HOMA-IR, GSSG, and NO levels were higher in the SZ patients than in the healthy controls ($P < 0.001$). On the other hand, the serum SOD levels were lower in the SZ patients than in the healthy controls ($t = -3.703$, $P < 0.001$).

Table 3 shows that HOMA-IR, GSSG and NO were significantly correlated to the total cognitive function scores of the patient group ($r = -0.345$, -0.369, -0.444, respectively, $P < 0.05$, **Table 3**). In a multiple regressing model including HOMA-IR, GSSG, NO as predictors, and the total MCCB score as the dependent variable, we found that only HOMA-IR had an effect on the MCCB composite score ($t = -2.321$, $P < 0.05$).

Table 4 shows that within the patient groups, the NO levels were positively correlated with the general pathological score and total score in the PANSS assessment ($r = 0.323$,0.375, respectively, $P < 0.05$, **Table 4**). After controlling for age, gender, disease duration and smoking status, we found that these biological indicators (FINS, HOMA-IR, GSSG, NO, SOD, UA) showed no correlation with the scores of PANSS (positive symptom score, negative symptom score, general pathology score, and PANSS total score) ($P > 0.001$).

DISCUSSION

Previous studies have reported associations between IR, oxidative stress and the risk of type 2 diabetes (20), but there has been little research on the associations between IR, oxidative stress and SZ. In addition, few studies have focused on the association between IR, oxidative stress and cognitive function in SZ patients. Our study found that serum marker levels for insulin resistance and oxidative stress were increased compared to healthy controls in first-episode untreated SZ patients, and that the levels were associated with cognitive function impairment. We also found correlations between several indicators of oxidative stress and clinical symptoms in the SZ patient group. To our knowledge, the present study is the first to show associations between cognitive performance and markers of IR and oxidative stress in first-episode untreated SZ patients.

Serum FINS and HOMA-IR were increased in first-episode SZ patients, consistent with the previous study by Spelman (9). Other published studies have shown that the development of IR may be caused by decreased insulin tyrosine kinase receptor activity, abnormal insulin signaling, decreased glucose transportation, decreased glucose phosphorylation and decreased glycogen synthase activity (29). As a result of IR, the activity of the cholinergic system in the hippocampus and other brain regions is significantly decreased, which can lead to neuronal degeneration and aggravated cognitive impairment. Our results showed that FINS levels and HOMA-IR were positively correlated with the severity of cognitive impairment in SZ patients. This effect may be related to the following aspects:

TABLE 1 | Demographic and clinical characteristics of the study sample.

Characteristics	Patients	Healthy controls	Group comparison	
	***N* = 90**	***N* = 70**		
	Mean (SD)	**Mean (SD)**	***t*/χ2**	***p***
Age (years)	21.5 ± 7.7	23.4 ± 5.4	−1.592	0.114
Education (years)	10.4 ± 2.6	11.1 ± 2.4	−1.63	0.106
BMI (kg/m^2)	21.5 ± 2.2	21.1 ± 2.4	1.052	0.295
Disease duration (months)	5.9 ± 6.3			
	N (%)	*N* (%)		
Gender			−0.397	0.692
Male	44 (48.9)	32 (45.7)		
Female	46 (51.1)	38 (54.3)		
Smoking status			1.056	0.293
Yes	5 (0.06)	7 (0.10)		
No	85 (0.94)	63 (0.90)		
PANSS-total	84.2 ± 12.7			
PANSS-positive	23.2 ± 4.8			
PANSS-negative	22.4 ± 5.9			
PANSS-general	38.5 ± 7.8			
MCCB composite score	28.6 ± 16.5	45.1 ± 19.4	−5.790	0.000
SOP	30.6 ± 9.4	51.7 ± 10.0	−12,334	0.000
CPT-IP	32.5 ± 10.9	50.7 ± 9.2	−10.201	0.000
WMS-III	38.8 ± 9.4	55.6 ± 10.6	−9.632	0.000
HVLT	37.6 ± 7.6	50.7 ± 10.0	−8.465	0.000
BVMT	39.8 ± 10.1	57.2 ± 12.1	−8.925	0.000
NAB	40.1 ± 9.8	46.1 ± 10.5	−3.351	0.001
MSCEIT	39.0 ± 11.6	56.3 ± 15.1	−7.375	0.000

PANSS-positive, Positive Symptom; PANSS-negative, Negative Symptom; PANSS-general, General Pathological Score; PANSS-total, PANSS Total Score.

TABLE 2 | Comparison of insulin resistance and oxidative stress measures between the two groups.

Items	Patient group (*N* = 90)	Healthy control group (*N* = 70)	*t*	*P*
BIOCHEMICAL MEASURES				
FPG (mmol/L)	4.4 ± 0.5	4.3 ± 0.7	1.448	0.150
FINS (mmol/L)	11.1 ± 3.3	6.1 ± 1.1	10.338	0.000
HOMA-IR (mU/L)	2.2 ± 0.7	1.2 ± 0.3	10.790	0.000
GSSG	30.9 ± 7.6	16.8 ± 5.8	11.667	0.000
NO	103.9 ± 54.5	32.2 ± 33.6	8.839	0.000
SOD	198.6 ± 43.1	231.7 ± 29.1	−3.703	0.000
UA	292.2 ± 87.5	273.6 ± 60.1	1.303	0.195

FPG, fasting plasma glucose; FINS, fasting insulin; HOMA-IR, insulin resistance index; GSSG, oxidized glutathione; NO, nitric oxide; SOD, superoxide dismutase; UA, uric acid.

(1) IR may be accompanied by insulin-like growth factor-1 (IGF-1) resistance, which competitively inhibits the binding of insulin to the insulin receptor (30). Other studies have reported elevated plasma FINS levels and decreased IGF-1 in SZ patients (31). (2) High FINS levels can disturb the insulin signaling pathway and decrease metalloproteinase (IDE) levels, leading to intracellular and extracellular Amyloid- β deposition, and causing neuronal degeneration. (3) IR can promote neuronal inflammation by enhancing the release of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, and TNF-α. (4) Long-term IR can increase the content of highly active oxidative groups and decrease the activity of antioxidant enzymes, causing neuronal cell apoptosis (32).

In the present study, we found that the levels of oxidized products (GSSG, and NO) were significantly increased in SZ patients, while SOD levels were significantly decreased, and that these levels were correlated to some cognitive function domains. Numerous studies have revealed an imbalance between the oxidative and antioxidant systems in SZ patients (33). Evidenced

TABLE 3 | Insulin resistance, oxidative stress, and cognitive function.

Indicators	HOMA-IR		GSSG		NO		SOD		UA	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
MCCB composite score (Patient group)	−0.345	0.001	−0.369	0.003	−0.444	0.000	0.200	0.143	−0.113	0.350
MCCB composite score (Healthy control group)	0.020	0.891	−0.153	0.230	0.001	0.997	0.002	0.992	0.118	0.412

TABLE 4 | Correlation of FINS, HOMA-IR, GSSG, NO, SOD, and UA values with PANSS in patient group ($N = 90$).

Indicators	FINS		HOMA-IR		GSSG		NO		SOD		UA	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
PANSS-positive	0.041	0.763	0.146	0.283	0.119	0.401	0.159	0.260	−0.081	0.591	−0.191	0.176
PANSS-negative	0.012	0.930	0.020	0.886	−0.082	0.562	0.213	0.130	−0.125	0.409	0.012	0.931
PANSS-general	0.078	0.568	0.013	0.924	0.011	0.936	0.323	0.020	0.024	0.874	−0.142	0.317
PANSS-total	0.065	0.636	0.059	0.663	0.016	0.908	0.375	0.006	−0.083	0.585	−0.138	0.331

by increased GSSG and NO levels accompanied by decreased SOD levels (34), and such an imbalance may correlate with impaired cognitive functions (12). NO, as a signaling molecule, may be involved in the impairment of cognitive function in SZ patients through the following mechanisms: (1) activation of the hypothalamus- pituitary-adrenal axis, secretion of prolactin, and corticosteroids, and elevation of hormones levels and thus activating the dopaminergic neurons in the hypothalamus, causing positive symptoms and cognitive impairment (35); (2) NMDA receptor activation, which is involved in the release of Glu and dopamine, and causes cognitive deficits and mental disorders (36); and (3) reduction in nitrite ions and superoxide anions, which react to the formation of peroxynitrite anions, leading to neurotoxic effects.

Previous studies have suggested that the antioxidant defense system is disrupted in SZ patients, and excessive free radical production and oxidative stress damage response may be involved in the pathogenesis of SZ (22, 23). SOD and UA are both effective indicators of antioxidant capacity, and SOD as an enzyme can effectively scavenge oxygen free radicals in the body, representing an important component of the free radical defense system. Our results showed that SOD levels were significantly decreased in SZ patients compared with healthy control subjects, which was consistent with the study by Reyazuddin (37), indicating that there may be a high level of oxidative stress in SZ patients. Other studies noted that the presence of the oxidative stress indicators SOD and NO in first-episode SZ patients is associated with cognitive impairment, consistent with the results of the present study. Our study did not find a correlation between UA levels and cognitive function impairment in SZ patients. whether SZ patients have a lower level of UA remains controversial (38–40).

Hyperglycemia, which mediates oxidative stress by directly or indirectly activating the diacylglycerol (DAG) -PKC pathway, is the main source of oxidative stress (41). Studies have shown that the PKC-D subtype is a potential activator of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and the activation of NADPH oxidase can increase the production of reactive oxygen species(ROS) (42). Normally, insulin receptor substrates (IRS-1 and IRS-2) are mainly distributed in low density microsomes (LDMs). However, but upon activating these receptors, phosphatidylinositol 3-kinase (P13K) is recruited to LDMs (43). Oxidative stress can interfere with P13K migration from the cytoplasm to LDMs, thereby inducing IR. Thus, oxidative stress reduces the recruitment of P13K by interfering with the phosphorylation of insulin receptor substrates, thereby inducing IR. On the other hand, High levels of free fatty acids lead to an increase in ROS and reactive nitrogen species (RNS), thereby activating the oxidative stress mechanisms that damage DNA, proteins and lipids. Additionally, high levels of free fatty acids can activate a range of intracellular signaling pathways that are closely related to IR and other cellular functions. The dysregulation of free fatty acids leads to neurodegenerative disease by promoting the phosphorylation of tau in the hippocampus (44). Insulin inhibits the activity of glycogen synthesis kinase-3β (GSK-3β), which phosphorylates tau, pyruvate dehydrogenase, and glycogen synthase. GSK-3β activation under insulin deficiency or IR promotes the phosphorylation of tau and the inactivation of pyruvate dehydrogenase and glycogen, affecting energy metabolism and acetylcholine synthesis. Thus, the interplay between IR and oxidative stress factors can synergistically impair cognitive functions in the SZ patients (45).

This study has several advantages. Our results were based on a relatively large number of drug-naïve SZ patients. The status of being drug-naïve and first episodes are important in removing the impact of medications on the associations between IR and oxidative stress and cognitive impairment in SZ patients. However, the present study also has limitations. Our results were based on analysis of cross-sectional samples. Future

follow-up studies that have measurements at multiple time points may strengthen our conclusions. In addition, unmeasured confounders in the present study may have some impact on our results thus future replication studies are warranted.

In summary, in the present work, we report abnormal IR and oxidative stress factors in first-episode untreated SZ patients, and show how they are correlated to on cognitive function. There was an association between IR and oxidative stress in the patients. The current findings need to be replicate, but suggest that IR may be a peripheral biological marker of cognitive dysfunction development in SZ patients, and could play a role in the pathological disease processes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study will not be made publicly available this data is confidential.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XS and YY contributed the conception and the design of the study. YM and HL were in charge of conducting clinical assessment and collecting fasting blood samples. QT, YM, and XY undertook the statistical analysis. QT, YM, and YY wrote the draft of the paper. YM revised the paper. XH, YW, OA, and XF were responsible for supervision and reviewing. All authors approved the submitted version.

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Genetic Determinants of Gating Functions: Do We Get Closer to Understanding Schizophrenia Etiopathogenesis?

Rastislav Rovný[1], *Dominika Besterciová*[1] *and Igor Riečanský*[1,2]*

[1] *Department of Behavioural Neuroscience, Institute of Normal and Pathological Physiology, Centre of Experimental Medicine, Slovak Academy of Sciences, Bratislava, Slovakia,* [2] *Social, Cognitive and Affective Neuroscience Unit, Department of Cognition, Emotion, and Methods in Psychology, Faculty of Psychology, University of Vienna, Vienna, Austria*

Deficits in the gating of sensory stimuli, i.e., the ability to suppress the processing of irrelevant sensory input, are considered to play an important role in the pathogenesis of several neuropsychiatric disorders, in particular schizophrenia. Gating is disrupted both in schizophrenia patients and their unaffected relatives, suggesting that gating deficit may represent a biomarker associated with a genetic liability to the disorder. To assess the strength of the evidence for the etiopathogenetic links between genetic variation, gating efficiency, and schizophrenia, we carried out a systematic review of human genetic association studies of sensory gating (suppression of the P50 component of the auditory event-related brain potential) and sensorimotor gating (prepulse inhibition of the acoustic startle response). Sixty-three full-text articles met the eligibility criteria for inclusion in the review. In total, 117 genetic variants were reported to be associated with gating functions: 33 variants for sensory gating, 80 variants for sensorimotor gating, and four variants for both sensory and sensorimotor gating. However, only five of these associations (four for prepulse inhibition—CHRNA3 rs1317286, COMT rs4680, HTR2A rs6311, and TCF4 rs9960767, and one for P50 suppression—CHRNA7 rs67158670) were consistently replicated in independent samples. Although these variants and genes were all implicated in schizophrenia in research studies, only two polymorphisms (*HTR2A* rs6311 and *TCF4* rs9960767) were also reported to be associated with schizophrenia at a meta-analytic or genome-wide level of evidence. Thus, although gating is widely considered as an important endophenotype of schizophrenia, these findings demonstrate that evidence for a common genetic etiology of impaired gating functions and schizophrenia is yet unsatisfactory, warranting further studies in this field.

Keywords: schizophrenia, endophenotypes, intermediate phenotype, prepulse inhibition, P50, sensory gating, sensorimotor gating, startle reflex

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Xiancang Ma,
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Alex Hatzimanolis,
National and Kapodistrian University of Athens, Greece

***Correspondence:**
Igor Riečanský
igor.riecansky@univie.ac.at

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INTRODUCTION

Sensory and sensorimotor gating are conceptualized as basic cognitive processes that regulate the processing of sensory input by the brain. It has been suggested that gating represents a filtering mechanism, preventing distraction and sensory overload, or a protective mechanism, securing uninterrupted processing of stimuli (1–4). Importantly, it has been

further postulated that disrupted gating may contribute to information processing deficits, cognitive fragmentation, and thought disorder, the hallmark feature of schizophrenia psychosis (5–8).

Sensory gating is routinely examined by measuring the electroencephalographic event-related potentials (ERPs) during a paired-pulse paradigm (7). The paradigm comprises trials with two identical auditory stimuli of the same intensity, a conditioning stimulus (S1) and a testing stimulus (S2), that are presented successively with an interstimulus interval of 500 ms (9, 10). Auditory stimuli elicit an ERP, which is characterized by a positive peak ∼40–90 ms after stimulus onset, known as P50 wave. It has been suggested that response to S1 triggers an inhibitory mechanism that results in a reduced amplitude of the P50 wave after the presentation of S2. The diminution of the P50 wave to S2 relative to that elicited by S1, called P50 suppression or P50 gating, is the operational definition of sensory gating (7, 10, 11). Other well-established, but less commonly assessed, measures of sensory gating include the suppression of the N100 and P200 ERP waves (12, 13). The most widely used measure of sensorimotor gating, on the other hand, is prepulse inhibition (PPI) of the acoustic startle reflex. During the PPI paradigm, the presentation of a sudden and intense auditory startling stimulus (pulse) is preceded (usually 30–120 ms) by a weaker non-startling stimulus (prepulse). This leads to a reduction in the startle reflex also known as PPI. In humans, PPI is commonly quantified by measuring the eye-blink component of the startle reflex using electromyography of the periocular muscles (14–16).

Both PPI and P50 gating are robustly reduced in schizophrenia spectrum disorders [e.g., (17–20)], but also several other psychiatric conditions, in particular, bipolar disorder and obsessive–compulsive disorder [e.g., (21–28), for review see e.g., (29, 30)]. Deficits in PPI and P50 gating were reported not only in psychiatric patients but also in their unaffected first-degree relatives [(19, 31–33), for a recent review of PPI studies see ref. (34)]. Several studies have demonstrated a significant heritability of these measures, ranging 29–58% for PPI and 10–68% for P50 gating (31, 35–42). Given these attributes, including a high test–retest reliability, PPI and P50 suppression deficits are considered as important endophenotypes of neuropsychiatric disorders (20, 37), i.e., intermediate phenotypes (or markers) that are associated with disorders but are simpler in terms of the genetic and neurobiological architecture (43–45). Endophenotypes represent an important approach to deal with the complexity and polygenic nature of mental disorders such as schizophrenia. It is supposed that studying the genetic architecture of endophenotypes and their relationship with biological processes impaired in neuropsychiatric disorders may contribute to a better understanding of the underlying pathophysiology [e.g., (46)]. The genetic basis of gating in humans has been intensively studied over the last decades, and it has become apparent that a significant genetic component is involved in both PPI and P50 suppression. Despite an extensive and rapidly growing body of literature on the relationship between genotype and gating in humans, the underlying genetic architecture of these endophenotypes remains elusive due to fragmentary evidence and lack of verification. Recently, Quednow et al. (47) carried out a systematic review (and a meta-analysis) of human association studies of PPI (sensorimotor gating). However, a similar assessment of sensory gating studies is lacking, as is an integrative review of genetic determinants of both sensory and sensorimotor gating functions. The aim of this work was thus to evaluate current knowledge regarding the etiopathogenetic links between genetic variation, gating efficiency, and schizophrenia. For this purpose, we carried out a systematic review of published genetic association studies assessing the relationship between genetic variation and the efficiency of sensory and sensorimotor gating in humans. Furthermore, we critically assessed the reliability of these findings by examining the quality of the studies, the number of replications, and the relative number of positive and negative results. Finally, we evaluated the evidence for genetic mechanisms shared between gating and schizophrenia.

MATERIALS AND METHODS

Study Design

The review process followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (48). The Covidence online software (Covidence systematic review software, Veritas Health Innovation, Melbourne, Australia; available at www.covidence.org) was used to facilitate the process of screening, paper selection, and data extraction.

Search Strategy

To identify eligible studies, we performed a systematic, comprehensive search of the published literature using Pubmed and Scopus until October 2019. The electronic databases were searched using the combination of Boolean operators and the following key words: sensorimotor gating, sensory gating, prepulse inhibition, P50, startle, polymorphism, gene and human, among others (for exact search phrases utilized, see **Supplementary Data 1**). Also, a secondary search of relevant articles was performed by screening the references of included full-text papers.

Inclusion Criteria and Study Selection

To be included in the review a study had to meet the following inclusion criteria: (1) study design of a candidate gene association study (CGAS), genome-wide association study (GWAS), or included these studies as a part of more complex study design (e.g., pharmacogenetic study), (2) study enrolled human subjects (healthy participants or psychiatric patients), (3) study outcomes included sensorimotor gating as indexed by PPI or sensory gating indices such as P50, N100, or P200 suppression, and (4) the report was written in English. Only original research papers were included; other article types, such as reviews, meta-analyses, case reports, editorials, and commentaries, were excluded.

The search results were imported to the Covidence, and after removing duplicates, titles and abstracts of identified studies were independently screened by the first and the second author (RR and DB). At this stage, only irrelevant studies that obviously did not meet the inclusion criteria were excluded from the review. Next, for the remaining potentially eligible papers, the same two authors independently assessed full texts to select only those

articles that meet all of the abovementioned inclusion criteria. Any disagreement between the two reviewers was discussed and resolved by consensus. If needed, the third author (IR) was involved to reach a decision.

Data Extraction

The following information was extracted from the selected studies: name of the first author, affiliation of the first author, year of publication, country, study design, sample characteristics (sample size, mean age, sex ratio, race/ethnicity, inclusion/exclusion criteria and population—healthy vs. psychiatric patients), parameters of the auditory stimulation (duration and intensity of acoustic stimuli, background sound intensity, and sound frequency), electrode placement, statistical test used, description of assessed polymorphisms (polymorphism type, reference number/label, chromosomal position, closest gene, reference and minor allele frequency, functional consequence, and association with disorders), and study outcomes of interest (genotype effects on PPI and sensory gating across all samples including *p*-value, effect size, direction of the effect, mean values, and standard deviations). The data extraction was carried out by DB and RR, working independently and in duplicate, using the Covidence data extraction tool. All data extraction forms from both reviewers were inspected for potential errors and compiled by RR.

Quality Assessment

The quality of genetic studies (Q-Genie) 11-item tool was used to evaluate the quality of all included studies. We opted for this tool since it was specifically developed and validated to facilitate the assessment of the global quality of genetic association studies, and it proved to be valid and reliable for both expert and non-expert raters (49). The quality assessment was conducted by DB and RR, working independently and in duplicate. The final quality score for each study was calculated by averaging the respective scores from the two reviewers. Following the Q-Genie scoring system, scores below 33 indicate poor-quality studies, scores between 33 and 40 indicate studies of moderate quality, and scores above 40 indicate good-quality studies. The degree of interobserver agreement was tested using the Cohen's kappa coefficient (κ), with values categorized as poor ($\leq$0.20), fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80), and almost perfect agreement (>0.80) (50). The Cohen's kappa coefficient was computed by using the FREQ procedure in SAS Studio software (SAS University Edition, release 3.8, SAS Institute, Cary, NC, USA).

RESULTS

Identification of Relevant Studies

The systematic search yielded 1,820 potentially relevant references. After removing 369 duplicates and 1,326 irrelevant articles identified by screening abstracts, the full texts of the remaining 125 papers were assessed for eligibility. Of them, excluded were 39 studies that did not meet the inclusion criteria and 16 duplicates not captured by Covidence, leaving 70 papers. Based on the Q-Genie scoring system, 41 out of the 70 relevant studies (58.6%) were rated high quality, 22 (31.4%) moderate, and 7 (10.0%) poor. A weighted kappa value of 0.55, 95% CI (0.50–0.60) indicates a moderate agreement between the two raters (DB and RR). Poor-quality studies were excluded from the systematic review due to concerns about the validity of results, leaving 63 eligible papers. No additional papers were identified by screening the references of the included articles. The process of study selection is depicted in the PRISMA flow diagram below (**Figure 1**).

Basic Description of the Included Studies

The final selection included 53 CGAS, 3 GWAS, and seven pharmacogenetic studies (25, 52–113). These studies investigated in total 63 independent sample groups: 36 samples of healthy individuals, 20 patient samples (16 with schizophrenia), and seven samples involving both patients and healthy individuals. Sensorimotor gating (PPI) was assessed in 41 studies, sensory gating (P50 or N100 suppression, for simplicity thereafter referred to as P50 gating) in 18 studies, and four studies assessed both measures. A short summary of the basic characteristics of the studies is provided in **Table 1**; details for each study included in this review are provided in **Supplementary Table 1**.

Identification and Description of Genetic Polymorphisms

Data extraction from the eligible studies resulted in the identification of 201 polymorphisms located within or close to 77 genes. Association with PPI was tested for 125 polymorphisms. Among them, 84 variants, within or close to 37 genes, were reported as significantly ($p < 0.05$) associated with PPI in at least one sample. Association with P50 gating was investigated for 109 polymorphisms, of which 37, located within or close to 13 genes, were significantly associated with this measure in at least one sample. Association with both PPI and P50 gating was investigated in 54 variants and a significant association with both measures was reported for four polymorphisms (*COMT* rs4680, rs165599, *ANKK1* rs1800497, and *TCF4* rs9960767). A vast majority of the variants were single nucleotide polymorphisms (SNPs, **Table 2**; for a detailed summary see **Supplementary Tables 2–5**).

To provide insight into the involved biological mechanism, we conducted an enrichment analysis using the Gene Ontology Resource (114–116). The associated variants were annotated by dbSNP and clustered based on the overrepresentation of the corresponding genes in the Gene Ontology classification section Biological Processes. The results of this analysis are provided in **Table 3**. Associations were considered as consistent (reliable) if a significant association with PPI or P50 gating was reported in at least two independent samples, and the number of reported significant associations was higher than the number of null findings. For both PPI and P50 gating, the reported positive associations included several genes involved in neurodevelopmental processes and/or cellular signaling (in particular glutamatergic, dopaminergic, serotoninergic, and cholinergic neurotransmission). However, most of the polymorphisms for which positive associations were reported were explored in only one published study (PPI:

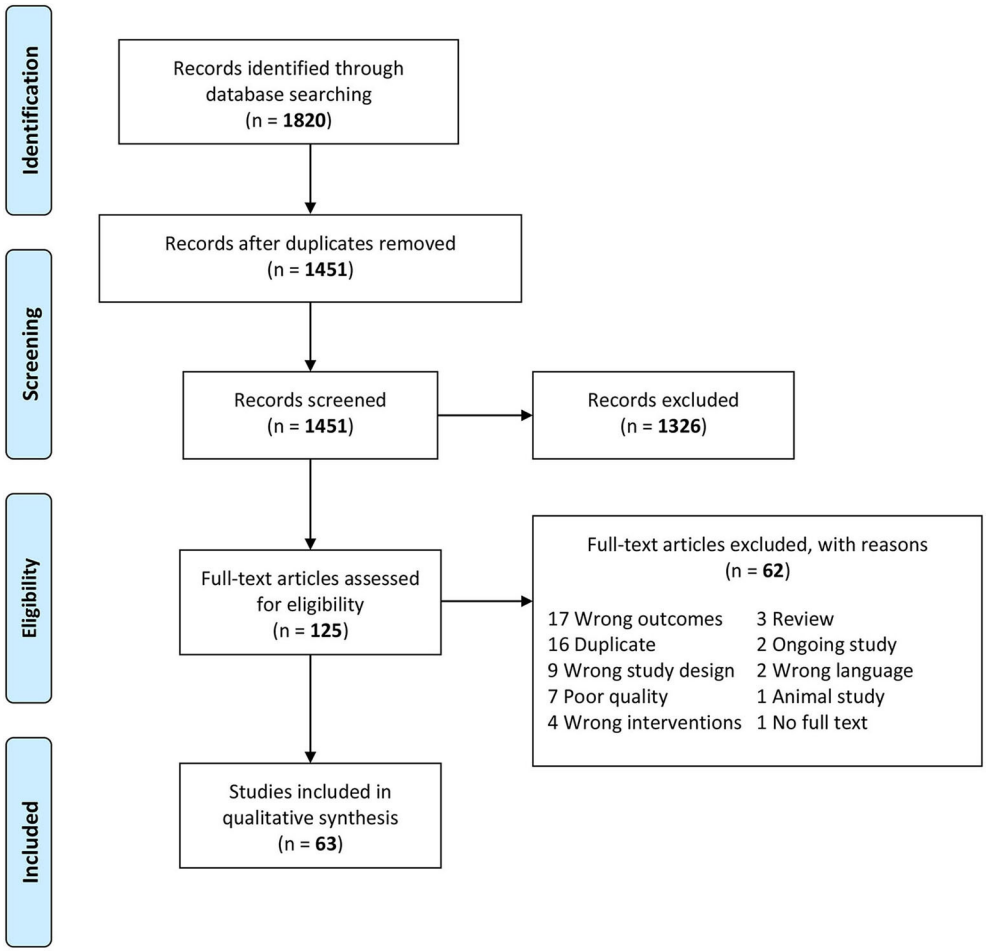

FIGURE 1 | The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram of the study selection process (51).

TABLE 1 | Overview of the basic characteristics of the studies included in the review (25, 52–113).

Study design	No. of studies	No. of samples				Sample size			Gating measure		
		Total	Healthy	Patients (SZ)	Mixed	Mean	SD	Range	PPI	P50	Both
CGAS	53	53	28	20 (16)	5	153	244	23–1,821	34	15	4
GWAS	3	4	2	0	2	719	385	306–1,212	2	1	0
Pharmacogenetic	7	6	6	0	0	57	41	23–114	5	2	0
Total	63	63	36	20 (16)	7				41	18	4

CGAS, candidate gene association study; GWAS, genome-wide association study; SZ, patients with schizophrenia; PPI, prepulse inhibition of the acoustic startle reflex; P50, suppression of wave P50 or N100 of the auditory evoked potential.

TABLE 2 | Overview of the reported associations with gating measures.

Gating measure	No. of investigated variants[a]	Reported associations		Positive associations[c]		
		Sig.[b]	Nonsig.	SNP	NonSNP	Genes
PPI	125	84 (3)	100	65	19	37
P50	109	37 (9)	85	30	7	13

[a]*Includes only variants investigated in candidate gene association studies and pharmacogenetic studies.* [b]*Associations reported as significant ($p < 0.05$), number in parentheses: no. of significant associations reported in genome-wide association studies.* [c]*No. of variants/genes positively associated with gating function in at least one study. PPI, prepulse inhibition of the acoustic startle reflex; P50, suppression of wave P50 or N100 of the auditory evoked potential; SNP, single nucleotide polymorphisms; non-SNP, includes copy number variants, combined genotype, haplotypes, genetic interaction, indels, short tandem repeats.*

TABLE 3 | Summary of genes and genetic variants associated with gating functions.

Gene ontology category (Section biological processes)	Genes with positive associations	Reliable associations[a]
Nervous system development	PPI: *AUTS2*, *AVPR1A*, *CTNNA2*, *ERBB4*, *KCNQ2*, *NCAM1*, *NGF*, *NOS1*, *NRG1*, *OXTR*, *RELN*, *TCF4*, *TSPAN2* P50: *DISC1*, *ERBB4*, *FLRT2*, *TCF4*	PPI: *TCF4* rs9960767
Synaptic transmission, glutamatergic	PPI: *GRID2*, *GRIK3*, *GRIN2A*, *GRIN3A*, *GRIN3B* P50: *GRID2*, *GRIK4*	
Synaptic transmission, cholinergic	PPI: *CHRNA3*, *CHRNA4*, *CHRNA7* P50: *CHRNA7*[c], *CHRFAM7A*	PPI: *CHRNA3* rs1317286 P50: *CHRFAM7A* rs67158670
GPCR signaling pathway, coupled to cyclic nucleotide second messenger	PPI: DRD2, *DRD3*, *HTR1A*[c], *HTR2A* P50: *GRM3*	PPI: *HTR2A* rs6311
Regulation of calcium ion transport	PPI: *CAMK2A*, *FMR1*, *NOS1AP* P50: *CACNAC1*	
Neurotransmitter reuptake	PPI: *SLC1A2*, *SLC6A3* P50: *SLC6A3*	
Dopamine metabolic process	PPI: *COMT*, *DAO*, *DBH* P50: *COMT*	PPI: *COMT* rs4680
Serotonin metabolic process	PPI: *TPH2*[b]	
Proline metabolic process	PPI: *PRODH*	
Unclassified	PPI: *ANKK1, KPNA4* P50: *ANKK1*	

[a]*Criteria of reliability: significant association was reported in at least two independent samples and the number of reported significant associations was higher than number of reported null results.* [b]*A significant association only at the level of haplotype, not single polymorphism.* [c]*A significant association only at the level of combined genotype of two or more variants. GPCR, G protein-coupled receptor; for details of the reported associations, see* ***Supplementary Tables 2–5****.*

64.3%, P50: 81.1%). Applying our criterion of reliability, only four associations with PPI (*CHRNA3* rs1317286, *COMT* rs4680, *HTR2A* rs6311, and *TCF4* rs9960767) and one with P50 gating (*CHRNA7* rs67158670) can be considered as consistent.

Among the polymorphisms positively associated with PPI, 22 (26.2%) are functional variants, i.e., related to the level of gene expression or the biological function of the protein products (as reported in the reviewed studies). For the remaining 62 (73.8%) variants, no direct functional consequences were reported. For P50 gating, 4 (10.8%) polymorphisms positively associated with this measure are functional and 33 (89.2%) are without known functional consequences. To examine the potential functional role of the positively associated variants, we carried out an *in silico* analysis using the HaploReg resource (117). The results of this analysis showed that a substantial proportion of polymorphisms that were associated with PPI (41 SNPs) and P50 gating (14 SNPs) overlap with regulatory motifs such as promoter/enhancer histone marks or DNase I hypersensitive sites (for a detailed description see **Supplementary Tables 2–5**).

DISCUSSION

In this paper, we reviewed the available data on the relationship between genetic variability and sensory information filtering in humans. More specifically, we summarized, in a systematic manner, findings from genetic association studies published in peer-reviewed journals, examining the effect of common genetic variants on two well-established parameters of gating functions, PPI and P50/N100 ERP suppression (jointly referred to as P50 gating), deficits of which are considered as schizophrenia endophenotypes. We found that association with PPI or P50 gating was reported for variants located within or near 37

and 13 genes, respectively, which are involved in a variety of biological processes, mostly related to neurotransmission and neurodevelopment. However, most of the polymorphisms positively associated with PPI and/or P50 gating were examined in only one study or were not consistently replicated in other studies. According to our criteria for reliability (i.e., association confirmed in at least two independent samples and positive outcomes outnumbering negative results), only four polymorphisms within four genes for PPI (*CHRNA3* rs1317286, *COMT* rs4680, *HTR2A* rs6311, and *TCF4* rs9960767) and one polymorphism for P50 gating (*CHRNA7* rs67158670) can be considered as reliable or consistent across the studies. Two of them (*COMT* rs4680 and *TCF4* rs9960767) were identified as significantly associated with PPI also by Quednow et al. (47), who included 16 independent samples into a meta-analysis. Although a large number of reported associations were with non-coding polymorphisms, our analysis shows that a substantial proportion of them may play a role in gene expression by affecting the binding of transcription factors or chromatin remodeling. However, since enhancers may activate transcription of their target genes over considerable distances, up to hundreds or even thousands of kilobases, caution should be taken when making inferences about the functional connection between non-coding variants in these regions and target genes (118).

Replication of Association Results

From the 35 associations that were tested in more than one study, only 10 polymorphisms were reported to be significantly associated with gating in two or more studies. Low statistical power in some studies could increase the probability of false-negative results and unsuccessful replications. Although we excluded studies whose quality was evaluated as poor according to the Q-Genie scoring system, yet in 12 of 63 studies that fulfilled the criteria to be included in this review, sample size was lower than 50. Furthermore, a considerable number of negative replication results (14 of 30) come from samples that differed in ethnicity compared to the initial studies reporting positive results. Notably, in addition to genetic diversity, difference in startle response and PPI across ethnic groups (119) could decrease the number of successful replications. On the other hand, the non-replications seem not to be due to diversity in stimulation parameters since these did not substantially differ between almost all studies that had yielded discrepant outcomes. In the light of considerable heritability of gating functions (31, 35–42), the low number of reliably assessed genetic associations clearly indicates that, despite the relatively large number of genetic studies, current knowledge on the genetic architecture of gating functions remains very limited. Next, we will focus our discussion on the variants/genes consistently associated with sensory and/or sensorimotor gating functions.

Catechol-O-Methyltransferase

Catechol-o-methyltransferase (COMT) is an enzyme degrading catecholamines. A single nucleotide G-A substitution at codon 158 results in a change from valine to methionine (Val158Met) causing a missense mutation with a lower metabolic activity of the enzyme. This polymorphism significantly affects dopamine turnover in the prefrontal cortex (PFC, Val allele associated with reduced PFC dopamine levels), PFC activity, and executive functions in healthy humans [for review see e.g., (120, 121)]. Numerous studies reported *COMT* Val158Met polymorphism to be related with liability to schizophrenia and several other mental disorders, but a recent meta-analysis did not confirm a significant association with schizophrenia (122). The Val allele was associated with weaker PPI in six of seven studies included in our review. In agreement with these reports, a study by Giakoumaki et al. (63) has shown that administration of a COMT inhibitor tolcapone increased PPI in Val allele carriers. As highlighted by the meta-analysis by Quednow et al. (47), the association of PPI with *COMT* Val158Met polymorphism is stronger in men than in women. Interestingly, a similar pattern of sex-dependent effects of this variation was also reported for response inhibition and linked with the activity of the PFC [(123), see also (124)]. Given the putative role of the PFC in the modulation of sensorimotor gating (125–128), it could be speculated that the prefrontal circuitry is also involved in the sex-specific effects of *COMT* genotype on PPI, which remains to be established in future studies. The evidence for the association of *COMT* Val158Met with P50 gating is less consistent, as a significant association was reported in seven and non-significant in nine studied samples.

Another *COMT* polymorphism, rs165599, has not fulfilled our reliability criteria but was reported to be significantly associated with both PPI and P50 gating (only in one study each). Functional consequences of this variation are less clear, although there is evidence indicating its relationship with COMT mRNA levels in the brain of healthy humans, and IQ and the presence of psychotic symptoms in patients with 22q11 deletion syndrome (129, 130). A large case-control study reported its association with schizophrenia in women but not in men, suggesting that this SNP confers a sex-specific genetic component in schizophrenia (131). Notably, rs165599 and rs4680 are both part of a three-marker haplotype (together with rs2075507) that has been implicated in COMT protein level, PFC function in obsessive–compulsive disorder and attention-deficit hyperactivity disorder (129, 132, 133). This haplotype was significantly associated with P50 in a sample of patients with bipolar disorder (but not in healthy controls) (25). Its relationship with PPI has not been studied yet, as far as we are informed.

To sum up, there is considerable evidence that genetic variability of *COMT* affects gating functions, which fits with the proposed role of dopamine in the PFC (134). Interestingly, however, disruption of PPI following administration of dopamine agonists in rodents has been attributed to modulation of striatal rather than cortical circuitry (135, 136). In humans, on the other hand, the effects of dopamine agonists on PPI are less evident and reliable (137). Given the importance of PPI to study the neurobiology of schizophrenia in animal models, it would be desirable in future studies to shed more light on the specific roles of dopamine in cortical and striatal processing related to gating in humans and rodents.

Serotonin 2A Receptor

The *HTR2A* gene encodes a G-protein-coupled serotonin 2A receptor (5-HT2AR). In humans, 5-HT2AR is widely expressed throughout the brain with particularly high density in the

neocortex (138). 5-HT2AR has been implicated in multiple brain functions such as learning, memory, and cognition [for review see (139)]. Importantly, several lines of evidence implicate 5-HT2AR in the pathophysiology of psychiatric disorders. First, genetic variants in the *HTR2A* gene and functional abnormalities of 5-HT2AR are associated with many psychiatric disorders including schizophrenia [for review see (140)]. Second, 5-HT2AR antagonists produce antipsychotic and antidepressant-like effects, whereas agonists have psychotomimetic properties including PPI-disruptive effects (140, 141). *HTR2A* rs6311 (also known as −1438A/G) is a functional SNP, which lies upstream of the *HTR2A* promoter region and alters its activity (142). Meta-analyses confirmed the association of this polymorphism with schizophrenia and obsessive–compulsive disorder (143–145). Given the involvement of serotonin in multiple neurobiological processes, warranted are further studies of the role of 5-HT2AR in gating and its relationship with schizophrenia.

Nicotinic Acetylcholine Receptor

A lot of research implicates signaling via nicotinic acetylcholine receptor (nAChR) in gating, schizophrenia, and nicotine dependence [for review see (146)]. It is well established in rodents and humans that the agonist of nAChR nicotine enhances PPI and P50 gating [for review see (147)]. In humans, sensorimotor gating efficiency was found to be inversely related to nicotine dependence (148). Smoking and nicotine dependence are highly prevalent in schizophrenia, and it has been proposed that tobacco is used by the patients as self-medication to alleviate the symptoms, in particular the impairment of cognitive functions (149). Moreover, recent research indicates that nicotine dependence and schizophrenia may share a part of their genetic liability [for review see (150)]. Across the reviewed studies, consistent associations were reported between PPI and variation in *CHRNA3* gene as well as between P50 gating and *CHRFAM7A* gene.

CHRNA3 is a part of a *CHRNA5–CHRNA3–CHRNB4* gene cluster on chromosome 15 (15q25 region), encoding α5, α3, and β4 subunits of the nAChR, linked in previous studies to nicotine dependence as well as schizophrenia (151–153). Our search specifically points to *CHRNA3* rs1317286, which was reported to be associated with nicotine dependence in a GWAS (154). The analysis using HaploReg indicates that this SNP overlaps with enhancer histone marks and may thus play a role in *CHRNA3* transcription. However, due to high linkage disequilibrium, it is difficult to determine causative variants in the *CHRNA5–CHRNA3–CHRNB4* cluster, which is under complex and coordinated regulatory control (155). Interestingly, TCF4 (see below) has been identified as one of the regulators of gene expression at this locus (156).

CHRFAM7A is a partial duplication of a gene encoding α7 nAChR, *CHRNA7*. Translation of CHRFAM7A is low, but it seems to negatively regulate α7 nAChR function [for review see (157)]. The P50 gating-associated polymorphism of *CHRFAM7A* denoted as rs67158670 (or *CHRFAM7AΔ2bp*) is a 2-bp deletion in exon 6. This mutation causes a frameshift in translation, resulting in a truncated protein, which is even a more potent inhibitor of α7 nAChR (157). Reduced expression of *CHRNA7* was found in the frontal cortex of schizophrenia patients post-mortem (158) and smoking counteracts this deficit (159). In addition to the association with P50 gating, studies reported association of *CHRFAM7AΔ2bp* with schizophrenia, bipolar disorder, and episodic memory [for review see (157)]. The impact of *CHRFAM7AΔ2bp* on brain development is debated, and research in this direction could bring new discoveries of the pathomechanistic links between gating deficits and schizophrenia.

Transcription Factor 4

The *TCF4* gene codes for a basic helix–loop–helix protein, transcription factor 4, which belongs to a subclass of transcriptional regulators termed E-proteins. E-proteins bind to a specific promoter element known as the Ephrussi-box (E-box) to regulate transcription of target genes in various tissues including the brain [for review see (160)]. Although the precise physiological function of TCF4 is not yet fully understood, a recent study demonstrated that binding sites for TCF4 are present in a large number of genes involved in nervous system development, ion transport, and signal transduction (156). Moreover, this study also showed that TCF4 binding sites are found in many susceptibility genes implicated in common neurodevelopmental disorders including schizophrenia and autism spectrum disorders. Notably, several SNPs in *TCF4* itself have been directly linked to schizophrenia, underscoring the possible role of this gene in schizophrenia pathogenesis (161). Our analysis points to a reliable association of *TCF4* rs9960767 with PPI. Notably, Quednow et al. (90) reported that the effect of this polymorphism on PPI is moderated by smoking behavior, which fits with the regulatory role of TCF4 on the *CHRNA5–CHRNA3–CHRNB4* cluster (156). The association of this variation with schizophrenia was confirmed at a meta-analytic and genome-wide level [for review see (162)]. *TCF4* rs9960767 is located within intron 3 of the *TCF4* gene and has no direct obvious functional consequences. Neither is there evidence of its linkage disequilibrium with other common non-synonymous polymorphisms or causal variants, which alter TCF4 mRNA expression in adult human brain (163). Williams et al. (163) suggested that rs9960767 may exert effects on *TCF4* expression in a developmental context. Our findings support this notion as the HaploReg analysis indicates that rs9960767 may affect putative binding sites of transcription factors Foxa and STAT in the brain germinal matrix, which plays a critical role during brain development. All these findings indicate that sensorimotor gating deficit is a constituent of the neurodevelopmental insult, which is assumed to play a crucial role in the pathogenesis of schizophrenia (164).

Common Genetic Factors of Gating Functions

Four polymorphisms out of 45 variants studied so far were reported to be significantly associated with both PPI and P50 gating. Given our criteria of reliability, however, none of these associations was reliable for both measures. Evidence for common genetic mechanisms underlying both sensory and sensorimotor gating thus remains elusive. Although sensory and

sensorimotor gating represent related concepts, the hallmark of which is inhibition, the relationship between PPI and P50 suppression is not fully understood. Correlation between the magnitude of PPI and P50 suppression seems weak since most studies found no significant relationship between the two measures (165–171). Furthermore, PPI primarily relies on the processing in the brainstem and the basal ganglia, which is modulated by the cerebral cortex (125–128, 135, 172–176), while the sources of P50 ERP and P50 suppression are thought to be localized predominantly in the hippocampus, the temporal and the frontal lobes (167, 177–179). Given the importance of PPI and P50 gating in psychiatry, further research is warranted to clarify the relationship between these two phenomena in more detail at both the cognitive/psychological and neurobiological levels.

CONCLUSION

Our review identified a considerable number of genetic variants associated with PPI or P50 gating in previous studies. However, a critical evaluation of the reports shows associations of only five polymorphisms (four for PPI and one for P50 gating) as consistently replicated across the studies. From these, only two variants (*HTR2A* rs6311 and *TCF4* rs9960767, both associated with PPI) also show a reliable association with schizophrenia (meta-analytic or genome-wide evidence). Although deficits in sensory and sensorimotor gating are widely considered as important endophenotypes of schizophrenia, the evidence for the common genetic etiology of the impaired gating functions and schizophrenia thus remains limited, and further large-scale studies are warranted to advance our understanding of this complex problem.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

IR provided the concept and design of the study. DB and RR collected and analyzed the data. RR, IR, and DB wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.550225/full#supplementary-material

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Roles of 5,10-Methylenetetrahydrofolate Reductase C677T Polymorphisms in First-Episode, Drug-Naive Adult Patients With Depression

Zhuoqing Li[1†], Bo He[2†], Jian Xu[3†], Nan Dai[1], Liangliang Ping[4], Cong Zhou[1], Zonglin Shen[1], Xiufeng Xu[1] and Yuqi Cheng[1]*

[1] Department of Psychiatry, First Affiliated Hospital of Kunming Medical University, Kunming, China, [2] Department of Medical Imaging, First Affiliated Hospital of Kunming Medical University, Kunming, China, [3] Department of Internal Medicine, First Affiliated Hospital of Kunming Medical University, Kunming, China, [4] Department of Psychiatry, Xianyue Hospital, Xiamen, China

5,10-Methylenetetrahydrofolate reductase (MTHFR) gene C677T polymorphism is considered as a predisposition and promising genetic candidate to major depressive disorder (MDD), as it is associated with impaired one-carbon cycles, which may be involved in the pathogenesis of depression. Cortical thickness (CT) and subcortical structure volumes have been extensively studied in MDD and have been proposed as one of the phenotypes for MDD. We intend to discuss the association between CT, subcortical structure volume, and *MTHFR* C677T polymorphism in first-episode, treatment-naive patients with MDD. In this study, 127 adult patients with MDD and 101 age- and gender-matched healthy controls (HCs) were included. All subjects underwent T1-weighted MRI, *MTHFR* C677T genotyping, and FreeSurfer software-based morphological analysis. MDD patients have been detected to have significantly decreased volumes in the left nucleus accumbens ($P < 0.001$). The *MTHFR* 677 T allele carriers manifested with thinner CT in the left caudal anterior cingulate cortex (cACC, $P = 0.009$) compared with CC genotype. There were significant genotype-by-diagnosis interactions for the CT in the left cACC ($P = 0.009$), isthmus cingulate ($P = 0.002$), medial orbitofrontal lobe ($P = 0.012$), posterior cingulate ($P = 0.030$), and the right lateral orbitofrontal lobe ($P = 0.012$). We also found a trend in the interaction effect on the volume of the left putamen ($P = 0.050$). Our results revealed that *MTHFR* C677T polymorphism may be involved in the dysfunction of limbic–cortical–striatal–pallidal–thalamic (LCSPT) circuits mediating emotion processing, which may contribute to pathogenesis of MDD.

Keywords: major depressive disorder, MTHFR C677T, cortical thickness, subcortical structure volume, anterior cingulate cortex

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Nadia Cattane, Centro San Giovanni di Dio Fatebenefratelli (IRCCS), Italy
Xinhua Shen, Third People's Hospital of Huzhou, China

***Correspondence:**
Yuqi Cheng
yuqicheng@126.com

† These authors have contributed equally to this work

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INTRODUCTION

Major depressive disorder (MDD) is a chronic persistent complex mental disorder with high morbidity. The etiology and pathogenesis of MDD are still unclear. Current evidence has indicated that MDD is mainly the outcome of the genetic and environment interactions, such as adverse experiences in childhood, especially childhood maltreatment (1). Family, adoption, and monozygotic- and dizygotic-twin studies have supported that both genetic and environmental factors and their interconnection contribute to MDD, and the heritability of MDD was about 37% (2, 3) Previous multiple meta-analyses have shown that 5,10-methylenetetrahydrofolate reductase (*MTHFR*) C677T confers a predisposition to depression (4–7).

The MTHFR enzyme mainly expresses in the brain and fetal and frontal cortex, and it is an irreversible rate-limiting enzyme in the essential methyl cycle of the body. MTHFR critically catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine (Hcy) remethylation to methionine, which then is activated as *S*-adenosyl methionine (SAM). *MTHFR* C677T has a prominent function in one-carbon metabolism (1-CM); the consequences of perturbed 1-CM include reduced remethylation of total Hcy (tHcy) into methionine, elevated Hcy levels, and a reduction in cellular methylation potential (8). Research on *MTHFR* C677T (9) has shown that MTHFR activity decreased in a T allele dose-dependent form. Defects in the MTHFR enzyme can cause methionine synthesis obstacles for Hcy. The synthesis of SAM from methionine in the organism decreases, and the methylation reaction decreases. High Hcy levels, through triggering the *N*-methyl-D-aspartate receptor (10), lead to monoamine neurotransmitter synthesis being blocked and essential amino acid levels being reduced in the brain tissue, especially neurotransmitters implicated in depression (such as serotonin, norepinephrine, glutamate, and γ-aminobutyric acid).

A meta-analysis substantiated that *MTHFR* C677T had the most prominent risk effect of depression in Asians (11); this effect is more prominent in the Chinese population (12). However, the results were inconsistent with some previous studies. For example, a meta-analysis study including only European Caucasian populations did not find that *MTHFR* C677T polymorphism was associated with recurrent depression (13), and there was a study suggesting that the C677T genotype may be a protector for MDD (14), yet most evidence from meta-analyses strongly suggested that the T allele or TT genotype tends to be a risk effect to MDD (5, 15). The inconsistent results may be due to clinical heterogeneity, ethnicity, geography, age, size of the sample, medication confounding factors, lack of statistical efficiencies, and/or the interaction of *MTHFR* C677T with other genes. Gene × gene studies deduced that by affecting the methyl donor SAM, *MTHFR* C677T exhibited a coordinated effect with some genes which may be involved in the monoamine neurotransmitter, dopaminergic pathway, and regulation of neuroplasticity, via diminished methyl (15). For instance, 5-HTT can remove 5-HT from the synaptic cleft, which in turn determines the amount and duration of postsynaptic membrane receptor-mediated signaling. *5-HTT* is often a target gene for epigenetic modification methylation, regulating the relationship between stress and depression (16, 17). *MTHFR* C677T can cause a decrease in the methyl level of the CpG island of the SLC6A4 promoter, increasing the expression and promoting the increase of 5-HTT activity and thus the reduction of synaptic 5-HT. Similarly, *MTHFR* C677T boosts the expression of the *COMT* gene and enhances the action of methylase to block the dopaminergic signaling pathway (18). Besides, the *MTHFR* C677T allele attenuates the degradation of norepinephrine and dopamine induced by high-efficiency methylases such as the COMT Val risk gene. The interaction between the *MTHFR* C677T and *COMT* gene determines the final effect of the genes, which will significantly increase the risk of depression (19) and correlate with the decrease in the volume of the putamen of elderly depression (20). On the other hand, gene × environmental studies have found that *MTHFR* C677T downregulates the expression of the *Sirtuin1* gene (*SIRT1*) with axonal growth, neurogenesis, and dendritic branching via high Hcy, hence accelerating endothelial progenitor cell aging (21). The level of DNA methyl in the promoter region of the glucocorticoid receptor gene (*NR3C1*) was observed to be reduced in MDD patients (22). Under stress, the DNA methylation of specific alleles in the glucocorticoid responsive element of the FKBP5 gene polymorphism was reduced, resulting in increased expression of the corresponding gene, glucocorticoid receptor inactivation, and glucocorticoid resistance (23). In short, *MTHFR* C677T may participate in the development of depression through epigenetic DNA methylation modifications, metabolic disorders, and neurotransmitter disturbances together with some genes and/or environments (such as stress). In addition, the MTHFR defect caused by *MTHFR* C677T can be restored to normal by available supplements (24), which is clinically significant.

Genetic imaging may be an advanced method to screen for reliable and heritable biomarkers in neuropsychiatric disorders (25). Previous studies have found that *MTHFR* C677T polymorphism with high Hcy level was associated with decreased cortical thickness (CT), subcortical gray matter (GM), and white matter volume and/or density in patients with mental illness (18, 20, 26, 27). The refinement/efficiency of the connections between prefrontal and subcortical structures could alter the emotion processing and regulation (28). Thus, we speculated that *MTHFR* C677T may affect brain connections and be associated with depression. However, little is known about the role of *MTHFR* C677T polymorphism in the MDD.

In this study, we intend to explore the potential role of *MTHFR* C677T polymorphism on the brain in first-onset adult depression, mainly in terms of the structure of brain gray matter. We used the FreeSurfer brain imaging technology to measure cerebral cortex thickness and subcortical gray matter volume to evaluate changes in brain gray matter structure.

MATERIALS AND METHODS

Participants

Two experienced attending psychiatrists selected the patients with depression that met the inclusion criteria from the

psychiatric clinic of the First Affiliated Hospital of Kunming Medical University. We collected some basic information about all subjects, such as age, gender, education level, ethnicity, dominant hand, and patients' course of disease. The inclusion criteria were as follows: (1) meeting *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition (DSM-IV) (29), diagnostic criteria for MDD, using the Structured Clinical Interview for DSM-IV Axis I disorders (SCID-1) (30) to improve its validity; (2) adults aged 18–45 years; (3) first-onset and untreated depression (without drug, physical, and standard psychological treatment); (4) a total score of $\geq$17 in the 17-item Hamilton Depression Rating Scale (HAMD-17) (31); and (5) being a Chinese Han, right handed, and a Kunming native. The exclusion criteria were as follows: (1) suffering from mental illnesses other than depression; (2) history of alcoholism, traumatic brain injury, or severe physical diseases; (3) and any contraindication for MRI. The healthy control (HC) participants were generally healthy, were aged 18–45 years, were recruited from local communities with SCID-1, and were well-matched to the patient group on gender, age, ethnicity, dextromanuality, and birthplace. All subjects underwent HAMD-17 (31), Hamilton Anxiety Scale (32) (HAMA) evaluation, *MTHFR* C677T genotyping, and T1-weighted MRI scans. Data of an abnormal brain structure visible to the naked eye or poor-quality images affected by head movement were eliminated. For detailed data, refer to **Table 1**. The study was approved by the Institutional Review Board of Kunming Medical University, Yunnan Province, China. All participants gave written informed consent.

Measurements

Treatment guidelines for depression suggest that it is important to consider severity when selecting a patient's initial treatment modality. HAMD has been the most frequently used scale to subdivide patients into severity groups and examine the treatment implications of symptom severity (31). HAMA is often used to assess anxiety symptoms and severity (32). Patients with depression are often with or without anxiety symptoms, and the two often have comorbidities. In this study, we mainly study patients with depression with or without anxiety. Thus, two professional psychologists evaluated HAMD-17 and HAMA scales on all subjects. The cutoff score on the HAMD that maximized the sum of sensitivity and specificity was 17 for the comparison of mild vs. moderate depression and 24 for the comparison of moderate vs. severe depression (33).

Genotyping

Genomic DNA was extracted from the subject's vein using the AxyPrep^{TM} Blood Genomic DNA Miniprep Kit and stored at -80°C until use. All the extracted genes were sent to the Beijing Huada Biological Company for genotyping. Huada used the principle of MassARRAY technology, with its core matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) technology, along with the corresponding Sequenom to detect the *MTHFR* C677T locus genotype. The genotyping completion rate in our study was 100%. Genotype frequencies of *MTHFR* C677T were used to calculate the Hardy–Weinberg equilibrium, applying the online software SHEsis (34). The details are described in **Table 1**.

MRI Data Acquisition

MRI data were acquired at Philips Achieva 3.0-T TX. Restraining foam pads were used to minimize head motion. 3D T1-weighted MRI-prepared rapid gradient-echo (MP-RAGE) was used with the following parameters to obtain brain structure data: 7,380-ms repetition time, 3.4-ms echo time, 250 $\times$ 250-mm field of view, 256 $\times$ 256 matrix size, 1.2-mm slice thickness, 230 coronal slices without gap, 90° flip angle, and 6-min 53-s scan duration time. T1- and T2-weighted magnetic resonance images were checked first to exclude those with significant structural abnormalities.

Image Processing

All primary DICOM images were converted into the NIfTI format using an MRI conversion software (http://lcni.uoregon.edu/downloads/mriconvert/mriconvert-and-mcverter). All the structural data were analyzed with the FreeSurfer 5.3 Development Version (Massachusetts General Hospital, Boston, http://surfer.nmr.mgh.harvard.edu). Cortical reconstructions were visually inspected to check for the accuracy of the automatic segmentation of the gray/white matter boundary, and no scans required manual editing. The average CT was defined as the shortest distance from the known apex of the cortex between the pial surface and the gray/white matter junction (35). A Gaussian kernel of 10-mm full width at half-maximum was applied to the subjects' CT maps. Each hemisphere was automatically parcellated into 34 distinct cortical regions according to the Desikan–Killiany atlas (36). The CT value was measured by calculating the distance between the white matter and pial surfaces at 160,000 vertices in each hemisphere in FreeSurfer. We used the automatic segmentation pipeline in FreeSurfer to calculate the subcortical volume data including volumes of the bilateral putamen, caudate nucleus, globus pallidus, nucleus accumbens (NAC), thalamus, amygdala, and hippocampus. We extracted CT values of the 34 regions and seven subcortical volumes' data in each hemisphere for subsequent statistical analysis.

Statistical Analyses

In the main analysis, CT and volume values were compared between MDD, HC, and *MTHFR* C677T genotype groups (CC vs. TT+TC), and then the genotype-by-diagnosis interaction was further investigated. The genotype groups were based on the dominant model (comparing risk allele with the non-risk allele carriers) in accordance with previous imaging studies on the *MTHFR* gene (37). The general linear model (GLM), two-way analysis of covariance (ANCOVA), and full factorial model were performed in SPSS 17.0, evaluating the main effect of "diagnosis," "genotype," and the interactive effect of "diagnosis $\times$ genotype." CT/subcortical volume values were selected as the dependent variables, diagnosis and genotype as the independent variables, and age, gender, years of education, and estimated total intracranial volume (eTIV) as covariates, according to the statistical procedure used in a previous similar study (38). In the primary

TABLE 1 | Demographics and clinical characteristics of patients with MDD and HC.

	MDD ($n = 127$)	HC ($n = 101$)	χ^2/t	*P*-Value
Gender (male/female)	43/84	45/56	2.716	0.099
Age (year)	31.39 ± 7.87	29.83 ± 5.84	1.711	0.088
Years of education	12.22 ± 4.16	16.15 ± 4.32	−6.964	<0.001
eTIV (mm^3)	1188703.41 ± 389654.26	1331694.02 ± 181989.74	−3.663	<0.001
HAMD score	23.81 ± 5.01	0.39 ± 0.63	52.165	<0.001
HAMA score	22.96 ± 6.01	0.66 ± 0.77	41.506	<0.001
Illness duration (months)	12.31 ± 16.60			
***MTHFR* gene polymorphism C677T**				
CC	39	40	3.663	0.160
CT	69	42		
TT	19	19		
HWE for MDD			1.658	0.198
HWE for HC			1.726	0.189
CC	39	40	1.966	0.161
CT+TT	88	61		
C(allele frequency)	147	122	0.296	0.587
T(allele frequency)	107	80		

MDD, major depressive disorder; HC, healthy control; eTIV, Estimated Total Intracranial Volume; HAMD, Hamilton Depression-17 item scale; HAMA, Hamilton Anxiety Scale; HWE, Hardy-Weinberg equilibrium test; CC, CC genotype of C677T; TT + TC, TT or TC genotype of C677T.
Data are means ± standard deviation for age, years of education, eTIV, HAMD, and HAMA scores, and duration of illness.
The P-values for distributions of gender, MTHFR C677T genotype, C/T allele frequency, were obtained by chi-square test. The P-values for comparisons of age, years of education, eTIV, HAMD, and HAMA scores, and duration of illness were obtained using independent t-tests. Allele frequencies (C/T allele): MDD patients 0.58/0.42, HC subjects 0.60/0.40.

TABLE 2 | Demographics and clinical characteristics of the subjects with *MTHFR* T-carrier and C-homozygous.

	TT+CT ($n = 149$)	CC ($n = 79$)	χ^2/t	*P*-Value
Gender (male/female)	69/80	19/60	10.792	0.001
Age (year)	29.89 ± 6.97	32.22 ± 7.06	−2.384	0.018
Years of education	14.07 ± 4.71	13.75 ± 4.56	0.504	0.615
Ethnic Han (%)	100%	100%		
Dextromanuality (%)	100%	100%		
eTIV (mm^3)	1.2570E6 ± 3.4432E5	1.2426E6 ± 1.2570E6	0.318	0.751
HAMD score	14.107 ± 12.044	12.165 ± 12.614	1.140	0.255
HAMA score	13.738 ± 11.847	11.810 ± 12.1540	1.159	0.248

eTIV, Estimated Total Intracranial Volume; HAMD, Hamilton Depression-17 item scale; HAMA, Hamilton Anxiety Scale; T-carrier, The TT genotype or CT genotype of MTHFR C677T polymorphism; C-homozygous, CC genotype of MTHFR C677T polymorphism.
Data are means ± standard deviation for age, years of education, eTIV, HAMD, and HAMA scores.
The P-value for distributions of gender was obtained by chi-square test. The P-values for comparisons of age, years of education, eTIV, HAMD, and HAMA scores, were obtained using independent t-tests.

analysis, the test level was set to $\alpha = 0.05$, two-sided test. Regions with statistically significant differences in the above ANCOVA analysis were further analyzed in pairs. As an exploratory investigations study, according to a previous research (ref), we set up multiple correction levels according to previous imaging genetic studies (18). Therefore, the multiple comparisons' correction test level for this study was relaxed to $\alpha_{\text{diagnosis}} = 0.01$, $\alpha_{\text{genotype}} = 0.05$, and $\alpha_{\text{interaction}} = 0.05$, two-sided test.

Secondly, we aimed to analyze the specific role of the C677T allele in brain structural changes of depression. Brain regions with statistically significant differences in the analysis of diagnosis × genotype interactions were chosen as regions of interest (ROIs), and the *post-hoc* tests for ROIs were performed later. With reference to a previous study (38), we compared the ROIs between MDD patients and the HC participants within each genotype group using a one-way ANCOVA, controlling for the same covariates as the main analysis.

TABLE 3 | The illness duration and disease severity assessment of different genotypes in MDD.

	CT+TT ($n = 88$)	CC ($n = 39$)	*t*	*P*-Value
HAMD	23.58 ± 4.98	24.33 ± 5.11	0.781	0.436
HAMA	22.77 ± 6.05	23.40 ± 5.95	0.463	0.644
Illness duration (months)	12.74 ± 18.05	11.34 ± 12.89	0.438	0.662

Data are means ± standard deviation for HAMD, HAMA scores, and illness duration.

TABLE 4 | Statistically significant diagnostic main effect, genotype main effect, and diagnosis × genotype interaction effect in the respect of the whole cortical thickness and subcortical gray matter volume.

Region	Diagnosis MDD ($n = 127$) vs. HC ($n = 101$)			Genotype CC ($n = 79$) vs. TT+CT ($n = 149$)			Diagnosis × Genotype	
	F	*P*		*F*	*P*		*F*	*P*
Cortical thickness (mm)								
L caudal anterior cingulate	1.102	0.295		6.933	0.009**	CC>CT+TT	6.926	0.009**
L rostral anterior cingulate	3.225	0.074		4.090	0.044*	CC>CT+TT	2.611	0.108
L frontal pole	0.998	0.319		4.099	0.044*	CC>CT+TT	0.578	0.448
L cuneus	1.560	0.213		6.646	0.011*	CC<CT+TT	0.060	0.808
L pericalcarine	0.349	0.555		6.565	0.011*	CC<CT+TT	1.380	0.241
L isthmuscingulate	0.001	0.971		0.238	0.626		10.257	0.002**
L medial orbitofrontal	0.669	0.414		0.093	0.761		6.457	0.012*
L posterior cingulate	0.223	0.637		1.258	0.263		4.751	0.030*
L caudal middle frontal	4.181	0.042*	HC>MDD	1.195	0.276		0.025	0.875
R inferiorparietal	4.442	0.036*	HC>MDD	0.298	0.586		0.499	0.481
R inferiortemporal	5.343	0.022*	HC>MDD	1.649	0.200		0.005	0.945
R postcentral	4.057	0.045*	HC>MDD	0.082	0.774		0.027	0.869
R lateral orbitofrontal	5.818	0.017*	HC<MDD	0.010	0.920		6.452	0.012*
R caudal anterior cingulate	0.015	0.903		5.663	0.018*	CC>CT+TT	1.449	0.230
R supramarginal	1.342	0.248		4.425	0.037*	CC>CT+TT	3.094	0.080
R pericalcarine	0.919	0.339		4.961	0.027*	CC<CT+TT	0.011	0.916
Volume of subcortical gray matter (mm^3)								
L putamen	1.080	0.300		0.016	0.900		3.872	0.050*
L amygdala	5.130	0.024*	MDD<HC	0.778	0.379		1.327	0.251
L nucleus accumbens	14.968	0.000***[a]	MDD<HC	0.532	0.466		3.169	0.076

MDD, major depressive disorder; HC, healthy control; T-carrier, The TT genotype or CT genotype of MTHFR C677T polymorphism; C-homozygous, CC genotype of MTHFR C677T polymorphism; L, left hemisphere; R, right hemisphere.

$P < 0.05$, $P < 0.01$**, $P < 0.001$***.*

The F and P-values were obtained using two-way analysis of covariance adjusted for age, gender, years of education, and eTIV as covariates.

Bonferroni correction was applied in the analyses of diagnosis effect (MDD vs. HC), genotype effect (TT + CT vs. HC) and diagnosis-by-genotype interaction.

[a]*Cortical regions that remained significant after Bonferroni correction are marked with an asterisk.*

The clinical features of depression in the MDD group (e.g., duration of disease, HAMD score, and HAMA score) were examined in relation to CT or subcortical volume values of ROIs. We conducted partial correlation analysis, controlling for age, gender, years of education, eTIV, and genotype. A height threshold of $P < 0.05$ was set.

The distributions of age, years of education, eTIV, HAMD score, and HAMA score between MDD and HC groups or *MTHFR* C677T genotype TT+CT vs. CC, as well as the duration of disease between genotype groups in patients with MDD, were analyzed using independent *t*-tests. The chi-square test was used to compare the gender and genotype distributions. The significance threshold was set to $P < 0.05$. Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL).

RESULTS

Demographic and Genotypic Characteristics

We could not find any significant difference between the patients and the HCs in terms of age, gender, and genotype distribution except for years of education ($t = -6.964$, $P < 0.001$), HAMD ($t = 52.165$, $P < 0.001$), HAMA ($t = 41.506$, $P < 0.001$), and eTIV ($t = -3.663$, $P < 0.001$). Comparing years of education, eTIV, HAMD score, and HAMA score between

TABLE 5 | *Post-hoc* analysis of the ROIs according to the interaction of "diagnosis × *MTHFR* C677T genotype."

Region	TT+CT				CC			
	MDD	HC	Mean Difference	F	MDD	HC	Mean Difference	F
Cortical thickness (mm)								
L cACC	2.399 ± 0.036	2.572 ± 0.044	−0.173	8.356**	2.632 ± 0.053	2.539 ± 0.052	0.093	1.409
L ISTC	2.475 ± 0.020	2.545 ± 0.024	−0.070	4.444*	2.563 ± 0.033	2.479 ± 0.033	0.084	2.903
L mOFC	2.341 ± 0.021	2.425 ± 0.025	−0.084	5.968*	2.396 ± 0.024	2.345 ± 0.024	0.052	2.054
L PCC	2.496 ± 0.017	2.555 ± 0.021	−0.059	4.031*	2.556 ± 0.030	2.505 ± 0.030	0.051	1.285
R lOFC	2.574 ± 0.018	2.578 ± 0.023	−0.004	0.015	2.621 ± 0.021	2.499 ± 0.021	0.122	15.127***[a]
Volume of subcortical gray matter (mm^3)								
L Pt	6.676E3 ± 0.089E3	6.588E3 ± 0.110E3	−0.170E3	0.343	6.247E3 ± 0.126E3	6.639E3 ± 0.125E3	−0.391E3	4.386*

MDD, major depressive disorder; HC, healthy control; T-carrier, The TT genotype or CT genotype of MTHFR C677T polymorphism; C-homozygous, CC genotype of MTHFR C677T; L, left hemisphere; R, right hemisphere; cACC, caudal anterior cingulate cortex; ISTC, isthmuscingulate; mOFC, the medial orbitofrontal cortex; PCC, posterior cingulate cortex; lOFC, the lateral orbitofrontal cortex; Pt, putamen.

[a]Data are means ± standard error (values of cortical thickness and volume of subcortical). The F and P-values were obtained using one-way analysis of covariance adjusted for age, gender, years of education, and eTIV as covariates as covariates.

P < 0.05, P < 0.01**, P < 0.001***.*

genotype groups of TT+CT and CC, we could not find any significant difference, except for age ($t = -2.384$, $P = 0.018$) and gender ($\chi^2 = 10.792$, $P = 0.001$). Compared to C homozygote, the T carrier group appeared younger and had more males. There was no significant difference between the genotype of CC and T carriers within the MDD group with regard to illness duration, HAMD score, and HAMA score. Detailed information is presented in **Tables 1**–**3**.

Differences on CT and Volume of Subcortical Values in the Whole Brain According to Diagnosis and *MTHFR* C677T Genotype

The statistically significant results of the 2 × 2 ANCOVA and full factorial model between MDD and HC and between T carrier and C-homozygous groups on CT and volume values (CT values of the 68 regions and 14 subcortical volumes' data in whole brain) are described in **Table 4**. Patients with MDD showed significantly smaller volume of the subcortical NAC ($F = 14.968$, $P < 0.001$, which remained significant after Bonferroni correction) in the left hemisphere compared with the HC group. However, no significant difference in CT values ($P > 0.01$) between patients with MDD and the HC group was found. In the comparison of genotype groups, *MTHFR* C677T allele carriers exhibited significantly reduced CT in the left caudal anterior cingulate cortex (cACC, $F = 6.933$, $P = 0.009$), rostral anterior cingulate cortex (rACC, $F = 4.090$, $P = 0.044$), frontal pole ($F = 4.099$, $P = 0.044$), right cACC ($F = 5.663$, $P = 0.018$), and supramarginal gyrus ($F = 4.425$, $P = 0.037$) compared with the C-homozygous group. In contrast, *MTHFR* C677T allele carriers manifested significantly increased CT in the left cuneus ($F = 6.646$, $P = 0.011$) and pericalcarine (left, $F = 6.565$, $P = 0.011$; right, $F = 4.961$, $P = 0.027$) in hemispheres on both sides. T allele carriers and the C-homozygous group did not differ significantly in terms of subcortical volume ($P > 0.05$). Significant diagnosis-by-genotype interaction effects on CT presented in the cACC ($F = 6.926$, $P = 0.009$), isthmus cingulate (ISTC, $F = 10.257$, $P = 0.002$), medial orbitofrontal cortex (mOFC, $F = 6.457$, $P = 0.012$), and posterior cingulate cortex (PCC, $F = 4.751$, $P = 0.030$) in the left hemisphere and in the right lateral orbitofrontal cortex (lOFC, $F = 6.452$, $P = 0.012$).

Moreover, we found a significant trend in interaction effect on the volume of the left putamen (Pt, $F = 3.872$, $P = 0.05$). We used the brain regions with statistically significant diagnosis-by-genotype interactions as ROIs for *post-hoc* analysis. These brain regions included the left cACC, ISTC, mOFC, PCC, and Pt and the right lOFC. We compared CT or volume values extracted from ROIs between MDD patients and HC within each genotype group using a one-way ANCOVA; covariates included those used in the main analysis. In the C-homozygous group, patients appeared to have significantly higher CT values in the right lOFC ($F = 15.127$, $P < 0.001$) and smaller volume values in the left Pt ($F = 4.386$, $P = 0.040$) compared with the HC group. However, there was no significant difference between the T carrier within MDD and HC groups in the above brain regions ($P > 0.05$). In the T allele carrier group, CT values of the left cACC ($F = 8.356$, $P = 0.004$), ISTC ($F = 4.444$, $P = 0.037$), mOFC ($F = 5.968$, $P = 0.016$), and PCC ($F = 4.031$, $P = 0.047$) in depressed patients were significantly thinner than those in the HC group. Likewise, we could not find any significant difference between the C-homozygous group with MDD and HC groups in the above four regions ($P > 0.05$). The detailed data are described in **Table 5**. The post-analysis of CT in ROIs is shown in **Figure 1**, and the volume values is displayed in **Figure 2**.

Correlation of CT or Subcortical Volume With Illness Duration, Depression, and Anxiety Severity

We investigated correlations between the course of the disease, HAMD score, HAMA score, and CT/volume values of ROIs in MDD patients using partial correlation analysis separately.

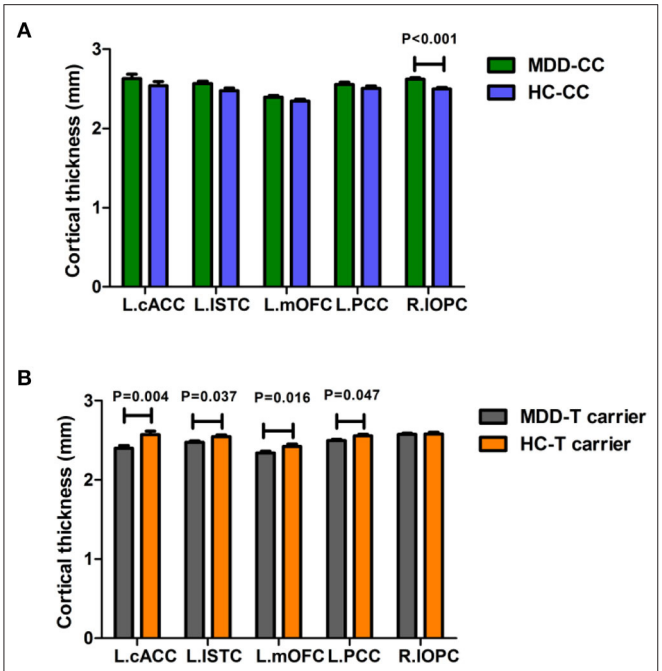

FIGURE 1 | Comparison of the cortical thickness values of the left cACC, lSTC, mOFC, PCC, and the right lOFC between MDD patients and the healthy control within each genotype. **(A)** In the C-homozygous group, cortical thickness in the lOFC of patients with MDD exhibited a significantly greater than that in the healthy controls. **(B)** While in the T-carrier group, patients showed significantly thinner cortical thickness in the left cACC, lSTC, mOFC, and PCC than healthy subjects. (The green bar, the CC genotype of the MDD group; the blue bar, the CC genotype of the HC group; the gray bar, T-carrier of the MDD group; the yellow bar, HC group T-carrier). MDD, major depressive disorder; HC, healthy controls; L, left hemisphere; R, right hemisphere; cACC, caudal anterior cingulate cortex; lSTC, isthmuscingulate; mOFC, the medial orbitofrontal cortex; PCC, posterior cingulate cortex; lOFC, the lateral orbitofrontal cortex. Data are means ± standard error.

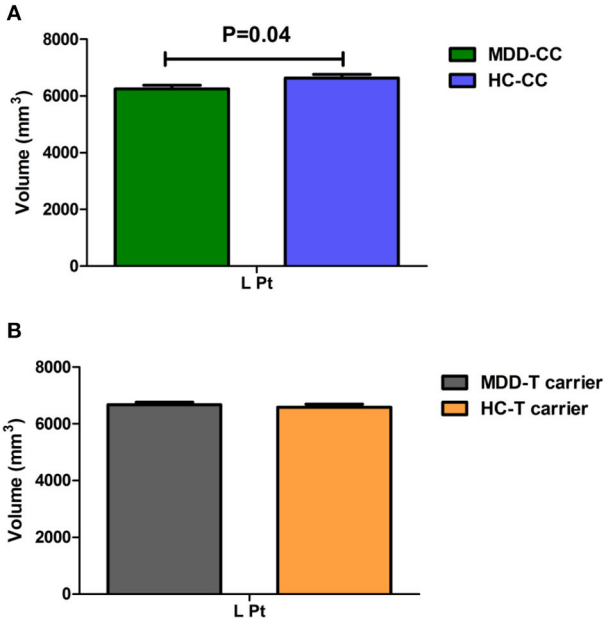

FIGURE 2 | Comparison of the volume value of the left putamen (Pt) between MDD patients and the healthy control within each genotype. **(A)** In the C-homozygous group, MDD patients showed a significantly decreased volume value in the Pt ($P = 0.040$) than healthy controls. **(B)** While In the T allele carrier group, volume value did not differ between patients with MDD and healthy controls. (The green bar, the CC genotype of the MDD group; the blue bar, the CC genotype of the HC group; the gray bar, T-carrier of the MDD group; the yellow bar, HC group T-carrier.). MDD, major depressive disorder; HC, healthy controls; L, left hemisphere; R, right hemisphere; Pt, putamen. Data are means ± standard error.

Consequently, we could not find any significant correlations (all $P > 0.5$). The data are described in detail in **Table 6**.

DISCUSSION

This study firstly investigated the role of *MTHFR* gene polymorphism C677T in pathophysiological processes of MDD in the Han Chinese population in southern China. We observed a significant reduction in the volume of the left NAC in first-episode MDD. Jan Wacker et al. (39) applied multiple methods of the rest-state fMRI, task fMRI, and volume-based measurement to investigate the association between anhedonia, NAC, and rACC. Their findings suggested that anhedonia was associated with reduced reward stimuli in NAC during task fMRI, decreased volume in NAC, and increased rest-state fMRI activity in cACC, which are involved in positive experience-related brain areas. Our research supports structural abnormality of the NAC-prefrontal cortex (PFC) in depression.

Meanwhile, we also found that *MTHFR* C677T allele carriers significantly decreased CT in the left cACC, rACC, frontal pole, right cACC, and supramarginal gyrus compared with the C-homozygous group. There were some studies showing that the C677T allele was associated with gray matter density reduction in the PFC (21), frontoparietal lobe cortex, parieto-occipital lobe cortex, orbitofrontal cortex (OFC), middle frontal gyrus, cingulum, and inferior parietal lobule; volume deficit of the parahippocampus and putamen; or increased volume of the putamen (26, 27, 40, 41), similar to our results. Based on a similar cytoarchitecture and common circuitry and functions, rACC has an integrated effect on the ventral ACC responsible for emotions and the dorsal ACC responsible for cognition function. Therefore, combined with the pathophysiological changes related to *MTHFR* C677T, the *MTHFR* T allele may cause a corresponding physiopathological change by reducing the *MTHFR* enzyme activity, which is related to the reduction of rACC gray matter and the reduction of CT and then participates in the emotional-cognitive changes of depression. Moreover, our research found for the first time that the C677T allele carriers exhibited increased CT in the left cuneus and bilateral pericalcarine. As stated by van Eijndhoven et al. (41), if the critical area (such as OFC) was damaged, other related brain regions [mainly the parts of the default mode network (DMN)] will be compensated by activation or expansion. We also found correlations between C677T risk allele carriers and increased CT

TABLE 6 | Analysis the correlation between cortical thickness, volume of subcortical values with clinical status of disease.

Region	Illness duration (months)		HAMD		HAMA	
	r-value	*P-value*	*r-value*	*P-value*	*r-value*	*P-value*
Cortical thickness (mm)						
L cACC	−0.012	0.893	0.112	0.210	0.142	0.111
L ISTC	0.010	0.912	0.106	0.235	0.114	0.203
L mOFC	0.000	0.996	−0.049	0.581	−0.076	0.394
L PCC	0.034	0.704	−0.020	0.826	−0.059	0.511
R lOFC	0.128	0.151	0.127	0.155	0.105	0.241
Volume of subcortical gray matter (mm^3)						
L Pt	−0.048	0.589	−0.101	0.257	−0.149	0.094

L, left hemisphere; R, right hemisphere; cACC, caudal anterior cingulate cortex; ISTC, isthmuscingulate; mOFC, the medial orbitofrontal cortex; PCC, posterior cingulate cortex; lOFC, the lateral orbitofrontal cortex; Pt, putamen.

The r and P-values were obtained using partial correlation analysis, controlling age, gender, years of education, eTIV, and MTHFR C677T genotype as covariates.

of the cuneus and pericalcarine. This may be interpreted as a compensatory increase.

Furthermore, our study verified that there were significant interactions between *MTHFR* C677T and a diagnosis of MDD on CT of the left cACC, ISTC, mOFC, PCC, and the right lOFC. Further, T allele carrier patients yielded reduced CT values in the left cACC, ISTC, mOFC, and PCC compared with T allele carrier HCs. In contrast with the *MTHFR* C-homozygous HCs, the C-homozygous patients demonstrated an increased CT value in the right lOFC. However, in terms of subcortical volume, only the left putamen volume tended to be smaller in C-homozygous MDD patients than in the C-homozygous control group. In short, we could hypothesize that the *MTHFR* C677T allele is a risk factor for the thinning of CT of MDD patients in the left cACC, ISTC, mOFC, and PCC; the C677T homozygote is a protective factor for increased lOFC CT and also allows the volume of the putamen to manifest a decreasing trend in depression. These changed brain regions are included in the structural covariance networks (SCNs), which are defined as having both structural and functional connectivity and modulated by *MTHFR* C677T. Our explanation for these results is that abnormalities in the CT or subcortical volume accompanied by genetic risk conferred by *MTHFR* C677T might be related to the disturbances of neural circuitry involved in emotion processing, which could contribute to a depressive mood. The histopathological alterations within and between structures in DMN and the limbic–cortical–striatal–pallidal–thalamic (LCSPT) circuits may promote dysregulation in emotional behavior and other cognitive aspects of depressive syndromes (40). Growing evidence suggest that *MTHFR* enzyme deficiency caused by *MTHFR* C677T is frequently accompanied by changes in methyl patterns and metabolic disturbance, resulting in methylation of choline and hampered monoamine neurotransmitters (42). Defective *MTHFR* may associate with a series of central nervous system (CNS) cell molecular changes, which negatively affect neuroplasticity in adults.

In sum, these results supported previous literatures and our hypotheses and indicated that *MTHFR* C677T may be involved in one of the pathological mechanisms of depression by affecting the brain structure of LCSPT, especially the thickness of the cACC. In addition, a recent meta-analysis concluded that folate may hold value as an adjuvant treatment for individuals with depression (43). As far as we know, this is the first research investigating the relationship between genetic variants of *MTHFR* C677T and the cortex–subcortical structural changes in patients with MDD.

There are some limitations in the present study. First, our sample is still limited. Second, this study did not measure plasma Hcy, folate, SAM, methyl, etc. at the beginning of the study. Third, we did not find significant differences in HAMD and HAMA scores between *MTHFR* T carriers and C-homozygous groups. Fourth, important environmental factors related to depression, such as childhood trauma, were not included in the study design. In the future, it is necessary to improve the research design to confirm the results.

DATA AVAILABILITY STATEMENT

This article contains previously unpublished data and datasets are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Kunming Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZL and YC: substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data. BH, JX, LP, CZ, and ZS: drafting the article or revising it critically for important intellectual content. XX, YC, and ND: final approval of the version to be published. All authors contributed to the article and approved the submitted version.

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BDNF Gene's Role in Schizophrenia: From Risk Allele to Methylation Implications

Xiaoqian Fu[1], *Jun Wang*[2], *Jianbin Du*[2], *Jing Sun*[3], *Ancha Baranova*[4,5] *and Fuquan Zhang*[3,6*]

[1] Department of Clinical Psychology, Suzhou Guangji Hospital, The Affiliated Guangji Hospital of Soochow University, Suzhou, China, [2] Department of Psychiatry, Wuxi Mental Health Center of Nanjing Medical University, Wuxi, China, [3] Department of Psychiatry, The Affiliated Brain Hospital of Nanjing Medical University, Nanjing, China, [4] School of Systems Biology, George Mason University, Fairfax, VA, United States, [5] Research Centre for Medical Genetics, Moscow, Russia, [6] Institute of Neuropsychiatry, The Affiliated Brain Hospital of Nanjing Medical University, Nanjing, China

Background: Schizophrenia (SZ) is a severe chronic mental disorder with complex genetic mechanisms. Brain-derived neurotrophic factor (BDNF) is one of promising candidate genes for SZ, and rs6265 is a non-synonymous single nucleotide polymorphism (SNP) in BDNF.

Methods: In this study, we performed a case-control association study of rs6265 in a cohort of Han Chinese population from eastern China, including 1,407 SZ patients and 1,136 healthy controls; and carried out a cis-mQTL (Methylation Quantitative Trait Loci) analysis for BDNF rs6265.

Results: We found a positive association of rs6265 with SZ ($P = 0.037$), with the minor allele (A) of rs6265 conferring a protecting effect for SZ (OR = 0.89). Furthermore, cis-mQTL analysis indicates that rs6265 is associated with several methylation loci surrounding BDNF.

Conclusions: Together, our findings provide further evidence to support the involvement of BDNF gene in the genesis of SZ.

Keywords: schizophrenia, BDNF, single nucleotide polymorphism, rs6265, methylation

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***Correspondence:**
Fuquan Zhang
zhangfq@njmu.edu.cn

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INTRODUCTION

Schizophrenia (SZ) is a serious mental disorder featured with profound disruption in emotion and cognition, affecting the most basic human properties such as language, thought, perception, and so on. The neurodevelopmental hypothesis is one of dominant hypotheses for SZ study in the last two decades, which supposes that SZ originates from developmental disorders of the nervous system early in brain development, long before the onset of the illness (1).

Brain-derived neurotrophic factor (BDNF) is believed to be involved in the pathophysiology of SZ and has been widely studied as a marker of neuropsychiatric diseases (2). The expression or functional changes of BDNF are proven associates of the pathophysiological process of several brain diseases, including mental diseases and neuro degenerative diseases (3). Animal studies have demonstrated the importance of BDNF in neurodevelopment and survival. Most of the homozygous BDNF mutant mice die within 2 days of birth, with some surviving 2 to 4 weeks. They exhibit distinct behavioral phenotypes as well as lack motor coordination and balance

(4). BDNF affects cell level maturation, survival, diffusion, and synaptic function by activating intracellular signaling cascades, including mitogen-activated protein kinase/extracellular signal regulated protein kinase (MAPK/ERK), phosphatidylinositol 3-kinase, and phospholipase Cc pathways (5–7).

The single nucleotide polymorphism (SNP) rs6265 in *BDNF*, also known as Val66Met or G189A replacement at codon 66 in the pro-region of BDNF, alters the classification of BDNF protein and its availability in the synaptic cleft (2). The rs6265 variant interferes with the activity-dependent secretion of BDNF by inhibiting the sorting of BDNF into secretory granules, thereby affecting its function (8). An association of rs6265 polymorphism with the changes in hippocampal structure and function has been replicated in both human and mice (8, 9). The BDNF rs6265 knock-in mice show decreased BDNF expression, reduced hippocampal neurogenesis (10), decreased hippocampal volume, and abnormal morphology of hippocampal neurons (9). It has been suggested that rs6265 genotypes in the hippocampus and infra limbic medial prefrontal cortex may affect NMDA receptor-mediated neurotransmission and plasticity, which are associated with the production of positive or negative symptoms of schizophrenia (11, 12). Moreover, there is increasing evidence that rs6265 modifies both the clinical presentation and genetic risk architecture of schizophrenia, possibly by influencing cognitive function, brain morphology, age of onset, and treatment response (13, 14).

Prenatal stress is deemed to be a risk factor for SZ as a neurodevelopmental disorder (15). Dong et al. (15) reported both the SZ-like behavioral abnormalities in adult offsprings of mice exposed to prenatal stress mice and the molecular changes in the postmortem brains of SZ patients, with expression levels of DNA-methyltransferase 1 (DNMT1) and 10-11-translocation hydroxylase being significantly increased in the frontal cortex and hippocampus. Moreover, the corresponding reduction of *BDNF* transcription levels, along with enrichment of 5-methylcytosine and 5-hydroxymethylcytosine in the regulatory regions of *BDNF* gene, were observed, pointing at important role of *epi*genetic modification of *BDNF* in the phenotype and pathogenesis of SZ. It's worth noting that epigenetic modifications, including DNA methylation of the *BDNF* promoter, are significantly related to the pathophysiology of psychiatric disorders (4).

To further elucidate the role of the *BDNF* gene as a risk allele or regulator in SZ, we performed a case-control association study of rs6265 in a cohort of Han Chinese population from eastern China, including 1,407 SZ patients and 1,136 healthy controls, and carried out a cis-mQTL (Methylation Quantitative Trait Loci) analysis for BDNF rs6265.

MATERIALS AND METHODS

Subjects

All subjects were unrelated Han Chinese recruited from China. In the patient group, the diagnosis of SZ was in line with criteria in the Diagnostic and Statistical Manual of Mental Disorders, Fourth edition (DSM-IV) and confirmed by two or more experienced psychiatrists using the Structured Clinical Interview for DSM-IV (SCID-I). Exclusion criteria included the presence of other mood or neurodevelopmental disorders, epilepsy, or intellectual disability. For the choice of healthy controls, the Structured Clinical Interview for DSM-IV, Non-patients edition (SCID-NP) was used to interview members of an unrelated general population and exclude those with mental illness by professional psychiatrists.

For genetic association analysis, our study sample includes 1,407 SZ patients (874 men and 533 women, aged 45.8 ± 11.5 years) and 1,136 healthy controls (633 men and 503 women, aged 44.9 ± 10.3 years). Healthy subjects were recruited through advertisement. This study was approved by the Ethics Committees of the Wuxi Health Mental Center, and either patients or their guardians signed informed consents.

Genotyping

Peripheral blood samples were collected from all subjects. Blood samples were collected from all participants using K2EDTA tubes and a Blood Genotyping DNA Extraction Kit. The genotype of the SNP was analyzed by the Shanghai Biowing Applied Biotechnology Co. Ltd. (www.biowing.com.cn) using the Ligase Detection Reaction-Polymerase Chain Reaction method. Genomic DNA extracted from blood samples was first subjected to multiplex RCR to obtain a PCR product including SNPs. The PCR product and LDR probes were then subjected to multiplex LDR reaction, with a DNA sequencer to detect the products (1).

Statistical and Bioinformatics Analysis

Genetic association tests were analyzed using PLINK v1.07 (16). The data obtained from SZ patients and healthy controls was compared. SNP association analyses were performed to test for possible associations between SNP rs6265 in BDNF and SZ using Plink v1.07. The two-tailed Fisher's exact test was used to compare the polymorphisms' distributions and testing their significance at $p < 0.05$ (17). The p-values were adjusted by false discovery rate correction for multiple test analysis (17). The allele frequencies and genotype distribution of rs6265 were calculated for the SZ cases and healthy controls and were analyzed for association by Logistic regression with the assumption of an additive genetic model, and Odds ratios (OR) with 95% confidence intervals were calculated (18). We performed the cis-mQTL in the methylation dataset (19) using Genevar 3.3 (20), with which we analyzed the association of rs6265 genotypes with neighboring methylations within 100 Kb distance. Associations between DNA methylation levels and probabilities of imputed genotypes were tested in samples of related individuals by a two-step analysis (19). That is, estimating a linear mixed model of methylation levels, covariates, and a kinship matrix, and then a score test. Age, beadchip, BS conversion efficiency, and BS-treated DNA input were cofactors. Cis analysis was limited to SNPs located within 100 kb of either side of the probe location and false discovery rate for the cis analysis was calculated with the q value package (19).

RESULTS

Genetic Association

Genetic association analysis was performed in a study sample comprised of 1,407 patients (874 men and 533 women, aged 45.8 ± 11.5 years) and 1,136 unrelated healthy controls (633 men and 503 women, aged 44.9 ± 10.3 years).

In both the patient and the control groups, genotypic distributions of rs6265 had not deviated from Hardy-Weinberg equilibrium (HWE) ($P > 0.05$). Allelic distribution of rs6265 was associated with SZ ($P = 0.037$), with the minor allele (A) of rs6265 conferring a protecting effect for SZ (OR = 0.89). Specifically, OR = 0.89 indicates that minor allele (A) of rs6265 is negatively correlated with SZ, and reduces the risk of SZ in its carriers (**Table 1**).

cis-mQTL Analysis

When the associations of rs6265 genotypes with neighboring methylations within 100 Kb distance were studied using the cis-mQTL analysis, an association of rs6265 with 4 methylation loci near or within BDNF was detected (**Figure 1**).

DISCUSSION

SZ is one of most disabling mental disorders; it affects the whole brain functions. Although the origin of this disorder remains unclear, a bulk of evidence supports that the abnormalities of early brain development play an important role in the pathogenesis of SZ.

In recent years, researches have focused on the effects of BDNF on brain development in the early stages of psychosis. For example, a significant correlation between the rs6265 polymorphism of *BDNF* and psychosis risk was found (21). An obvious association between the *BDNF* rs6265 genotype and the onset age of psychosis has also been demonstrated (22). Serum BDNF levels in first-episode psychosis patients were found to be much lower than those in the control group (23). Cumulatively, these findings indicate that BDNF may play an important role in the early stages of SZ.

For genetic association analysis, we found a positive association between rs6265 and SZ, with the minor A-allele of rs6265 conferring a protecting effect for SZ. A meta-analysis found that individuals with the Met/Met genotype had a 19% increased risk of developing SZ compared to individuals with the Val/Met genotype (24), a conclusion consistent with our results. However, it has been suggested that the non-mutated 66Val allele may be a risk locus for SZ, while the 66Met allele may actually be protective (14). For example, it was found that the Val gene conveyed risk in 321 Scottish SZ-spectrum probands (25). Moreover, a significant association was found between Val/Val genotype frequency and male patients with chronic SZ (26). The reasons for the inconsistencies in results may include differences in sample size, ethnic heterogeneity, etc., which still need further investigation.

A meta-analysis of imaging studies in neuropsychiatric patients with SZ, bipolar disorder, major depression or anxiety, showed that psychiatric patients with different BDNF rs6265 genotypes, such as Val/Val homozygous and Met-carrier both had smaller hippocampal volume as compared to that in healthy controls (2). Considering that rs6265 SNP was not associated with decreased hippocampal volume in neuropsychiatric patients, it followed that the Met allele might not be a risk allele (A/Met) for SZ (2). This hypothesis supports our findings pointing that allele A of rs6265 confers a protective effect for SZ. Due to a wide range of psychiatric disorders included in the meta-analysis cited above, whether this conclusion holds for the association between SZ and rs6265 genotype required further investigation.

At present, the body of literature describing results of the risk association studies of rs6265 and SZ remain contradictory due to lack of strong evidence and small sample sizes (14, 27). In view of larger sample size (1,406 SZ patients and 1,136 healthy controls), our findings may be important for bringing more clarity to the association between rs6265 and SZ.

cis-mQTL analysis indicated an association of rs6265 with several methylation loci within *BDNF*. As our study strongly suggests that rs6265 is associated with SZ, mQTL findings may

TABLE 1 | Genetic association of rs6265 with schizophrenia.

Trait	A (freq)	G (freq)	OR (95%CI)	*P*
Schizophrenia	1,339 (0.476)	1,475 (0.524)	0.89 (0.80–0.99)	0.037
Control	1,148 (0.505)	1,124 (0.495)		

freq, frequency.

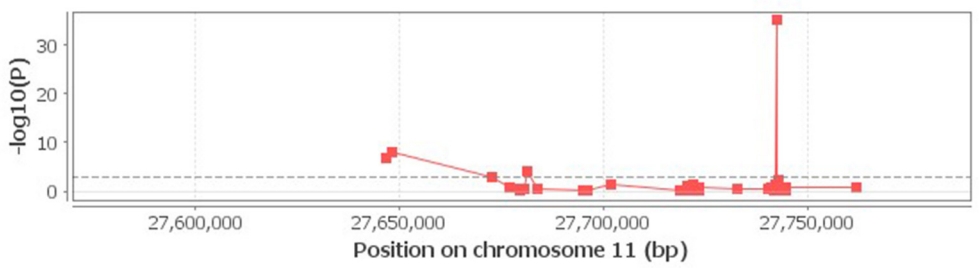

FIGURE 1 | Correlation of rs6265 with neighboring methylations. The 4 loci above the dash lime are deemed to be significant ($P < 0.001$).

be used to support the role of BDNF related methylation in the etiology of SZ. The peripheral blood level of DNA methylation of *BDNF* in SZ were accessed previously, with more methylated alleles and lower expression levels of BDNF found in the patient group than in the control group (28). Our findings are consistent with those observation, suggesting that BDNF related methylation may play an important role in SZ. Ikegame et al. (4) put forth that the down-regulation of *BDNF* levels is commonly associated with the increase in DNA methylation of the *BDNF* promoter. Cell and animal models have shown that the expression of BDNF in the neurons may be regulated by DNA methylation of specific promoters, but the mechanism of elevated DNA methylation at those specific sites is still elusive (4).

One previous study investigated peripheral blood lymphocytes of SZ patients and found the changes in DNA methylation of *BDNF* promoter I, thus, connecting the epigenetic alteration of *BDNF* locus in peripheral blood cells to the pathophysiology of SZ (29). Dong et al. (30) have shown that mice born from dams stressed during pregnancy develop behavioral deficits similar to those detected in adult SZ patients. Moreover, they showed that clozapine treatment reverses both the behavioral deficits and 5-methylcytosine and 5-hydroxy methylcytosine changes at *BDNF* promoters, as well as reduces mRNA and protein expression of this gene (30). This provides further evidence that BDNF-related methylation may play an important role in the pathophysiology of SZ.

On the other hand, in a study of the prefrontal cortex samples from 25 SZ patients and 25 healthy controls, the risk of SZ was independent of *BDNF* mRNA expression levels and the differences in DNA methylation of its promoter (31). Another study examined the DNA methylation levels of *BDNF*-encoded exons and three different promoter regions in the prefrontal cortex of SZ patients and found no significant differences in DNA methylation in patients with schizophrenia when their brain regions were compared to that of the controls (32). In sum, the above studies did not find an association between *BDNF* and DNA methylation in SZ. Since these studies were made using samples from the prefrontal cortex of the postmortem brains, these conclusions are vulnerable to possible bias due to their postmortem character, precluding direct comparisons with the conclusions made using living samples from the cells peripheral blood.

Kundakovic et al. (33) have explored a model of environmental exposure to bisphenol A in pregnant mice. Persistent changes of DNA methylation were detected in regions related to the transcription of *BDNF* gene in the hippocampus and blood of exposed pups. These changes were consistent with those in human umbilical cord blood of the newborns of exposed mothers, suggesting that the DNA methylation of *BDNF* in blood may serve as indicator for the DNA methylation of *BDNF* in the brain and the biomarker of behavioral vulnerability. Here we studied first-episode SZ patients and found an association between *BDNF* polymorphism rs6265 and methylation within *BDNF* locus. Our findings suggest that abnormal methylation of *BDNF*-related regions occurs early in SZ, pointing at the utility of these minimally-invasive for the detection of early SZ.

Many studies have found that epigenetic abnormalities in the SZ postmortem brain and the peripheral blood lymphocytes of SZ patients parallel each other. For example, in both types of samples, activities of cytosine modify DNMT1 and ten-eleven methylcytosine dioxygenase 1 increase, while amounts of BDNF-encoding mRNA, which is highly sensitive to the levels of its DNA methylation, decrease (34–41). In addition, studies have shown that, in SZ patients, increased DNMT expression may be seen as a probable cause of the concomitant decrease in *BDNF* expression, mediated by the DNMT-dependent elevation of cytosine methylation levels in *BDNF* promoter (36, 42). Davies et al. (43) showed that the DNA methylation patterns in the brain and the blood are highly correlated, including that in genes related to neural differentiation and neurodevelopment, such as BDNF. Furthermore, a similarity between brain- and lymphocyte-specifics changes of DNMT and ten-eleven methylcytosine dioxygenase 1 activities was shown (34, 44). In the case of SZ, the mechanisms of epigenetic regulation in the peripheral blood lymphocytes and the brain may be similar (34, 41, 43, 45). Therefore, the methylation levels in certain genes expressed in cells of the peripheral blood may be used as proxy biomarkers for early identification of SZ, and early interventions.

There are several limitations to consider in our study. The methylation data were derived from peripheral tissues rather than the brain itself, thus caution is needed when extrapolating the conclusions. Said that, detected abnormalities in peripheral tissue may serve as potential indicators of disease pathology even though they distinct from that in the brain. The changes in peripheral biomarkers may parallel pathological processes in the brain only in part, while in other part they would reflect the molecular reaction of the peripheral cells, which is secondary to the disease (46). Because both of these regulatory arms are reflective of the disease, "cause and consequences" arguments have lower applicability to biomarkers, which typically serve as indicators of association rather that causality. This study highlighted methylation biomarkers in the BDNF promoter as possible contributors for SZ detection in first-episode patients.

CONCLUSION

Our study supports the association between BDNF polymorphism rs6265 and SZ, as well as the relevance between rs6265 and BDNF methylation, providing further evidence to support the involvement of BDNF gene in the genesis of SZ.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: European Variation Archive, accession no: PRJEB41532 and ERZ1685357.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committees of the Wuxi Health Mental Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FZ designed the study and performed data analyses. XF, JW, JD, JS, and AB were responsible for manuscript writing and modification. All authors reviewed and approved the final manuscript.

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Inhibitory Top-Down Control Deficits in Schizophrenia With Auditory Verbal Hallucinations: A Go/NoGo Task

Qiaoling Sun[1], *Yehua Fang*[2], *Yongyan Shi*[1], *Lifeng Wang*[3], *Xuemei Peng*[1,4]* and *Liwen Tan*[1]*

[1] Department of Psychiatry, Mental Health Institute of the Second Xiangya Hospital, Central South University, Changsha, China, [2] Department of Clinical Psychology, Zhuzhou Central Hospital, Zhuzhou, China, [3] Department of Clinical Psychology, The Third Xiangya Hospital of Central South University, Changsha, China, [4] Department Psychology, Xiangtan Central Hospital, Xiangtan, China

Objective: Auditory verbal hallucinations (AVH), with unclear mechanisms, cause extreme distresses to schizophrenia patients. Deficits of inhibitory top-down control may be linked to AVH. Therefore, in this study, we focused on inhibitory top-down control in schizophrenia patients with AVH.

Method: The present study recruited 40 schizophrenia patients, including 20 AVH patients and 20 non-AVH patients, and 23 healthy controls. We employed event-related potentials to investigate the N2 and P3 amplitude and latency differences among these participants during a Go/NoGo task.

Results: Relative to healthy controls, the two patient groups observed longer reaction time (RT) and reduced accuracy. The two patient groups had smaller NoGo P3 amplitude than the healthy controls, and the AVH patients showed smaller NoGo P3 amplitude than the non-AVH patients. In all the groups, the parietal area showed smaller NoGo P3 than frontal and central areas. However, no significant difference was found in N2 and Go P3 amplitude between the three groups.

Conclusions: AVH patients might have worse inhibitory top-down control, which might be involved in the occurrence of AVH. Hopefully, our results could enhance understanding of the pathology of AVH.

Keywords: P3, event-related potential, schizophrenia, auditory verbal hallucination, inhibitory top-down control

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Xiancang Ma,
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Ning Hua Wang,
Fourth Military Medical University, China

***Correspondence:**
Liwen Tan
tanliwen@csu.edu.cn
Xuemei Peng
yutianqile@163.com

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INTRODUCTION

Auditory verbal hallucinations (AVH) are vivid perceptions of sound that occur without corresponding external stimuli and have a strong sense of reality. It occurs in 60–80% of schizophrenia patients (1) and causes multiple dysfunctions and poor control of behaviors (2, 3). Schizophrenia patients with AVH may have an increased tendency toward violent behaviors or acts (4–6), which may pose a threat and serious burden to society and their families.

Controlling and eliminating symptoms of hearing voices is difficult in treatment. Many efforts have been devoted to the treatment of auditory hallucinations, but the results still remain unsatisfactory (7). Studies have been conducted to investigate the efficacy of antipsychotic medications for AVH in schizophrenia patients, which exhibited a significant treatment effect of

several typical and atypical antipsychotics (8, 9). However, there are still a considerable minority of schizophrenia patients showing no treatment effect of antipsychotics (9) and AVH can be drug-resistant and become chronic in around 25% of schizophrenia patients (10). Brain stimulation and psychological intervention are also applied in the treatment for AVH, but the curative effect is not ideal. For example, transcranial magnetic stimulation may reduce the frequency and severity of AVH, but the efficacy effect size of 1 Hz transcranial magnetic stimulation was just 0.44, supported by meta-analysis (11). And cognitive-behavioral therapy, which is considered as the most investigated psychological intervention of AVH, has an average effect size of 0.44 (12).

Schizophrenia patients experiencing AVH usually report that they have been hearing words, sentences and conversations which often comment on their thoughts. Healthy individuals who experience this are typically aware that the "voices" they hear are false perceptions and originate from their mind. In addition, they seem to be able to cope with this false perceptual experience by recruiting inhibitory control functions. However, hallucinating schizophrenia patients seem to focus on "voices" and appear less able to exhibit inhibitory control and thus are less able to attend to events around them (13). It is suggested that inhibitory top-down control functions should play an essential role in modulating experience of voice that originates from one's mind or an external source. In other words, deficits in inhibitory top-down control process may be important in AVH (14).

Neuroimaging studies have shown that AVH results from a variety of alterations in brain structure (15, 16). Findings of structural imaging studies converge on gray matter reductions in the superior temporal gyrus, insula and inferior frontal gyrus as well as abnormalities in the connecting white matter between these regions, which associated with the processing of auditory verbal stimuli and executive control functions. Paulik et al. conducted a study with 589 undergraduate students who were drawn into high- and low-predisposed groups using the Launay-Slade Hallucination Scale (LSHS). They found that compared with the low LSHS group, the high LSHS group showed significantly increased false alarms on critical "inhibitory" runs (17). And according to Waters et al. and Badcock et al., the damage level of inhibitory control ability was positively correlated with the auditory hallucination severity (18, 19).

Electrophysiological approaches also provide important insights into the underlying mechanisms of AVH. Especially the event-related potentials (ERP), which are real-time measures of neural activity with high temporal resolution and promising tools to explore brain dynamics that underlie deficits during task performance. ERP recordings in schizophrenia with AVH have shown deficits in a series of components, including the early, P50 (20) and mismatch negativity (21) and the later, P3. P3 is a measure of inhibitory control, which has been well-studied in schizophrenia, but only few studies have reported the relationship between P3 and AVH in schizophrenia (22). Top-down inhibitory control measured in ERP often via the dichotic listening test, and the study have found that more dysfunctional top-down inhibition seemed to mediate the association between impairment to affective theory of mind and severity of hallucinations (23). The Go/NoGo task is a classical paradigm, in which participants respond to the frequent "Go" stimuli as quickly as possible and avoid button pressing reaction in the infrequent "NoGo" stimuli (24, 25). N2 and P3 in the Go/NoGo task are closely related inhibitory control (26–28). Despite intensive investigations, the AVH remains a poorly understood feature of schizophrenia. Many studies found that schizophrenia patients showed deficits in inhibitory control (29–31), but most of these studies did not make further analysis with regard to symptoms. Only a few studies argued that patients experiencing no AVH may not have obvious deficits in inhibitory control (18). It is not clear whether the inhibitory control deficits in AVH patients stem from the disorder or the symptom. Additionally, results from Go/NoGo task may provide more support for inhibitory control deficits in AVH patients. Thus, in the present study, we aim to investigate inhibitory top-down control in schizophrenia patients with and without AVH using a Go/NoGo task.

MATERIALS AND METHODS

Participants

The schizophrenia patients were recruited from the Outpatient Department of Psychiatry, the Second Xiangya Hospital of Central South University, China. The study and its aims were explained to all the participants and informed consent from them was obtained. This manuscript of the informed consent was obtained in compliance with the Helsinki Declaration. The inclusion criteria are (a) met ICD-10 criteria for schizophrenia; (b) aged between 18 and 30 years; (c) normal or corrected-to-normal vision; (d) right-handed; and (e) education level > 9 years and able to complete the test. The exclusion criteria are (a) with a history of head injury resulting in loss of consciousness; (b) alcohol or drug dependence; and (c) had taken an ERP test before.

Twenty patients with AVH were asigned to the AVH group and 20 patients who had never experienced AVH were assigned to the non-AVH group. All the patients were assessed by two senior clinical psychiatrists using the Positive and Negative Syndrome Scale (PANSS) (32). The healthy controls ($n = 23$) were recruited from the local community by advertisement. The demographic and clinical characteristics of all the subjects are demonstrated in **Table 1**.

Stimuli and Task

Participants completed the Go/NoGo task in an electrically shielded, sound-attenuated room. Participants were positioned about 100 cm away from the screen. The Go/NoGo program was presented using the E-prime 2.0 software. The task begins with a non-informative cue (a small white cross) for 1,000 ms, and then the stimulus (K or X) was presented for 500 ms, followed by a blank screen for 500 ms. The "K" stimuli, as the Go stimulus, requires a button press response as quickly and accurately as possible, and its probability to appear is 2/3, with 240 times of appearance in total. The "X" stimuli, as the NoGo stimulus, requires non-response and its probability to appear is 1/3, with 120 times of appearance in total. The rare "X" stimuli are set to ensure that the NoGo reaction is the non-dominant response,

TABLE 1 | Demographic and clinical characteristics of AVH patients ($n = 20$), non-AVH patients ($n = 20$) and Healthy controls ($n = 23$).

Characteristics	AVH patients	Non-AVH patients	Healthy controls	Statistics values	*p*
Sex (male/female)	14/6	15/5	17/6	$\chi^2 = 0.142$	0.932
Age (years)	24.85 ± 5.57	25.10 ± 4.85	23.09 ± 3.01	1.278	0.286
Education (years)	13.00 ± 2.49	13.95 ± 2.82	15.22 ± 1.00	7.576	<0.001
Duration of illness (months)	25.45 ± 21.00	23.05 ± 25.76	-	0.323	0.749
PANSS-P3	4.45 ± 1.50	1.05 ± 0.22	-	10.000	<0.001
PANSS total score	61.55 ± 12.09	60.45 ± 18.26	-	0.224	0.824
PANSS positive score	18.05 ± 5.37	13.95 ± 3.95	-	2.749	0.009*
PANSS negative score	15.00 ± 6.52	16.95 ± 7.71	-	0.864	0.393
PANSS general psychopathology	28.50 ± 6.51	29.55 ± 9.26	-	0.415	0.681

*A hyphen "-" was used when the data were unavailable or there was no data. *p < 0.05.*

and thus more attention is needed to carry it out correctly. Only correct Go responses (press within 200–1,000 ms after a Go-stimulus) and NoGo responses (no press after a Nogo stimulus) were recorded.

Recording and Data Processing Procedures

Continuous electroencephalography (EEG) data were recorded using the BrainAmp MR (Brain Products, Germany). The electrode cap consists of 32 Ag/AgCl electrodes in accordance with a modified international 10 - 20 system. Vertical electro-oscillogram was recorded from one electrode fixed above the left eye, and the horizontal electro-oscillogram was recorded from one electrode fixed at the outer canthus of the right eye. The reference electrode was at FCz. All signals were digitalized with a sample rate of 500 Hz and with a frequency band from 0.1 to 100 Hz. Electrical impedance for each site was below 5 kΩ throughout the experiment. Offline data were processed with the Brain Vision Analyzer 2.0 system (Brain Products GmbH, Germany). EEG data were referenced to the average of mastoids (TP9 and TP10). EEG signals were bandpass filtered using a 0.5 to 30 Hz (50 Hz notch) rate. Eye movements and eye blinks were removed using an independent component analysis. Artifact rejection procedures were applied to all epochs (−200 ms pre-stimulus to 1,000 ms post-stimulus), with a baseline correction from −200 ms to 0 ms pre-stimulus. Epochs were averaged using only correct attempts according to the condition (Go, NoGo). The N2 and P3 amplitude were defined as the global maximum value to baseline at signal subject level (N2, 200–300 ms post-stimulus; P3, 300–500 ms post-stimulus).

Statistical Analysis

Statistical analyses were completed using the SPSS19.0 software package (Statistics Product and Service Solutions), Chinese version. Sex differences between groups were analyzed using χ^2 test. Differences in age between the three groups were assessed using one-way ANOVA. Education difference was evaluated using Welch's ANOVA, and Games-Howell was used for the *post-hoc* test. Clinical differences between two patient groups were evaluated using *t*-test. Amplitude and latency of N2 and P3 were evaluated with repeated-measures ANOVA, with group (AVH patients, Non-AVH patients, Healthy controls) as between-subject variable, and trial category (Go, NoGo) and topographical site [Frontal (Fz, F3, F4), Central (Cz, C3, C4), Parietal (P3, Pz, P4)] as within-subject variables. Age and education were unconcerned covariates. For behavioral data, one-way ANOVA was used to analyze the response time of the correct Go attempts. The accuracy (i.e., button presses in the Go trials and no responses in the NoGo trials) were investigated using a repeated-measures ANOVA with trial category × group. The threshold for statistical significance was set at $p < 0.05$. Statistical analyses were adjusted for variance non-sphericity using the Greenhouse-Geisser correction (33). All the *post-hoc* analyses were adjusted using the Bonferroni adjustment.

RESULTS

Demographic Data

There were no significant differences ($p > 0.5$) in age and sex between all the groups but there was significant difference in the years of education ($\chi^2 = 7.576$, $p < 0.001$). Healthy controls had a higher education level than the AVH patients and non-AVH patients ($p < 0.05$). For the PANSS scale, the AVH patients had higher scores than non-AVH patients ($p < 0.005$) in only PANSS-P3 and PANSS positive symptoms. The two patient groups did not significantly differ in the duration of illness and the scores of other PANSS items.

Behavioral Data

The three groups differed significantly in Go reaction time (RT) ($F = 8.118$, $p = 0.001$). The Go RT of the healthy controls was significantly shorter than those of the two patient groups (AVH patients, $p = 0.018$; Non-AVH patients, $p = 0.005$) (**Figure 1**). The main effect of trial category revealed that all the participants made more accurate responses in the Go trials than in the NoGo trials ($F = 5.493$, $p = 0.022$). A main group effect was found ($F = 4.910$, $p = 0.011$), and healthy controls had a higher accuracy than the AVH patients ($p = 0.042$) and non-AVH patients ($p = 0.020$). No significant trial category × group interaction was found ($F = 0.604$, $p = 0.550$).

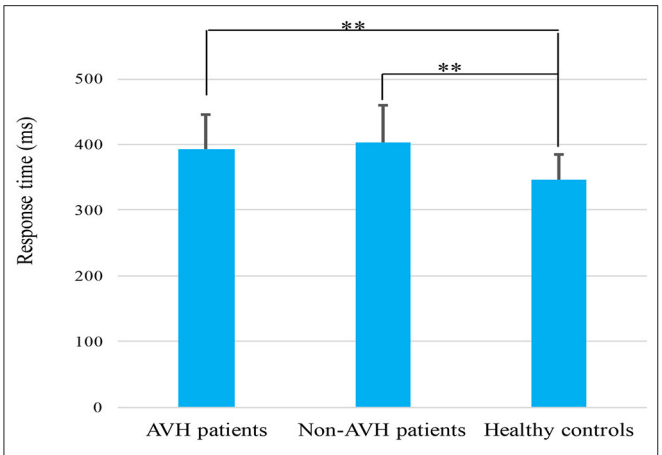

FIGURE 1 | Mean response time elicited by correct Go trials in AVH patients, non-AVH patients and healthy controls. Error bars denote the standard error. $^{**}p < 0.01$.

N2

For N2 amplitude, no significant main effect of group was found ($F = 0.269$, $p = 0.765$). The main effect of trial category ($F = 23.096$, $p < 0.001$) and topographical site ($F = 6.392$, $p = 0.002$) were found; however, a significant interaction of trial category × topographical site ($F = 12.534$, $p < 0.001$) was also observed. *Post-hoc* tests demonstrated that the frontal ($p < 0.001$) and central ($p < 0.001$) areas has higher NoGo N2 amplitude than the parietal area (**Figure 2**). With regard to N2 latency, the main effect of group was not significant ($F = 1.077$, $p = 0.347$), indicating that the N2 latency was not different among three groups. A significant main effect of trial category was observed ($F = 15.601$, $p < 0.001$); however, a significant interaction of trial category × topographical site ($F = 12.534$, $p < 0.001$) was also observed. *Post-hoc* tests demonstrated that the NoGo N2 latency was longer, compared to Go N2 latency in the frontal and central areas.

P3

In P3 amplitude, the main effect of group ($F = 10.419$, $p < 0.001$), trial category ($F = 38.215$, $p < 0.001$) and topographical site ($F = 9.303$, $p = 0.001$) were observed separately, and a significant trial category × topographical site × group interaction ($F = 4.530$, $p = 0.020$) was also observed. Simple effect and *post-hoc* tests revealed that the healthy controls had higher NoGo P3 amplitude than the two patient groups, and the non-AVH patients had higher NoGo P3 amplitude than the AVH patients in all the topographical sites (**Figure 2**, frontal, $p = 0.020$; central, $p = 0.046$; parietal, $p = 0.048$); no significant difference was found of Go P3 amplitude between the three groups; NoGo P3 amplitude was larger than Go P3 amplitude (**Figure 3**), but this effect was not significant in the central brain region of the AVH patients ($p = 0.865$) and in the parietal area of the non-AVH patients ($p = 0.544$); and in all the three groups parietal showed smaller NoGo P3 than the frontal (AVH patients, $p = 0.019$; non-AVH patients, $p = 0.008$; healthy controls, $p = 0.004$) and central (AVH patients, $p < 0.001$; non-AVH patients, $p < 0.001$; healthy controls, $p < 0.001$) areas. No group main effect was found with regard to P3 latency. For detailed results of repeated-measures ANOVA of N2 and P3, see **Supplementary Table**.

DISCUSSION

In line with previous reports (29, 34), the present study also found that the two schizophrenia groups had longer Go RT and lower accuracy than those of the healthy controls, which might indicate that schizophrenia patients have inefficient cognitive processing. No significant difference was found in Go P3 amplitude between the three groups. The NoGo P3 amplitude in healthy controls was larger than the two patient groups, and the AVH patients had the smallest NoGo P3 amplitude, indicating that the inhibitory control was weakened in schizophrenia patients and inhibitory top-down deficits may also be related to AVH.

Inhibitory control is impaired in schizophrenia patients (30, 31). Patients with schizophrenia showed a significantly increased duration of the voluntary response inhibition process compared to healthy controls (35). A meta-analysis using the stop-signal task confirmed an inhibition deficit of moderate size in schizophrenia (36). Furthermore, schizophrenia patients showed a combination of a moderate deficit in response time with a moderate deficit in omission errors (30) in a meta-analysis of research using a go/no-go task, Conners' continuous performance task and sustained attention to response task. There were some circuit abnormalities underlying the response inhibition impairment in schizophrenia (37) and inhibitory control deficits were correlated with poorer prognosis in schizophrenia (38).

Compared to patients with minimal hallucinatory behavior and healthy controls, patients with pronounced hallucinations showed poorer inhibitory top-down control (23). Hugdahl et al., using a dichotic listening paradigm, found that the more frequent the hallucinations appear, the less the patients were able to use cognitive control in the forced-left instruction condition, indicating that patients experiencing AVH fail to use executive functions and cognitive control to avoid their engaging in the "voices" (39). Many neuropsychological studies have found that inhibitory control plays an important role in the AVH of schizophrenia patients (18, 40). Recent findings from neuroimaging studies have revealed that AVH in schizophrenia is associated with alterations in brain structure and function (41–43), which may provide the neural substrates for the production of AVH. Dysfunction in these neural substrates may produce internal auditory signals, and then patients with deficits in top-down control fails to suppress such information, which contributes to the failure to control the frequency and onset of these auditory signals effectively. These results suggest that impaired top-down control is involved in the formation of hallucinations (14, 44).

NoGo N2 may reflect the confirmation and preparation stage of inhibitory control, whereas NoGo P3 may correspond to the execution stage (27, 28). The present study found that, compared with healthy controls and Non-AVH patients, ERP

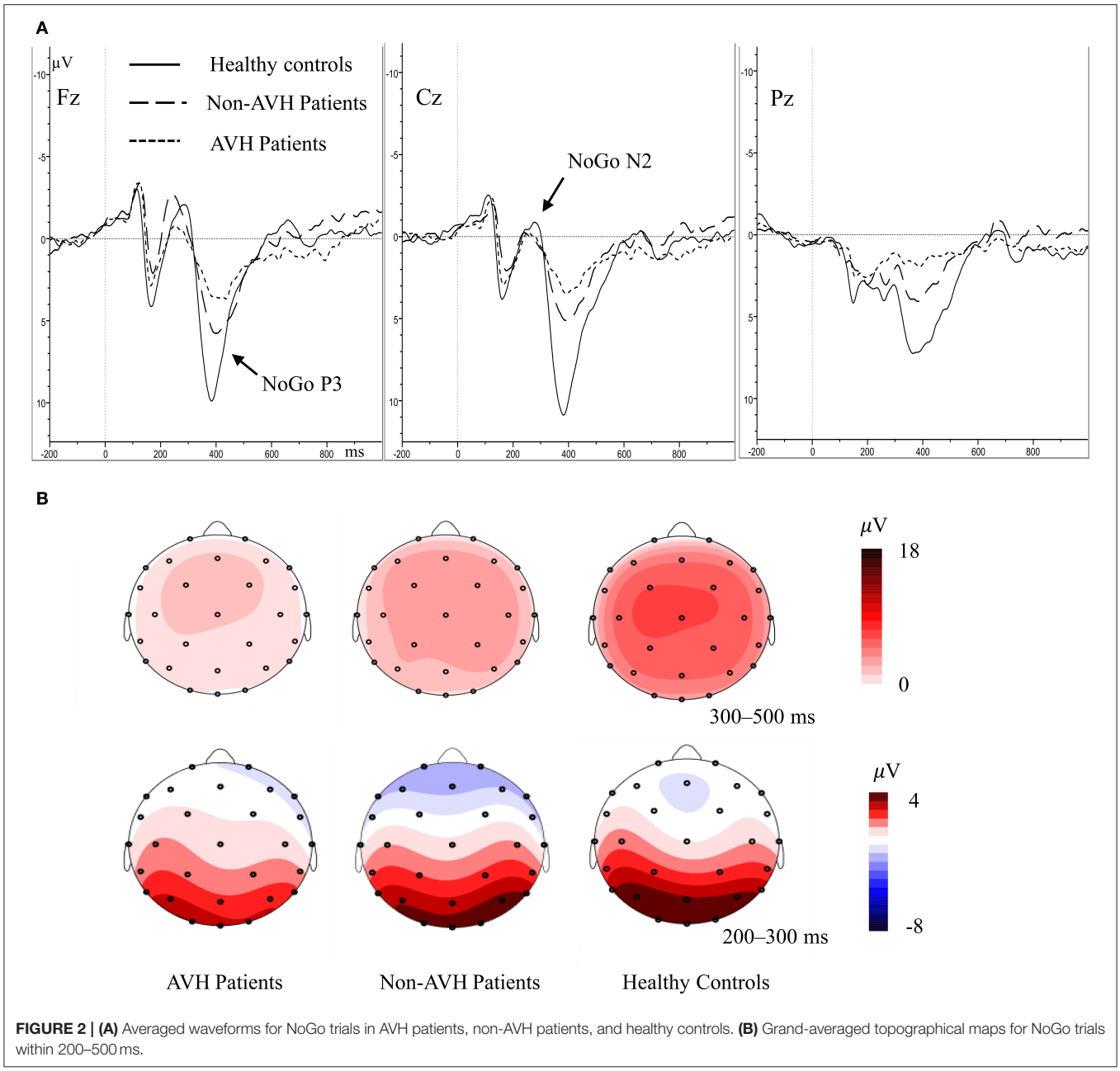

FIGURE 2 | (A) Averaged waveforms for NoGo trials in AVH patients, non-AVH patients, and healthy controls. **(B)** Grand-averaged topographical maps for NoGo trials within 200–500 ms.

abnormalities of AVH patients only appear in P3, which prompts that the neural mechanism of AVH may be related to the late inhibitory control process. The present study found no obvious inter-group difference in N2 latency, P3 latency and N2 amplitude between the AVH patients and healthy controls. RT is substantially prolonged in the AVH patients, but the latency of the P3 component is not, which may also suggest that the RT deficits arise from impairments in a late inhibitory control process (45).

In all the groups, the parietal area showed smaller NoGo P3 amplitude than the frontal and central area, prompting that NoGo P3 was mainly distributed in the frontal-central region, which was consisted with previous studies (29, 46). These findings suggest that AVH in schizophrenia patients may be associated with neuropathological abnormalities in frontal-central brain regions. Compared with non-AVH patients, AVH patients showed larger frontal gray matter volume (47) and decreased connection from the left inferior frontal gyrus to the left middle temporal gyrus (48). An abnormal structural network, including medial/inferior frontal areas, may reflect a neural signature of AVH in the expression of specific characteristics of AVH (49).

The results suggest us that it is possible to develop cognitive remediation that target top-down processing for AVH

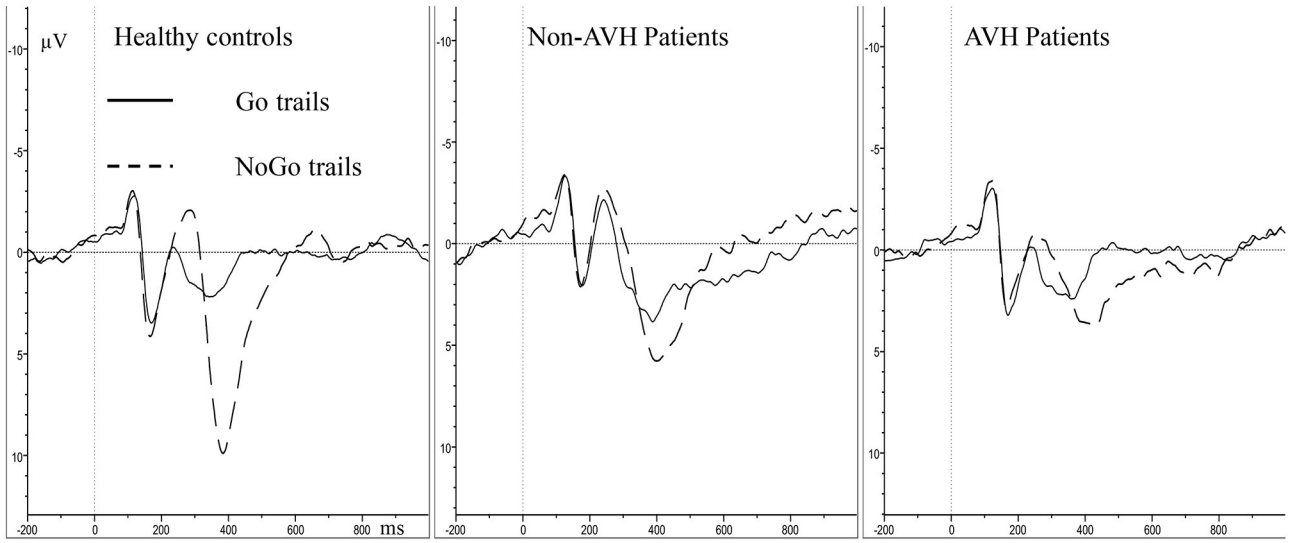

FIGURE 3 | Averaged waveforms at Fz in Go and NoGo trails.

in schizophrenia patients. Basic neuroscience research has elucidated that the behavioral and biological determinants of neurophysiological change mediated by alterations in synaptic connection and neural network function (which termed "neural plasticity"). Additionally studies (50, 51) have shown that neural plasticity in adults requires intensive practice. Cognitive remediation refers to a series of treatments aimed to enhancing neurocognitive abilities, which can be carried out in a "bottom-up," a "top-down" and a non-targeted training perspective (52). Training uses a "top-down" approach can train higher level cognitive processes, and this approach has been combined within broader training environments to simultaneously target both basic perceptual abilities, and higher level executive functions. The study use a computer-based training to enhances verbal memory in schizophrenia via a top-down and bottom-up approach have achieved good results (53). However, it still needs a lot of research to apply the top-down cognitive remediation to AVH in schizophrenia. Because the top-down mechanisms involved in AVH includes not only inhibitory control, but also other demains such as attention, prior knowledge/experience and emotional processes.

The present study has some limitations. Firstly, the sample size was relatively small, and the participants were predominantly male. Secondly, at the stage of research design, we set the education level $>$ 9 years, but unfortunately, we failed to match the education years among the three groups. Although we did covariate analysis, but there is no guarantee that the effect of education level during this population is linear. So the mismatched education may increase the false positive rate of the results. Thirdly, a clinical interview, instead of a structured interview was used in the diagnosis of mental disorders. Hence, the accuracy of the diagnosis might not be optimal. In addition, part of our patients was being prescribed atypical antipsychotic medications. Whether the medicines influenced performance on these tasks is unclear. Therefore, we cannot rule out a possible normalizing effect of medicines and studies of unmedicated patients are required to clarify this.

The present study shows that inhibitory control was impaired in schizophrenia patients. AVH in schizophrenia may also be related to deficits in late inhibitory control process and neuropathological abnormalities in frontal-central brain regions. Our study provided some evidence that inhibitory top-down control may be involved in the occurrence of AVH.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Biomedical Ethics Board of the Second Xiangya Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LT, QS, and XP conceived and designed the experiments. QS, XP, YS, YF, LW, and LT performed the experiments. QS and XP analyzed the data and drafted the manuscript. XP and YF made great contribution to the revision of the manuscript. All authors gave final approvement of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.544746/full#supplementary-material

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