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ORIGINAL ARTICLE

Convergent lines of evidence support *CAMKK2* as a schizophrenia susceptibility gene

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Genes that are differentially expressed between schizophrenia patients and healthy controls may have key roles in the pathogenesis of schizophrenia. We analyzed two large-scale genome-wide expression studies, which examined changes in gene expression in schizophrenia patients and their matched controls. We found calcium/calmodulin (CAM)-dependent protein kinase kinase 2 (*CAMKK*2) is significantly downregulated in individuals with schizophrenia in both studies. To seek the potential genetic variants that may regulate the expression of *CAMKK*2, we investigated the association between single-nucleotide polymorphisms (SNPs) within *CAMKK*2 and the expression level of *CAMKK*2. We found one SNP, rs1063843, which is located in intron 17 of *CAMKK*2, is strongly associated with the expression level of *CAMKK*2 in human brains ($P = 1.1 \times 10^{-6}$) and lymphoblastoid cell lines (the lowest $P = 8.4 \times 10^{-6}$). We further investigated the association between rs1063843 and schizophrenia in multiple independent populations (a total of 130 623 subjects) and found rs1063843 is significantly associated with schizophrenia ($P = 5.17 \times 10^{-5}$). Interestingly, we found the T allele of rs1063843, which is associated with lower expression level of *CAMKK*2, has a higher frequency in individuals with schizophrenia in all of the tested samples, suggesting rs1063843 may be a causal variant. We also found that rs1063843 is associated with cognitive function and personality in humans. In addition, protein–protein interaction (PPI) analysis revealed that *CAMKK*2 participates in a highly interconnected PPI network formed by top schizophrenia genes, which further supports the potential role of *CAMKK*2 in the pathogenesis of schizophrenia. Taken together, these converging lines of evidence strongly suggest that *CAMKK*2 may have pivotal roles in schizophrenia susceptibility.

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INTRODUCTION

Schizophrenia is a severe chronic psychiatric disorder which affects about 1% of the population worldwide. Family, twin and adoption studies have revealed a strong genetic component with estimates of heritability about 80%.[1] So far, numerous molecular genetic studies on schizophrenia have been performed, and many susceptibility genes have been identified through linkage analyses,[2,3] candidate gene association studies,[4,5] genome-wide association studies (GWAS)[6–16] and convergent functional genomics (CFG) analysis.[17] These promising candidates include

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*DISC*1,[18–20] *NRG*1,[21–23] *ZNF804A*,[16,24,25] *TCF4*,[8,15,26] *MIR*137[6] and genes from the MHC region.[14,15]

Though significant progress has been made in the genetic dissection of schizophrenia,[6–16,26] the pathophysiology of schizophrenia is still largely unknown. Alterations in gene expression may have a pivotal role in the pathogenesis of schizophrenia. To seek the potential genes that are differentially expressed between schizophrenia patients and healthy controls, many expression microarrays have been performed and numerous dysregulated genes have been identified.[27–30] However, we noticed that only very limited overlapping genes were identified among different expression studies, which is likely due to the use of different brain regions and quantitative methods. In addition, the quality of the analyzed samples and sample size may also influence the identification of dysregulated genes in individuals with schizophrenia. To identify genes that are consistently dysregulated (downregulated or upregulated) in the prefrontal cortex of schizophrenia patients from different expression studies, we compared the dysregulated genes from two recent expression studies.[27,30] We found three genes (*CAMKK*2, *CACNB*3 and *SNN*) were consistently downregulated in both expression studies, suggesting these genes are likely high-confidence downregulated genes in schizophrenia patients compared with normal controls. In addition, we also identified two genes (*WNK*1 and *ABCA*1) that were consistently upregulated in prefrontal cortex of individuals with schizophrenia in both studies. Among these consistently downregulated and upregulated genes, we focused on *CAMKK*2 as this gene is highly expressed in central nervous system[31] and it is involved in many important brain functions such as learning and memory,[32] two pivotal cognitive functions that are significantly impaired in individuals with schizophrenia.

Accumulating evidence supports that schizophrenia is a neurodevelopmental disorder characterized by abnormal brain development and impaired cognitive function;[33] therefore, genes affect brain development may also contribute risk for schizophrenia. *CAMKK*2 encodes calcium/calmodulin (CAM)-dependent protein kinase kinase 2, an important serine/threonine-specific protein kinase. *CAMKK*2 can promote neurite outgrowth during brain development and inhibition of *CAMKK*2 inhibits neurite outgrowth and causes axonal growth cone collapse.[34,35] In addition, transgenic studies performed in mice have shown that *CAMKK*2 is required for late long-term potentiation (LTP) in the hippocampus and spatial memory formation.[32] Furthermore, previous studies have revealed that *CAMKK*2 is required for the activation of hippocampal cyclic AMP-responsive element-binding protein (CREB) by spatial training,[32] a key step in long-term memory formation. These *in vitro* and transgenic studies consistently support the key roles of *CAMKK*2 in brain function, including neurodevelopment such as neurite outgrowth and cognitive function such as learning and memory. Finally, *CAMKK*2 regulates production of the appetite stimulating hormone neuropeptide Y, an important neuropeptide that has been found associated with schizophrenia,[36,37] further supporting the possible role of *CAMKK*2 in schizophrenia pathogenesis. These findings led us to hypothesize that *CAMKK*2 may be a risk gene for schizophrenia. To elucidate the potential role of *CAMKK*2 in schizophrenia, we systematically studied the association between *CAMKK*2 and schizophrenia by combining expression studies with large-scale genetic association studies. Complementary to our expression and genetic association studies, we also investigated the association between genetic variation in *CAMKK*2 and cognitive functions in humans. Finally, we performed protein–protein interaction (PPI) analysis by using a high-confidence pair-wise protein interaction database. Our consistent and convergent results strongly suggest that genetic variation in *CAMKK*2 may have a role in the etiology of schizophrenia and that *CAMKK*2 may be a new molecular target for anti-neuropsychiatric drugs.

MATERIALS AND METHODS

Expression data used in this study

We selected two well-characterized genome-wide expression studies[27,30] from recent publications, which were performed in individuals with schizophrenia and their matched controls. The first study measured the expression of over 30 000 mRNA transcripts in postmortem tissue from a brain region associated with the pathophysiology of schizophrenia (Brodmann area 10: anterior prefrontal cortex) in two large schizophrenia cohorts.[27] The second study performed a large-scale cross-study analysis of seven microarray data sets comprising a total of 153 schizophrenia samples and 153 normal controls.[30]

The inclusion criteria for the expression studies were as follows: First, we selected those studies that used brain tissues from the prefrontal cortex region. As the basis of cognitive and social behaviors, the prefrontal cortex is one of the best-studied brain regions and impaired function of prefrontal cortex has been consistently reported in individuals with schizophrenia.[38,39] Therefore, genes that are dysregulated in the prefrontal cortex may have crucial roles in the pathogenesis of schizophrenia. Second, expression studies with independent replications were given higher priority. Third, cross-study meta-analysis was also included as the power of meta-analysis is greatly increased due to significantly enlarged sample size. If a gene was found consistently downregulated or upregulated in brain tissues from different expression studies, it may represent an authentic differentially expressed gene in individuals with schizophrenia.

Correlation analysis between SNPs in *CAMKK*2 and the expression level of *CAMKK*2

To investigate the potential impact of the genetic variants in the identified dysregulated genes on their expression level, we examined the association between single-nucleotide polymorphisms (SNPs) within the dysregulated genes and their expression level using expression quantitative trait loci (eQTL) databases. We explored two well-characterized expression databases. The first expression database is Brain Cloud[40] (http://braincloud.jhmi.edu/), which contains genome-wide gene expression and genetic data from the human postmortem dorsolateral prefrontal cortex (DLPFC) of normal subjects ($N = 261$, including 113 Caucasian subjects and 148 African American individuals) across the lifespan. SNP-expression associations were conducted under linear models. The second database is from Genevar[41] (http://www.sanger.ac.uk/resources/software/genevar/). Among Genevar (there are several data sets in Genevar), we used the data set from Stranger *et al.*,[41] which correlated genome-wide gene expression in lymphoblastoid cell lines from a total of 726 individuals from 8 global populations from the HapMap3 project with HapMap3 SNPs located in the region cis to the genes. The number of individuals in each population, mRNA quantification and correlation between expression level and genotype can be found in the original study.[41] Briefly, the populations used in study of Stranger *et al.*[41] are: CEU: 109 Caucasians living in Utah USA, of northern and western European ancestry. CHB: 80 Han Chinese from Beijing, China. GIH: 82 Gujarati Indians in Houston, TX, USA. JPT: 82 Japanese in Tokyo, Japan. LWK: 82 Luhya in Webuye, Kenya. MEX: 45 Mexican ancestry in Los Angeles, CA, USA. MKK: 138 Maasai in Kinyawa, Kenya. YRI: 108 Yoruba in Ibadan, Nigeria.

Case–control subjects

We used the case–control subjects from the Schizophrenia Psychiatric GWAS Consortium (PGC) as our screening sample.[6] The schizophrenia PGC performed a large-scale meta-analysis by combining GWAS data from 17 independent studies. In total, 9394 schizophrenia cases and 12 462 controls from the Schizophrenia PGC were included in this study. All of the samples were of European ancestry. Further information about the study, including diagnostic assessments, genotyping, quality control and association analysis can be found in the original publication.[6]

For replication analysis, we recruited four independent schizophrenia case–control samples and one family sample from different locations. Detailed information on each sample, including diagnostic assessment, genotyping, quality control and association analysis has been published previously.[11,12,15,42–44] Briefly, the five samples included in replication study are: (1) the non-PGC SGENE-plus sample: this sample contained 1932 schizophrenia cases and 92 074 controls. (2) The German sample from MooDS SCZ consortium: this sample consisted of 1332 schizophrenia cases and 866 controls. (3) The Jewish–Israeli sample: this sample comprised 107 schizophrenia families with a total of 331 individuals. (4) The Chinese sample from Shanghai: this sample contained 3750 schizophrenia cases

and 6468 controls. (5) The Chinese sample from Beijing: this sample consisted of 746 schizophrenia cases and 1599 healthy controls. Subjects from non-PGC SGENE-plus, German and Israeli samples were of European ancestry and individuals from the Chinese samples were of Han Chinese origin. All subjects of replication samples showed no overlap with the Schizophrenia PGC samples. In total, 17 154 schizophrenia cases, 113 469 controls and 107 schizophrenia families were included in the meta-analysis. All studies were conducted under the appropriate ethical approvals, and written informed consent was obtained from all subjects.

Genetic association analysis between SNPs in *CAMKK*2 gene and schizophrenia susceptibility

We first investigated the association between rs1063843 and schizophrenia in the Schizophrenia PGC sample. The Schizophrenia PGC performed a meta-analysis of GWAS data sets through analyzing a large data set that consists of 9394 schizophrenia cases and 12 462 controls. All of the samples were genotyped by Affymetrix, Illumina or Perlegen high-throughput genotyping platforms. The association was performed by using logistic regression of imputed dosages with sample identifiers and three principal components as covariates. Detailed information on sample ascertainment and diagnosis, genotyping quality control, genomic control and statistical analyses can be found in the original study.[6] The association significance (*P*-value) between rs1063843 and schizophrenia was extracted from Ricopili (http://www.broadinstitute.org/mpg/ricopili/).

To further validate the association between rs1063843 and schizophrenia, we recruited five independent schizophrenia samples. Genotyping of rs1063843 in the different replication samples was mainly performed by Illumina and Affymetrix platforms. The association of rs1063843 with schizophrenia was conducted by logistic regression using PLINK [v1.07]. More detailed information on genotyping, quality control and statistical analyses can be found in the Supplementary Data.

Finally, we performed a meta-analysis by combining the genetic association data from six samples. The meta-analysis was conducted in the R package (metafor module) and PLINK (v1.07) using the Mantel–Haenszel method with fixed effects (inverse variance).

Cognitive function studies

We investigated the association between rs1063843 and cognitive functions in two independent samples. The first sample contained patients with schizophrenia (DSM-IV diagnosed) and healthy subjects from Ireland (282 cases and 85 controls). All participants were of European ancestry. Cognitive functions known to be impaired in schizophrenia were assessed, including IQ, episodic memory, working memory and attention. Detailed information on sample description, cognitive and behavioral data acquisition, genotyping and statistical analysis can be found in the previous papers[45,46] and Supplementary Data.

The second sample consisted of 342 healthy (that is, no history of neurological and psychiatric disorders based on self-reports) Chinese college students from Beijing Normal University. Among them, 197 subjects were females and 145 individuals were males. Their age ranged from 18 to 23 years old. This experiment was approved by the IRB of the State Key Laboratory of Cognitive Neuroscience and Learning at Beijing Normal University, China. Written consent was obtained from all participants after a full explanation of the study procedure. Cognitive and behavioral measures included working memory, executive functions (assessed with the Attention Network Test, the Wisconsin Card Sorting Task, and a reversal learning test), IQ, personality and motivation traits (for example, intrinsic and extrinsic motivation) and so on. Detailed information on cognitive and behavioral data acquisition, genotyping and statistical analysis can be found in the previous studies[47,48] and Supplementary Data and Supplementary Table S1.

Linkage disequilibrium analysis

Linkage disequilibrium (r^2) between rs1063843 and the other studied SNPs (rs3794207 and rs1140886) was calculated using genotype data (CEU) from the 1000-genomes project and the Haploview[49] program. Haplotype blocks were defined according to the criteria of Gabriel *et al.*[50] (confidence intervals).

PPI analysis and assessment of the significance of the PPI network

To investigate whether CAMKK2 participates in the protein–PPI network that is formed by top schizophrenia susceptibility genes, we constructed a PPI network using the high-confidence protein interaction database (InWeb interaction database) developed by Lage *et al.*[51] InWeb contains 169 801 high-confidence pair-wise interactions that are defined by a rigorously tested signal to noise threshold by comparison with the well-established interactions from protein interaction databases, including MINT, BIND, IntAct and KEGG annotated PPIs (PPrel).

We selected two well-characterized data sets that contained top schizophrenia susceptibility genes. The first data set consisted of schizophrenia candidate genes that reached genome-wide significance level in recent GWASs of schizophrenia (Supplementary Table S2). The second data set was from a recent work of Ayalew *et al.*[17] Ayalew *et al.*[17] identified and prioritized 42 top schizophrenia candidate genes (Supplementary Table S3) by using a translational CFG approach, which was first developed by Niculescu *et al.*[52] CFG integrates GWAS data with other genetic and gene expression studies in humans and animal models. As CFG integrated many pivotal data sets from schizophrenia studies, the genes identified by Ayalew *et al.*[17] represent high-confidence candidate genes for schizophrenia. In fact, the CFG method was proved to be a powerful and promising approach to identify the psychosis-associated genes.[53–57] The top schizophrenia genes from these two data sets represent promising candidate genes for schizophrenia. Therefore, it is important to investigate whether *CAMKK*2 gene is involved in the network that is formed by proteins encoded by top schizophrenia susceptibility genes.

Protein products of top schizophrenia susceptibility genes (Supplementary Table S4) were used as seed proteins. If there is *in vitro* evidence of physical interaction between two seed proteins, these two proteins are linked by one edge. In a PPI network, the nodes represent proteins, while the edges represent physical interaction. DAPPLE (Disease Association Protein–Protein Link Evaluator, http://www.broadinstitute.org/mpg/dapple/dapple.php) was used to extract and reconstruct the PPI network.[58] Permutation testing was utilized to assess the significance of the network built from PPI data. More detailed information about PPI network construction and significance evaluation can be found in previous studies.[58,59]

*CAMKK*2 expression analysis in human tissues

We explored the temporal-spatial expression pattern of *CAMKK*2 in human tissues by using different sets of expression data. We first investigated the tissue-specific expression distributions of *CAMKK*2 in human tissues in Gene Enrichment Profiler, a database that contains gene expression data for 126 different cell types/tissues.[60] We also examined the expression profiling of *CAMKK*2 in Gene Atlas (http://biogps.org/#goto=welcome) database. Finally, we investigated the expression pattern of *CAMKK*2 in brain regions by utilizing the Human Brain Transcriptome[61] and the Brain Cloud databases.[40] More detailed information can be found in the Supplementary Data.

RESULTS

*CAMKK*2 is consistently downregulated in individuals with schizophrenia in different expression studies

To identify genes that were consistently downregulated in individuals with schizophrenia from different expression studies, we compared two well-characterized expression studies. The first study is from the work of Maycox *et al.*[27] Maycox *et al.*[27] first analyzed brain tissues from the anterior prefrontal cortex (Brodmann area 10; BA10) from 28 schizophrenia patients and compared these with 23 healthy control samples from the same region. They then compared their results with an independent study that is from the Harvard Brain Bank,[62] in which brain samples from the DLPFC (Brodmann area 9) were used. Maycox *et al.*[27] finally identified 49 genes that showed the same direction of disease-associated regulation in their samples and Harvard Brain Bank samples. Among these genes, 33 showed significant downregulation and 16 showed significant upregulation in schizophrenia subjects in both studies. The second study is from the recent work of Mistry *et al.*[30] Mistry *et al.*[30] conducted a cross-study meta-analysis of seven microarray data sets comprising a total of 153 schizophrenia samples and 153 normal controls. Their meta-analysis revealed 39 probes that are

consistently upregulated in individuals with schizophrenia and 86 probes that are significantly downregulated across studies.

By comparing the dysregulated genes identified by Maycox *et al.*[27] and Mistry *et al.*,[30] we identified five consistently dysregulated genes that showed the same direction of disease-associated regulation in both studies. Among these consistently dysregulated genes, three genes (*CAMKK*2, *CACNB*3 and *SNN*) were consistently downregulated in individuals with schizophrenia in both studies (that is, expression studies from Maycox *et al.*[27] and Mistry *et al.*[30]). Two genes (*WNK*1 and *ABCA*1) were consistently upregulated in schizophrenia patients in both studies. The *CACNB*3 gene encodes a regulatory beta subunit of the voltage-dependent calcium channel.[63] The *SNN* gene encodes the stannin protein, a highly conserved, 88-amino acid small protein.[64] *WNK*1, also known as WNK lysine deficient protein kinase 1, encodes a cytoplasmic serine–threonine kinase that is expressed in the distal nephron.[65] The *ABCA*1 gene encodes ATP-binding cassette transporter ABCA1, which is a major regulator of cellular cholesterol and phospholipid homeostasis.[66] The *CAMKK*2 gene encodes the CAM-dependent protein kinase kinase 2,[67] a member of the serine/threonine-specific protein kinase family. *CAMKK*2 has a critical role in the CAM-dependent kinase cascade by phosphorylating the downstream kinases CAM kinase I (*CAMK*1) and CAM kinase IV *(CAMK*IV). One study also showed the enzyme encoded by *CAMKK*2 can phosphorylate AMP-activated protein kinase.[68] The *CAMKK*2 gene has the strongest expression in brain[31] and it influences signaling cascades that are involved with learning and memory,[32,69] neuronal differentiation and migration,[70] neurite outgrowth[34,35,70] and synapse formation.

rs1063843 is strongly associated with the expression level of *CAMKK*2

To investigate the genetic mechanisms underlying the observed dysregulation of these five identified genes in individuals with schizophrenia, we first explored the potential association between genetic variants in these genes and their expression level in BrainCloud,[40] a well-characterized expression database based on brain tissues from the DLPFC of normal subjects. As dysregulation of these five identified genes was observed in the prefrontal cortex of schizophrenia patients, the BrainCloud (which used brain tissues from DLPFC) is an excellent resource to investigate whether the genetic variants in these genes are associated with their expression. To seek the most promising genetic variants that may regulate the expression level of these identified dysregulated genes, we only considered SNPs that were located within the gene (that is, exon and intron regions) or in potential regulatory regions (50 kb upstream and downstream flanking sequences). The threshold *P*-values of the potential cis-regulatory eQTL SNPs were set to 0.01.

We found no SNPs in *WNK*1, *CACNB*3 and *ABCA*1 showed significant association with their expression level based on above criteria. Therefore, these three genes were not considered for further study. For *SNN*, we found one SNP, rs7194034, which is located in 3′-UTR region, is significantly associated with the expression of *SNN* ($P = 0.003$). For *CAMKK*2, We found rs1063843, which located in intron 17 of *CAMKK*2 (Supplementary Figure S1), is significantly associated with the expression level of *CAMKK*2 in DLPFC of normal subjects ($P = 1.1 \times 10^{-6}$) (Figure 1a). In addition to rs1063843, two other SNPs (rs1140868 and rs3794207) also showed significant association with the expression of *CAMKK*2 in human brain (Supplementary Table S5).

To further validate the association between rs7194034 and *SNN* expression, and the correlations between rs1063843, rs1140886, rs3794207 and the expression level of *CAMKK*2, we investigated the associations between these identified SNPs and the expression level of *SNN* and *CAMKK*2 in Genevar,[41] which used lymphoblastoid cell lines from 726 Hapmap3 individuals. We found rs7194034 is not associated with the expression of *SNN* in lymphoblastoid cell lines (Supplementary Figure S2). Thus, *SNN* gene was not considered for further study.

Interestingly, we found rs1063843 is also highly significantly associated with the expression level of *CAMKK*2 in all of the populations except for GIH (82 Gujarati Indians in Houston, TX, USA) ($P = 0.079$) (Figure 1b). In addition, rs3794207 and rs11140886, which showed significant association with *CAMKK*2 expression in human brain tissues, are significantly associated with the expression level of *CAMKK*2 in lymphoblastoid cell lines in several populations from Hapmap (Supplementary Figures S3 and S4). Taken together, these consistent data support these three SNPs (rs1063843, rs3794207 and rs1140886) are significantly associated with the expression level of *CAMKK*2, suggesting these three SNPs may be authentic eQTL markers for the *CAMKK*2 gene.

rs1063843 is significantly associated with schizophrenia

Considering that the expression of *CAMKK*2 was significantly downregulated in schizophrenia patients and three SNPs (rs1063843, rs1140886 and rs3794207) are significantly associated with *CAMKK*2 expression, we hypothesized that these three eQTL SNPs may be associated with schizophrenia. To test this conjecture, we performed genetic association studies in multiple independent populations. We first tested the association between these eQTL SNPs (that is, rs1063843, rs1140886 and rs3794207) and schizophrenia in the Schizophrenia PGC sample (9394 cases and 12 462 controls). We found rs3794207 is not significantly associated with schizophrenia in this sample ($P = 0.40$). However, rs1063843 and rs1140886 showed suggestive association ($P = 0.029$ for rs1063843 and $P = 0.055$ for rs1140886) with schizophrenia in the Schizophrenia PGC sample (Table 1). As rs1063843 and rs1140886 are in high linkage disequilibrium ($r^2 = 0.78$ in CEU, based on data from the 1000-genomes project), we only followed-up rs1063843, which has the smallest *P*-value. To further validate the association between rs1063843 and schizophrenia, we performed a replication study in five independent schizophrenia samples: the non-PGC SGENE-plus sample, a German sample, a Jewish–Israeli sample, and two Chinese samples from Shanghai and Beijing, respectively. Intriguingly, we found rs1063843 is also significantly associated with schizophrenia in non-PGC SGENE-plus sample (1932 schizophrenia cases and 92 074 controls) ($P = 0.0032$) (Table 1). In the German sample (1332 schizophrenia cases and 866 controls), rs1063843 also showed a trend of association ($P = 0.078$) (Table 1). In the Jewish–Israel sample, the association between rs1063843 and schizophrenia did not reach significance level ($P = 0.53$), which is likely due to the small sample size (only 107 families), as the odds ratio is very close to those of the non-PGC SGENE-plus and the German samples. In the Chinese samples, the association did not reach significance ($P = 0.10$ for Shanghai sample and $P = 0.80$ for Beijing sample) (Table 1). More importantly, compared with controls, we found the T allele of rs1063843, which is associated with lower expression level of *CAMKK*2 (Figure 1), has a higher frequency in individuals with schizophrenia in all of the studied samples (Table 1), suggesting that the T allele of rs1063843 may be a causal risk variant (or it is highly linked with the causal variant) for schizophrenia. To further verify our results, we performed meta-analysis by combining all of the six studied samples. A test of heterogeneity showed there was no heterogeneity among the six studied samples ($P = 0.55$) (Table 1). The results of meta-analysis indicated rs1063843 is significantly associated with schizophrenia ($P = 5.17 \times 10^{-5}$) (Table 1), which strongly suggests that genetic variation in the *CAMKK*2 gene confers risk of schizophrenia.

rs1063843 and cognitive performance

Accumulating data indicate that schizophrenia susceptibility genes also influence cognitive function in humans.[45,71–73] Given

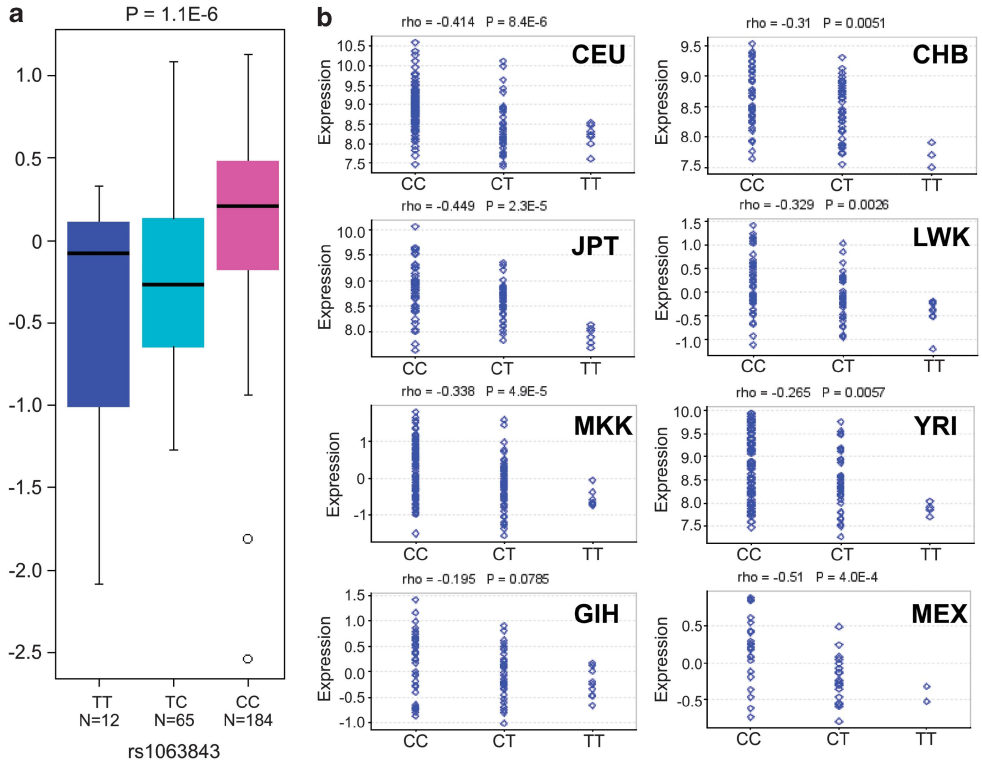

Figure 1. rs1063843 is significantly associated with the expression level of calcium/calmodulin-dependent protein kinase kinase 2 (*CAMKK2*). (**a**) rs1063843 is significantly associated with the expression level of *CAMKK2* in human postmortem dorsolateral prefrontal cortex (DLPFC) of normal subjects across the lifespan ($P = 1.1 \times 10^{-6}$). The individuals with TT genotype have lower *CAMKK2* expression level. (**b**) rs1063843 is significantly associated with *CAMKK2* expression in lymphoblastoid cell lines from individuals from 7 global populations. In GIH, rs1063843 also showed a trend of association ($P = 0.079$). TT genotype carriers have lower *CAMKK2* expression level in both DLPFC and lymphoblastoid cell lines.

Table 1. rs1063843 (in *CAMKK2* gene) is significantly associated with schizophrenia

		Polymorphism				
SNP ID	*Sample (cases/controls)*	*Allele 1*	*Allele 2*	*MAF*[a]	*OR*[b]	P-*value*[c]
rs1063843						
	Schizophrenia PGC (9394/12 462)	T	C	T (0.22)	1.06	$\mathbf{2.92 \times 10^{-2}}$
	Non-PGC SGENE-plus (1932/92 074)	T	C	T (0.19)	1.17	$\mathbf{3.16 \times 10^{-3}}$
	German (1332/866)	T	C	T (0.21)	1.14	7.76×10^{-2}
	Jewish–Israeli (107 families)	T	C	T (0.23)	1.17	5.29×10^{-1}
	Shanghai (3750/6468)	T	C	T (0.29)	1.07	1.04×10^{-1}
	Beijing (746/1599)	T	C	T (0.28)	1.02	8.03×10^{-1}
	Meta-analysis (107 families, 17 154/113 469)[d]	T	C	T (0.20)	1.08	$\mathbf{5.17 \times 10^{-5}}$

Abbreviations: CAMKK2, calcium/calmodulin-dependent protein kinase kinase 2; MAF, minor allele frequency; OR, odds ratio.
Test of heterogeneity, $P = 0.55$. Statistically significant *P*-values (< 0.05) are shown in bold. [a]MAF in controls. [b]OR is based on allele1. [c]Two-tailed *P*-values. [d]Meta-analysis was performed based on a fixed-effects model.

the important roles of *CAMKK2* in hippocampal function and CREB activation,[32,74] we hypothesized that *CAMKK2* may also associate with cognitive function in humans. First, previous studies have shown that the activation of CREB is required for long-term memory formation.[75–80] Intriguingly, CAMKK2 can phosphorylate CAM kinase I (CAMKI) and CAM kinase IV (CAMKIV) to increase the activity of these kinases, which then can activate CREB by phosphorylation at Serine 133.[81,82] A transgenic study has shown that loss of *CAMKK2* reduced CREB activation in mouse hippocampus and impaired spatial memory formation.[32] Second, *CAMKK2* also regulates the transcription of brain-derived neurotrophic factor, an important regulator of cognitive function.[83] Loss of *CAMKK2* gene resulted in decreased expression of brain-derived neurotrophic factor in both mRNA and protein level in mouse cerebellar granule cell neurons.[74] Considering the crucial role of CAMKK2 in CREB and brain-derived neurotrophic factor activation, it is likely that *CAMKK2* may regulate cognitive function in humans. To test this

hypothesis, we performed cognitive studies in independent samples.

We first investigated the association between rs1063843 and cognitive function in the Irish sample. We found rs1063843 is nominally associated with working memory ($F = 5.9$, $P = 0.02$) (Supplementary Table S6). Working memory is one of the best-studied cognitive functions known to be impaired in schizophrenia, therefore, the suggestive association between rs1063843 and variation in working memory performance in Irish sample implied that *CAMKK*2 may be an authentic schizophrenia susceptibility gene.

We further studied the impact of rs1063843 on cognitive function in the Chinese sample and found rs1063843 is nominally associated with executive functions (the Wisconsin Card Sorting Task) ($P = 0.03$) (Supplementary Table S7). In addition, rs1063843 also showed suggestive association with a motivation trait (that is, extrinsic motivation) ($P = 0.02$) (Supplementary Table S7). Finally, we analyzed SNPs around rs1063843 and their potential impact on cognitive function. Again, we found several other SNPs in *CAMKK*2 were also nominally associated with cognitive function in normal subjects. For example, we found rs1140886, which is also located in intron 17 of *CAMKK*2 (151 bp downstream of rs1063843) (Supplementary Figure S1) and is linked with rs1063843 ($r^2 = 0.68$ in CHB), was significantly associated with working memory ($P = 0.0079$) (Supplementary Table S7). Besides, rs2686346 (in intron 7) showed suggestive association with IQ (performance subscale) ($P = 0.019$) and scores on the reversal learning test ($P = 0.0013$) (Supplementary Table S7). Taken together, these results suggest that genetic variants (including rs1063843) in *CAMKK*2 may also be associated with cognitive performance (that is, working memory and executive function) in humans. Considering that these cognitive functions (for example, working memory and executive function) were impaired in schizophrenia patients, the suggestive association between genetic variation in *CAMKK*2 and cognitive performance provides further evidence that *CAMKK*2 may have pivotal roles in the pathogenesis of schizophrenia.

*CAMKK*2 participates in a highly interconnected PPI network formed by top schizophrenia genes

Recent studies support the notation that perturbations to a common but limited set of underlying molecular processes or pathways may modulate risk to schizophrenia.[59,84] To further test whether *CAMKK*2 is involved in schizophrenia, we constructed a PPI network by using the top schizophrenia genes (Supplementary Table S2–S4). If *CAMKK*2 is an authentic schizophrenia susceptibility gene, it may participate in the common molecular network that is formed by proteins encoded by top schizophrenia susceptibility genes.

We found the top schizophrenia susceptibility genes encode a densely interconnected PPI network (Figure 2). We tested this degree of interconnectivity by permutation ($n = 10\,000$ permutations) and found the direct PPI network of genes from these two data sets had significantly more edges than expected by chance ($P = 9.9 \times 10^{-5}$, corrected) (Figure 2). Intriguingly, we found *CAMKK*2 participates in the highly interconnected network formed by top schizophrenia susceptibility genes (Figure 2), implying *CAMKK*2 is involved in the common molecular network that modulates risk to schizophrenia. These PPI data further support the potential role of *CAMKK*2 gene in the pathogenesis of schizophrenia.

*CAMKK*2 is preferentially expressed in human brain tissues

Schizophrenia is a mental disorder that mainly originates from abnormal brain function, thus, if *CAMKK*2 is an authentic schizophrenia susceptibility gene, it may be expressed in the human brain. To test the biological plausibility of *CAMKK*2 in the pathogenesis of schizophrenia, we investigated temporal-spatial expression profiling of *CAMKK*2 in multiple human tissues. As expected, we found *CAMKK*2 is preferentially expressed in human brain tissues (Figure 3a). The expression level of *CAMKK*2 is very low or not detected in non-neuronal tissues, for example, immune and gastrointestinal tissues (Supplementary Figure S5). We further examined *CAMKK*2 expression in human brain tissues and found *CAMKK*2 is abundantly expressed in cerebellum, cerebellum peduncles, frontal cortex and whole brain (Figure 3b). Analysis of the expression of *CAMKK*2 in another expression database (Gene Atlas) revealed similar results (Supplementary Figure S6). Temporal expression analysis showed that the expression level of *CAMKK*2 is relatively low at early developmental stages (fetal age). As development continues, the expression of *CAMKK*2 is gradually increased in human brain (Figures 3c and d). Our expression analysis indicates that *CAMKK*2 is preferentially expressed in human brain tissues, which further support the potential role of *CAMKK*2 in brain function and schizophrenia susceptibility.

In summary, we present convergent and consistent evidence that supports *CAMKK*2 as a schizophrenia susceptibility gene. Considering that this evidence arises from different sources, for example, expression data from different groups of schizophrenia patients, genetic association data from six independent schizophrenia samples, cognitive data from European and Chinese populations, PPI and temporal-spatial expression data from PPI and brain transcriptome analyses, their convergence strongly supports the important roles of *CAMKK*2 in the pathogenesis of schizophrenia. Our results may also provide new insight into the pathogenesis of schizophrenia and a potential therapeutic target for schizophrenia.

DISCUSSION

Schizophrenia is a complex mental disorder that affects millions of individuals worldwide. Genetic studies, especially the advent of GWAS, have greatly promoted the progress of schizophrenia research. Though many promising candidate genes have been identified, the pathogenesis of schizophrenia is still largely unknown. Genes that are differentially expressed between schizophrenia patients and healthy controls may have key roles in the pathogenesis of schizophrenia. Therefore, investigating the most consistently differentially expressed genes may provide us with important information about the pathogenesis and potential treatment of schizophrenia. In this study, we present convergent lines of evidence that support *CAMKK*2 as a schizophrenia susceptibility gene. First, *CAMKK*2 is downregulated in the PFC of schizophrenia patients from different expression studies, suggesting it may be an authentic dysregulated gene in individuals with schizophrenia. Second, we found a genetic variant (rs1063843) in *CAMKK*2 that is highly associated with the expression level of *CAMKK*2 in human brains (DLPFC). Third, we further confirmed the association between rs1063843 and expression level of *CAMKK*2 in lymphoblastoid cell lines. These consistent results strongly support that rs1063843 is significantly associated with the *CAMKK*2 expression. Fourth, we found that rs1063843 is associated ($P = 0.029$) with schizophrenia in the Schizophrenia PGC sample. We further validated this association in replication samples. In fact, meta-analysis results indicate that rs1063843 is significantly associated with schizophrenia after Bonferroni correction ($P = 5.17 \times 10^{-5}$) for the three SNPs tested (uncorrected $P = 5.17 \times 10^{-5}$). Considering *CAMKK*2 expression is significantly downregulated in schizophrenia patients and that rs1063843 is an eQTL SNP, this consistent association data provide further support for the implication of *CAMKK*2 in schizophrenia. Fifth, we noticed the T allele of rs1063843, which is associated with lower expression level of *CAMKK*2, has a higher frequency in individuals with schizophrenia in all of the tested samples. This is quite interesting and strongly suggests rs1063843 may be a causal SNP or it is highly linked with a causal variant that contributes to

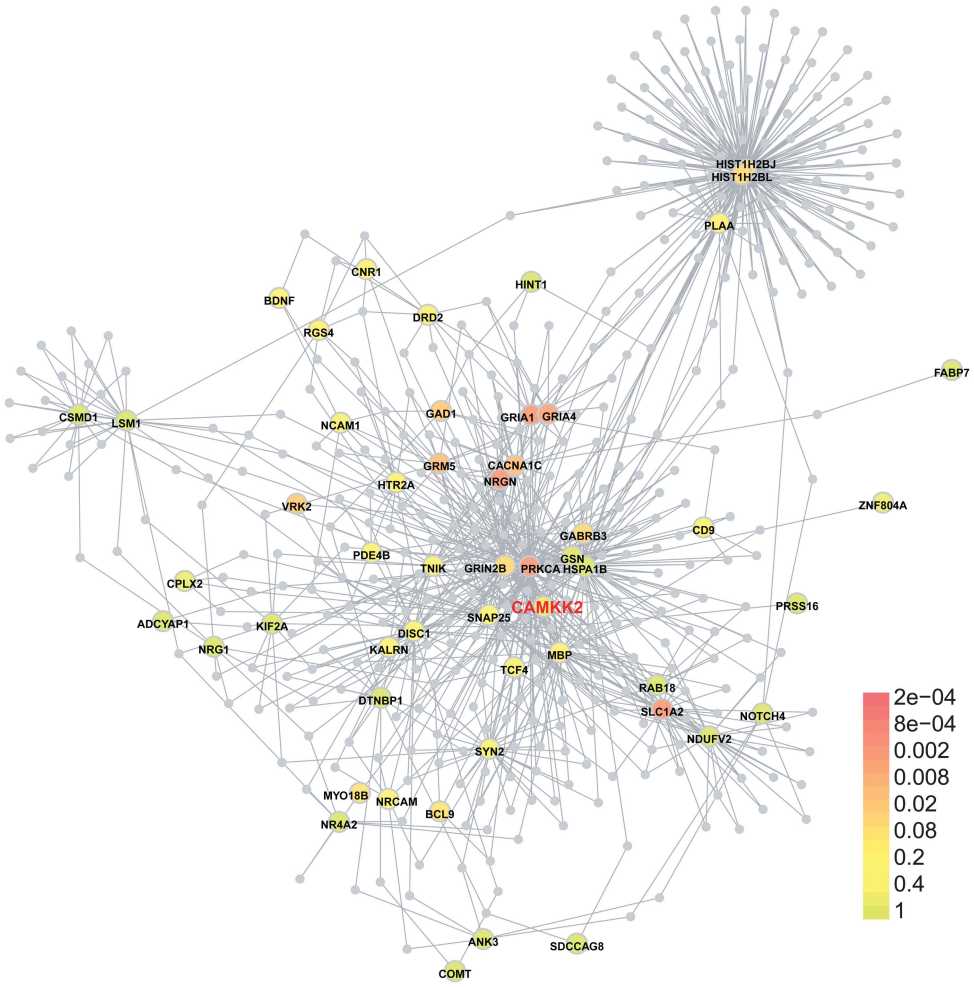

Figure 2. Calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) participates in a densely interconnected Protein–protein interaction (PPI) network formed by top schizophrenia susceptibility genes. Top genes from convergent functional genomics (CFG) and genome-wide association studies of schizophrenia were used to construct the PPI network. Proteins encoded by top schizophrenia susceptibility genes formed a highly interconnected network and CAMKK2 participates in this network, suggesting *CAMKK*2 is an important schizophrenia susceptibility gene that is involved in common molecular network that modulate risk to schizophrenia. The direct connectivity network is statistically highly significant (has more edges) compared with 10 000 random networks ($P = 9.9 \times 10^{-5}$, corrected), suggesting perturbations of common underlying molecular processes or pathways that modulate risk to schizophrenia.

risk of schizophrenia. Sixth, our cognitive data indicate that rs1063843 is also nominally associated with working memory in humans. Given that schizophrenia is a mental disorder with significant brain dysfunction and multiple previous studies have shown that schizophrenia susceptibility genes also influence cognitive function in humans, the suggestive association between rs1063843 and cognitive function provide further evidence that *CAMKK*2 is involved in schizophrenia. Seventh, our PPI analysis provides additional evidence that supports *CAMKK*2 as a schizophrenia susceptibility gene. Eighth, temporal-spatial expression analysis of *CAMKK*2 in human brain tissues also supports the potential role of *CAMKK*2 in schizophrenia. Finally, the study of animal models provides further support that *CAMKK*2 is associated with schizophrenia. *CAMKK*2 has the highest expression level in brain and it is associated with learning and memory.[32] Most importantly, in addition to dysregulation in schizophrenia patients, recent studies have revealed that *CAMKK*2 is also dysregulated in animal model of schizophrenia. Papaleo *et al.*[85] found the expression level of *CAMKK*2 was also significantly downregulated in *DTNBP*1-null mutant mice. Taken together, these evidences, both from gene expression studies in humans and animal models, consistently support the implication of *CAMKK*2 in the pathogenesis of schizophrenia. Therefore, the downregulation of *CAMKK*2 may have a role in impaired learning and memory of schizophrenia patients.

In addition, we also used the CFG method that is developed by Niculescu *et al.*[52] to evaluate the possible role of *CAMKK*2 in schizophrenia. To better understand the genetic and molecular mechanisms underlying psychosis, Niculescu *et al.*[52] developed the CFG approach to identify and prioritize top psychosis susceptibility genes by using multiple independent lines of evidence. We noticed the gene expression data from animal models and humans have pivotal roles in CFG. In fact, we found *CAMKK*2 has a high CFG score (5.0) when analyzed by CFG method, further supporting *CAMKK*2 is implicated in the pathogenesis of schizophrenia. Interestingly, we found *CAMK2A*, a member of the CAM-dependent protein kinases subfamily, was ranked in the top schizophrenia candidate genes identified by Le-Niculescu *et al.*,[86] which further supports that CAM kinase cascade may have important roles in schizophrenia susceptibility.

It should be noted that the two expression studies used in this study were not completely independent as Mistry *et al.*[30]

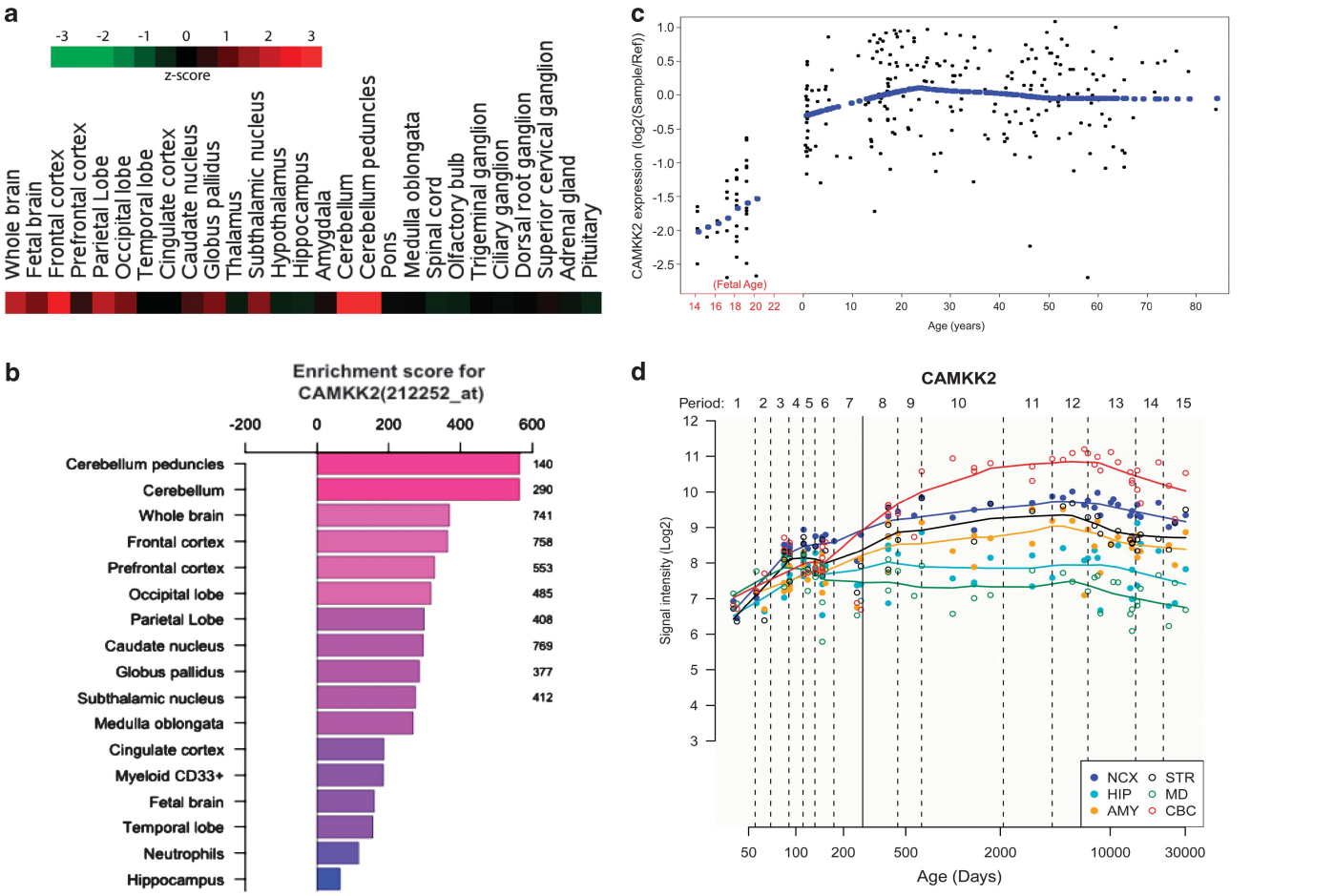

Figure 3. Temporal-spatial expression profiling of calcium/calmodulin-dependent protein kinase kinase 2 (*CAMKK2*) in human brain tissues. (**a**) *CAMKK2* is abundantly expressed in human brain tissues, with the highest expression level in cerebellum and frontal cortex. (**b**) Expression of *CAMKK2* is enriched in human brain tissues, the cerebellum, whole brain and frontal cortex have the highest enrichment scores. (**c**) Temporal expression profile of *CAMKK2* in human postmortem dorsolateral prefrontal cortex (DLPFC). The expression level of *CAMKK2* in human brain is relatively low at early developmental stage. As development continues, *CAMKK2* expression level is increased. (**d**) Temporal expression pattern of *CAMKK2* in different human brain regions. AMY, amygdala; CBC, cerebellar cortex; HIP, hippocampus; MD, mediodorsal nucleus of the thalamus; NCX, neocortex; STR, striatum.

performed a meta-analysis, and they included the study of Maycox *et al.*[27] However, as Mistry *et al.*[30] also used five other independent expression studies and only three genes (*CAMKK2*, *CACNB3* and *SNN*) were found to be consistently downregulated in individuals with schizophrenia in both studies (that is, expression studies from Maycox *et al.*[27] and Mistry *et al.*[30]), it suggests that the other five independent expression studies also support the downregulation of *CAMKK2* in schizophrenia cases. If the downregulated genes identified by Maycox *et al.*[27] were not supported by the other independent expression studies, their expression level will not be significantly downregulated in Mistry *et al.*'s[30] meta-analysis. For example, except for *CAMKK2*, *CACNB3* and *SNN*, many other downregulated genes identified by Maycox *et al.*[27] were not significantly downregulated in Mistry *et al.*'s[30] meta-analysis. Therefore, the differentially expressed genes identified in both studies may represent high-confidence dysregulated genes in schizophrenia patients compared with controls. Furthermore, our findings that genetic variant (rs1063843) in *CAMKK2* is associated with the expression level of *CAMKK2* and genetic association results also support the possible role of *CAMKK2* in pathogenesis of schizophrenia.

We identified three SNPs (rs1063843, rs1140886 and rs3794207) that are significantly associated with the expression level of *CAMKK2* in human brains and lymphoblastoid cell lines. However, only rs1063843 showed significant association with schizophrenia in Schizophrenia PGC sample. We therefore performed linkage disequilibrium analysis. We found rs1063843 is not linked with rs3794207 ($r^2 = 0.09$) in Europeans (Supplementary Figure S7). However, rs1063843 showed some degree of genetic linkage with rs1140886 ($r^2 = 0.78$) (Supplementary Figure S7). These results imply the causal risk variant for schizophrenia may be strongly correlated with rs1063843.

The suggestive associations between genetic variation in *CAMKK2* and cognitive performance are intriguing. However, more work is needed to validate this as rs1063843 only showed nominally significant association with cognitive performance. In summary, these consistent and convergent evidence support that *CAMKK2* is a novel schizophrenia susceptibility gene.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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