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RESEARCH ARTICLE

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A molecule-based genetic association approach implicates a range of voltage-gated calcium channels associated with schizophrenia

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Traditional genome-wide association studies (GWAS) have successfully detected genetic variants associated with schizophrenia. However, only a small fraction of heritability can be explained. Gene-set/pathway-based methods can overcome limitations arising from single nucleotide polymorphism (SNP)-based analysis, but most of them place constraints on size which may exclude highly specific and functional sets, like macromolecules. Voltage-gated calcium (Ca_v) channels, belonging to macromolecules, are composed of several subunits whose encoding genes are located far away or even on different chromosomes. We combined information about such molecules with GWAS data to investigate how functional channels associated with schizophrenia. We defined a biologically meaningful SNP-set based on channel structure and performed an association study by using a validated method: SNP-set (sequence) kernel association test. We identified eight subtypes of Ca_v channels significantly associated with schizophrenia from a subsample of published data ($N = 56{,}605$), including the L-type channels ($Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$), P-/Q-type $Ca_v2.1$, N-type $Ca_v2.2$, R-type $Ca_v2.3$, T-type $Ca_v3.1$, and $Ca_v3.3$. Only genes from $Ca_v1.2$ and $Ca_v3.3$ have been implicated by the largest GWAS ($N = 82{,}315$). Each subtype of Ca_v channels showed relatively high chip heritability, proportional to the size of its constituent gene regions. The results suggest that abnormalities of Ca_v channels may play an important role in the pathophysiology of schizophrenia and these channels may represent appropriate drug targets for therapeutics. Analyzing

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subunit-encoding genes of a macromolecule in aggregate is a complementary way to identify more genetic variants of polygenic diseases. This study offers the potential of power for discovery the biological mechanisms of schizophrenia.

KEYWORDS

channels, molecule-based GWAS, schizophrenia, SKAT, SNP-sets

1 | INTRODUCTION

Schizophrenia is a highly heritable complex disease (Lichtenstein et al., 2009). The biological underpinnings of schizophrenia remain an enigma, making prevention difficult and delaying development of better treatment alternatives (Van Os & Kapur, 2009). Recently, advances in technology and the establishment of an international consortium, the Psychiatric Genomics Consortium (PGC), have made it possible to perform genome-wide association studies (GWAS) involving more than a hundred thousand individuals. The latest study from PGC has reported 108 independent genomic regions associated with schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). However, the variants identified can only explain a small fraction of the estimated heritability (Giusti-Rodríguez & Sullivan, 2013; Goldstein 2009; Ripke et al., 2013; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), and the functional consequences of these variants remain largely uncharacterized. These problems may originate from inherent limitations of the GWAS methodology: The mass univariate testing approach requires an extremely stringent significance threshold to control false positives, thus reducing power; Genetic heterogeneity further complicate interpretation in large meta-analysis; Connecting SNP markers to the causal variants they represent is not straightforward; And, robust, efficient methods for detecting interactions among genetic variants remain elusive.

Gene-based, and gene-set/pathway-based methods provide promising alternatives to overcome certain limitations of GWAS (Askland, Read, O'Connell, & Moore, 2012). Typically, genetic variants within or near to a gene are aggregated and tested for associations with a disease (Liu et al., 2010). Gene-set/pathway-based analyses aggregate functionally related genes, providing a potentially powerful and biologically oriented bridge between genotypes and phenotypes (Ramanan, Shen, Moore, & Saykin, 2012; Wang, Li, & Hakonarson, 2010). These methods, complementary to GWAS, have several advantages: they can reduce the number of tests performed; they may reduce the impact of genetic heterogeneity across cohorts; and they can facilitate the interpretation of findings. On the other hand, they also have limitations: genes typically work in concert with one another (Liu et al., 2010), thus gene-based methods cannot take into account the joint effect among genes; the organization of pathways is typically derived from experiments of model organisms or predicted from mathematical models so uncertainties may be present (Bauer-Mehren, Furlong, & Sanz, 2009); the mechanism of the pathways is rarely clear (Khatri, Sirota, & Butte, 2012); and most published gene-set/pathway analyses place constraints on size from 10 to a few 100 genes (Ramanan et al., 2012). Restriction to pathways with more than 10 genes may exclude highly specific and potentially informative functional SNP-sets, like macromolecules.

A macromolecule is a very large molecule created by polymerization of multiple smaller subunits. Voltage-gated calcium (Ca_v) channels that belong to macromolecules are pore-forming membrane proteins involved in diverse physiological processes including depolarization of neuronal action potentials, neurotransmitter release, neuronal excitability, and intracellular signaling (Simms & Zamponi, 2014). Before interesting GWAS findings emerged, they have already received considerable physiological investigations in psychiatric and neurological disorders due to their importance to brain function (Catterall, 2000; Simms & Zamponi, 2014). Ca_v channels are key mediators of calcium entry into neurons (Turner, Anderson, & Zamponi, 2011) and calcium signaling is involved in major molecular hypothesis of schizophrenia such as dopamine, glutamatergic, and GABAergic hypothesis (Lidow, 2003). In fact, calcium signaling dysfunction has been suggested as a unifying pathological mechanism in schizophrenia (Lidow, 2003). Thus, Ca_v channels gene variants are of large interest in relationship to schizophrenia and we chose to perform the macromolecular analysis of functional Ca_v channels.

Recently, GWAS have identified several associated neuronal ion channel genes (e.g., *CACNA1C*, *CACNB2*, *CACNA1I*, *KCNB1*, *HCN1*, *CHRNA3*, *CHRNA5*, *CHRNB4*) (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Ripke et al., 2013). In particular, associations at *CACNA1C*, *CACNB2*, and *CACNA1I*, which encode Ca_v channel subunits, extend previous findings implicating members of Ca_v channels in schizophrenia (Hamshere et al., 2013; Ripke et al., 2013). Ca_v channels can either be monomers (one subunit), or heteromultimers (three or four subunits). Although, these subunits physically bind together to form a channel, their encoding genes are located in different regions of a chromosome or even on different chromosomes. For example, in the $Ca_v1.1$ channel (Bannister & Beam, 2013), the α_1 subunit gene *CACNA1S*, $\alpha_2\delta$ subunit gene *CACNA2D1*, β subunit gene *CACNB1*, and γ subunit gene *CACNG1* are located at chromosomal bands 1q32, 7q21-q22, 17q21-q22, and 17q24, respectively (Figure 1). Due to the limitations of gene-based and gene set-based analysis mentioned above, it is possible that taking the macromolecules (Ca_v channels) as a joint entity can explain more for the risk of schizophrenia than one single locus alone.

We defined a SNP-set from single channel genes and investigated how this biologically functional unit is associated with schizophrenia, using the accessible PGC schizophrenia GWAS data ($N = 56{,}605$:

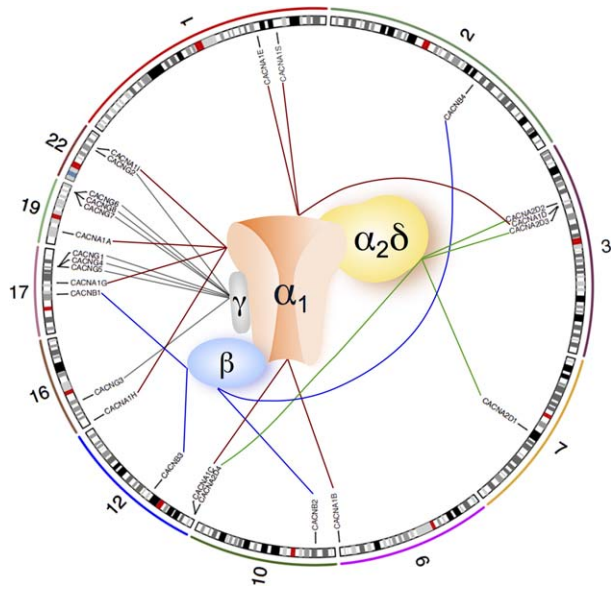

FIGURE 1 Molecular organization of voltage-gated calcium channels and chromosome locations of their subunit-coding genes. Most Ca_v channels are multi-subunit structure (containing three or four subunits, α_1, β, $\alpha_2\delta$, with or without γ subunits), but T-type Ca_v channels only have the α_1 subunit. In one specific channel, the subunits are physically bound together, but their encoding genes are localized far apart or even on different chromosomes. Nine autosomal genes (*CACNA1A*, *CACNA1B*, *CACNA1C*, *CACNA1D*, *CACNA1E*, *CACNA1G*, *CACNA1H*, *CACNA1I*, *CACNA1S*) encode α_1 subunit (connected by red lines), four genes (*CACNB1*, *CACNB2*, *CACNB3*, *CACNB4*) encode β subunits (connected by blue lines), four genes (*CACNA2D1*, *CACNA2D2*, *CACNA2D3*, *CACNA2D4*) encode $\alpha_2\delta$ subunit (connected by green lines), and eight genes (*CACNG1*, *CACNG2*, *CACNG3*, *CACNG4*, *CACNG5*, *CACNG6*, *CACNG7*, *CACNG8*) encode γ subunit (connected by gray lines). The numbers 1, 2, 3, 7, 9, 10, 12, 16, 17, 19, and 22 represent chromosome numbers [Color figure can be viewed at wileyonlinelibrary.com]

25,629 cases and 30,976 controls) divided into a discovery and a replication sample. We applied the SNP-set (sequence) kernel association test (SKAT) (Wu et al., 2010) and identified significant associations in eight subtypes of Ca_v channels ($Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, $Ca_v2.1$, $Ca_v2.2$, $Ca_v2.3$, $Ca_v3.1$, and $Ca_v3.3$). In contrast, only genes (*CACNA1C*, *CACNB2*, and *CACNA1I*) from two subtypes were implicated by the original GWAS despite its larger sample ($N = 82{,}315$). These findings show the potential of the macromolecule approach to identify the possible etiology of diseases, and suggest that abnormalities of Ca_v channels may play an important role in the pathophysiology of schizophrenia.

2 | MATERIALS AND METHODS

2.1 | Ca_v genes

A total of 26 genes encoding subunits of Ca_v channels can be classified into four groups (Table 1) according to the types of subunits they encode (Catterall, 2000; Simms & Zamponi, 2014). Genes *CACNA1A*, *CACNA1B*, *CACNA1C*, *CACNA1D*, *CACNA1E*, *CACNA1F*, *CACNA1G*, *CACNA1H*, *CACNA1I*, *CACNA1S* encode the α_1 subunits; *CACNA2D1*, *CACNA2D2*, *CACNA2D3*, *CACNA2D4* encode the $\alpha_2\delta$ subunits; *CACNB1*, *CACNB2*, *CACNB3*, *CACNB4* encode the β subunits; and *CACNG1*, *CACNG2*, *CACNG3*, *CACNG4*, *CACNG5*, *CACNG6*, *CACNG7*, *CACNG8* encode the γ subunits. We only analyzed genes located on the autosomes, so the gene *CACNA1F* on the X-chromosome was excluded.

2.2 | Genotype data

Due to IRB restrictions from some substudies in PGC, we used the largest accessible PGC schizophrenia data which contains 36 case-control substudies ($N = 56{,}605$; 25,629 cases and 30,976 controls compared

TABLE 1 Gene-level test result from discovery and validation stages

Gene name	Type of encoding subunit	Stage1 BH_SKAT *P*	Stage2 BH_SKAT *P*	Combined dataset BH_SKAT *P*
CACNA1A	α_{1A}	4.66E-01	3.80E-01	2.29E-01
CACNA1B	α_{1B}	7.15E-01	1.75E-01	1.75E-01
CACNA1C	α_{1C}	2.24E-04[a]	2.42E-12[a]	3.07E-18[a]
CACNA1D	α_{1D}	5.85E-01	7.63E-01	5.53E-01
CACNA1E	α_{1E}	4.48E-01	7.49E-02	8.85E-03[a]
CACNA1G	α_{1G}	8.94E-03[a]	1.75E-01	3.41E-03[a]
CACNA1H	α_{1H}	5.60E-01	8.16E-01	3.29E-01
CACNA1I	α_{1I}	3.75E-04[a]	2.32E-04[a]	9.88E-09[a]
CACNA1S	α_{1S}	2.13E-01	2.64E-01	1.26E-01
CACNA2D1	$\alpha_2\delta_1$	5.85E-01	8.42E-02	1.26E-01
CACNA2D2	$\alpha_2\delta_2$	5.60E-01	7.49E-02	1.94E-01
CACNA2D3	$\alpha_2\delta_3$	4.48E-01	8.42E-02	8.01E-02
CACNA2D4	$\alpha_2\delta_4$	4.48E-01	1.60E-01	7.15E-02
CACNB1	β_1	7.15E-01	1.75E-01	1.53E-01
CACNB2	β_2	3.55E-02[a]	6.73E-02	2.41E-05[a]
CACNB3	β_3	4.66E-01	1.92E-01	1.60E-01
CACNB4	β_4	6.68E-01	1.86E-01	1.75E-01
CACNG1	γ_1	6.48E-01	1.75E-01	1.53E-01
CACNG2	γ_2	4.48E-01	1.75E-01	2.91E-01
CACNG3	γ_3	4.66E-01	1.60E-01	1.26E-01
CACNG4	γ_4	8.47E-01	7.49E-02	1.26E-01
CACNG5	γ_5	4.66E-01	1.87E-01	1.26E-01
CACNG6	γ_6	4.66E-01	1.75E-01	4.98E-01
CACNG7	γ_7	5.75E-01	1.75E-01	1.26E-01
CACNG8	γ_8	4.66E-01	2.65E-01	1.75E-01

[a]*p*-Value <.05 after correction. Stage1: discovery phase; Stage 2: validation phase; BH: Benjamini Hochberg; SKAT: SNP-set (sequence) kernel association test.

to 52 sub-studies and $N = 82{,}315$ in the primary study) (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Quality control and imputation were performed by the PGC Statistical Analysis Group for each dataset separately. Briefly, SNP meets with following conditions were retained: SNP missingness <0.05, SNP Hardy–Weinberg equilibrium $p > 1 \times 10^{-6}$ in controls or $p > 1 \times 10^{-10}$ in cases. Samples with missing rate >0.05 were removed. After quality control, the remaining genotypes were imputed using SHAPEIT2/IMPUTE2 (Delaneau, Marchini, & Consortium, 2014; Howie et al., 2012) based on the full 1000 Genomes Project dataset (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). To evaluate the replicability of our analysis, we selected out the data used in the first phase of PGC (PGC1) as a discovery sample (10,616 cases and 10,315 controls), and used the rest as replication sample (15,013 cases and 20,661 controls). In addition, we also used combined samples from both discovery and replication stages. We first merged the best-guessed genotype data (imputation information score >0.8 and minor allele frequency >0.05) across 36 substudies, and then, performed the second round of quality controls using parameters SNP missingness <0.05 and minor allele frequency >0.05. To control the impact of population stratification on our analysis, we computed the first 20 principal components based on the merged and quality controlled genotype data by using the program EigenSoft (Price et al., 2006). Since, some Ca_v genes are close together in genomic position (e.g., *CACNG6*, *CACNG7*, and *CACNG8*), it is possible that some SNPs may be assigned to more than one genes. To avoid such undesired bias, we annotated SNPs to the closest gene (GENCODEv1.9) based on genomic positions that were derived from the human genome assembly build hg19 (Supporting Information Table S8). Then based on the SNPs list, the genotypes of the 25 Ca_v genes were extracted.

Ca_v channels can either be monomers (only the α_1 subunit), or heteromultimers (three subunits α_1, β, $\alpha_2\delta$; or four subunits α_1, β, $\alpha_2\delta$, γ). Great diversity of Ca_v channels allows them to fulfill highly specialized roles in specific neuronal subtypes (Simms & Zamponi, 2014). Thus, for each α_1 subunit (principal subunit for classifying subtypes of Ca_v channels), co-assembly of a variety of ancillary subunits (β, $\alpha_2\delta$, γ) exists (Table 2). In some Ca_v channels, the ancillary subunit types are not completely known. So for channel-level association analysis, we test all of the possible combinations based on the current literatures (Buraei & Yang, 2010; Catterall, 1996; Davies et al., 2010; Hofmann, Flockerzi, Kahl, & Wegener, 2014; Schlick, Flucher, & Obermair, 2010). According to different subunit gene combinations (three or four genes per set), genotypes of the genes consisting of a Ca_v channel were concatenated. Therefore, each SNP-set is corresponding to one functional channel that exists in nature.

2.3 | SNP-set (sequence) kernel association test

SKAT was used to test for association between a set of genetic variants and dichotomous or quantitative phenotypes. It uses the logistic kernel-machine regression modeling framework. SKAT aggregates individual score test statistics of SNPs in a SNP-set and computes SNP-set level *p*-values. SKAT can be used for common or/and rare variants

TABLE 2 Channel-level test results

Channel name	Subunits combination	Stage1 BH_SKAT *P*	Stage2 BH_SKAT *P*	Combined datasets BH_SKAT *P*
$Ca_v1.1$	$\alpha_{1S}\ \beta_1\ \alpha_2\delta_1\ \gamma_1$	4.63E-01	2.78E-02*	3.54E-02*
	$\alpha_{1C}\ \beta_1\ \alpha_2\delta_1$	9.56E-04*	5.85E-12*	1.51E-16*
	$\alpha_{1C}\ \beta_1\ \alpha_2\delta_2$	5.09E-05*	8.42E-14*	1.62E-19*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_1$	6.21E-05*	8.87E-13*	1.31E-20*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_1\ \gamma_1$	6.21E-05*	8.05E-13*	1.16E-20*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_1\ \gamma_2$	5.70E-05*	6.13E-13*	1.13E-20*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_1\ \gamma_3$	6.21E-05*	4.66E-13*	5.56E-21*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_1\ \gamma_4$	6.93E-05*	6.03E-13*	1.07E-20*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_1\ \gamma_5$	6.21E-05*	6.93E-13*	6.59E-21*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_1\ \gamma_6$	6.21E-05*	7.04E-13*	1.31E-20*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_1\ \gamma_7$	6.21E-05*	8.05E-13*	1.13E-20*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_1\ \gamma_8$	6.21E-05*	8.36E-13*	1.13E-20*
$Ca_v1.2$	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_2$	6.66E-06*	8.72E-14*	1.75E-22*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_2\ \gamma_1$	6.66E-06*	8.42E-14*	1.64E-22*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_2\ \gamma_2$	6.66E-06*	8.42E-14*	1.61E-22*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_2\ \gamma_3$	6.66E-06*	8.42E-14*	1.16E-22*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_2\ \gamma_4$	7.22E-06*	8.42E-14*	1.61E-22*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_2\ \gamma_5$	6.66E-06*	8.42E-14*	1.16E-22*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_2\ \gamma_6$	6.66E-06*	8.42E-14*	1.64E-22*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_2\ \gamma_7$	6.66E-06*	8.42E-14*	1.61E-22*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_2\ \gamma_8$	6.66E-06*	8.42E-14*	1.61E-22*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_3$	3.72E-05*	2.65E-12*	2.67E-20*
	$\alpha_{1C}\ \beta_2\ \alpha_2\delta_4$	6.66E-06*	1.49E-13*	1.16E-22*
	$\alpha_{1C}\ \beta_3\ \alpha_2\delta_1$	9.49E-04*	5.85E-12*	1.51E-16*
	$\alpha_{1C}\ \beta_3\ \alpha_2\delta_2$	5.09E-05*	8.42E-14*	1.62E-19*
	$\alpha_{1C}\ \beta_4\ \alpha_2\delta_1$	3.50E-03*	7.39E-11*	8.50E-15*
	$\alpha_{1C}\ \beta_4\ \alpha_2\delta_2$	7.72E-04*	4.67E-11*	6.26E-15*
	$\alpha_{1D}\ \beta_3\ \alpha_2\delta_1$	5.71E-01	6.08E-02	8.46E-02
	$\alpha_{1D}\ \beta_3\ \alpha_2\delta_2$	5.71E-01	1.85E-01	3.93E-01
	$\alpha_{1D}\ \beta_3\ \alpha_2\delta_3$	3.17E-01	5.79E-02	4.26E-02*
$Ca_v1.3$	$\alpha_{1D}\ \beta_3\ \alpha_2\delta_4$	4.39E-01	3.18E-01	1.21E-01
	$\alpha_{1D}\ \beta_4\ \alpha_2\delta_1$	6.41E-01	4.63E-02*	6.17E-02
	$\alpha_{1D}\ \beta_4\ \alpha_2\delta_2$	6.78E-01	1.22E-01	2.00E-01
	$\alpha_{1D}\ \beta_4\ \alpha_2\delta_3$	4.07E-01	4.36E-02*	3.54E-02*
	$\alpha_{1D}\ \beta_4\ \alpha_2\delta_4$	5.71E-01	1.72E-01	9.55E-02
	$\alpha_{1A}\ \beta_1\ \alpha_2\delta_1$	4.99E-01	4.00E-02*	4.42E-02*
	$\alpha_{1A}\ \beta_4\ \alpha_2\delta_1$	5.71E-01	3.28E-02*	3.72E-02*
$Ca_v2.1$	$\alpha_{1A}\ \beta_4\ \alpha_2\delta_2$	5.71E-01	7.90E-02	1.02E-01
	$\alpha_{1A}\ \beta_4\ \alpha_2\delta_3$	3.49E-01	3.27E-02*	2.13E-02*
	$\alpha_{1A}\ \beta_4\ \alpha_2\delta_4$	4.69E-01	1.13E-01	4.99E-02*
	$\alpha_{1B}\ \beta_1\ \alpha_2\delta_1$	6.41E-01	2.34E-02*	3.92E-02*
	$\alpha_{1B}\ \beta_1\ \alpha_2\delta_2$	7.14E-01	3.28E-02*	1.02E-01
	$\alpha_{1B}\ \beta_1\ \alpha_2\delta_3$	3.58E-01	2.34E-02*	2.16E-02*
	$\alpha_{1B}\ \beta_3\ \alpha_2\delta_1$	6.41E-01	2.34E-02*	3.92E-02*
$Ca_v2.2$	$\alpha_{1B}\ \beta_3\ \alpha_2\delta_2$	6.92E-01	3.28E-02*	1.02E-01
	$\alpha_{1B}\ \beta_3\ \alpha_2\delta_3$	3.58E-01	2.34E-02*	2.16E-02*
	$\alpha_{1B}\ \beta_4\ \alpha_2\delta_1$	6.90E-01	2.15E-02*	3.51E-02*
	$\alpha_{1B}\ \beta_4\ \alpha_2\delta_2$	7.66E-01	3.97E-02*	8.89E-02
	$\alpha_{1B}\ \beta_4\ \alpha_2\delta_3$	4.37E-01	2.15E-02*	1.92E-02*
	$\alpha_{1E}\ \beta_1\ \alpha_2\delta_1$	4.25E-01	5.15E-03*	1.47E-03*
$Ca_v2.3$	$\alpha_{1E}\ \beta_2\ \alpha_2\delta_1$	4.53E-02*	4.25E-04*	2.30E-07*
	$\alpha_{1E}\ \beta_3\ \alpha_2\delta_1$	4.25E-01	5.15E-03*	1.47E-03*
	$\alpha_{1E}\ \beta_4\ \alpha_2\delta_1$	4.82E-01	5.15E-03*	1.56E-03*
$Ca_v3.1$	α_{1G}	2.23E-03*	1.28E-01	1.05E-03*
$Ca_v3.2$	α_{1H}	4.82E-01	8.16E-01	3.08E-01
$Ca_v3.3$	α_{1I}	7.31E-05*	3.85E-05*	1.64E-09*

*p-Value <0.05 after corrections. Stage1: discovery phase; Stage 2: validation phase; BH: Benjamini Hochberg; SKAT: sequencing kernel association test.

(Ionita-Laza et al., 2013; Wu et al., 2010, 2011). In the current study, we focus on the common variants in line with the PGC schizophrenia study and used SKAT version 1.07 (Wu et al., 2010). The linear kernel with *beta* (*p*, 1.25), where *p* is the minor allele frequency of a SNP, was used. In our analysis, we carefully selected the cohort indicators and the first six principal components as covariates after comparing results including different number of principal components (3, 6, and 10) (Supporting Information Table S1). At the same time, to overcome the issue of the large number of degrees of freedom, SKAT employs a test that adaptively estimates the degrees of freedom by accounting for correlation (LD) among the SNPs (Wu et al., 2010). In this study, a SNP-set can be a collection of SNPs from a gene or several genes consisting of a heteromeric channel. The Benjamini Hochberg (BH) procedure was used to correct for multiple comparisons both in the Tables 1 and 2 (Hochberg & Benjamini, 1990; Wu et al., 2011).

2.4 | Estimate schizophrenia heritability contributed by Ca_v channels SNPs

Channels significantly associated with schizophrenia (Table 2; Supporting Information Table S6) were selected. For each subtype of Ca_v channel, all of the auxiliary subunit (β, $\alpha_2\delta$, γ) genes contributing to a significant association with schizophrenia were grouped with each α_1 gene. The following gene lists $Ca_v1.1$ (*CACNA1S*, *CACNA2D1*, *CACNB1*, *CACNG1*); $Ca_v1.2$ (*CACNA1C*, *CACNA2D1*, *CACNA2D2*, *CACNA2D3*, *CACNA2D4*, *CACNB1*, *CACNB2*, *CACNB3*, *CACNB4*, *CACNG1*, *CACNG2*, *CACNG3*, *CACNG4*, *CACNG5*, *CACNG6*, *CACNG7*, *CACNG8*); $Ca_v1.3$ (*CACNA1D*, *CACNA2D3*, *CACNB3*, *CACNB4*); $Ca_v2.1$ (*CACNA1A*, *CACNA2D1*, *CACNA2D3*, *CACNA2D4*, *CACNB1*, *CACNB4*); $Ca_v2.2$ (*CACNA1B*, *CACNA2D1*, *CACNA2D3*, *CACNB1*, *CACNB3*, *CACNB4*); $Ca_v2.3$ (*CACNA1E*, *CACNA2D1*, *CACNB1*, *CACNB2*, *CACNB3*, *CACNB4*); $Ca_v3.1$ (*CACNA1G*); $Ca_v3.3$ (*CACNA1I*) were used to extract genotype–phenotype data for estimating chip heritability by using the linear mixed method BOLT-REML (Loh et al., 2015). The level of enrichment for association with schizophrenia was represented by the ratio of proportion of chip heritability (from each subtype of channel) in total heritability (33%) (Ripke et al., 2013) to the proportion of their SNPs in all SNPs (9423850 variants, minor allele frequency >0.05) from the 1000 Genomes Project.

3 | RESULTS

3.1 | Association of Ca_v genes with schizophrenia (gene level)

Two genes, *CACNA1C* and *CACNA1I* significantly associate with schizophrenia in the discovery cohort (corrected $p < .05$) and in the replication cohort (corrected $p < .05$) both according to the SKAT) method (Table 1) and univariate analysis (Supporting Information Table S2). Within the combined sample (56,605 subjects) a further three genes were identified by the SKAT analysis: *CACNA1E*, *CACNA1G*, and *CACNB2*. *CACNA1C*, *CACNA1I*, and *CACNB2* were previously reported, whereas *CACNA1E* and *CACNA1G* have not been reported as schizophrenia candidates (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

3.2 | Association of Ca_v channels with schizophrenia (macromolecule level)

Macromolecule-level testing in the discovery cohort identified heteromers $Ca_v1.2$ (all possible subunits combinations), $Ca_v2.3$ (α_{1E} β_2 $\alpha_2\delta_1$), and monomers $Ca_v3.1$ (α_{1G}) and $Ca_v3.3$ (α_{1I}) as associated (corrected $p < .05$). All of them except $Ca_v3.1$ (α_{1G}) were replicated in the separate samples by SKAT analysis (Table 2). In the combined sample, heteromers $Ca_v1.1$ (α_{1S} β_1 $\alpha_2\delta_1$ γ_1); $Ca_v1.2$ (all possible subunits combinations); $Ca_v1.3$ (α_{1D} β_3 $\alpha_2\delta_3$, α_{1D} β_4 $\alpha_2\delta_3$); $Ca_v2.1$ (α_{1A} β_1 $\alpha_2\delta_1$, α_{1A} β_4 $\alpha_2\delta_1$, α_{1A} β_4 $\alpha_2\delta_3$, α_{1A} β_4 $\alpha_2\delta_4$); $Ca_v2.2$ (α_{1B} β_1 $\alpha_2\delta_1$, α_{1B} β_1 $\alpha_2\delta_3$, α_{1B} β_3 $\alpha_2\delta_1$, α_{1B} β_3 $\alpha_2\delta_3$, α_{1B} β_4 $\alpha_2\delta_1$, α_{1B} β_4 $\alpha_2\delta_3$); and $Ca_v2.3$ (α_{1E} β_1 $\alpha_2\delta_1$, α_{1E} β_2 $\alpha_2\delta_1$, α_{1E} β_3 $\alpha_2\delta_1$, α_{1E} β_4 $\alpha_2\delta_1$), and monomers $Ca_v3.1$ (α_{1G}) and $Ca_v3.3$ (α_{1I}) associate with the risk of schizophrenia (corrected $p < .05$) (Table 2).

3.3 | Chip heritability of Ca_v channels

We estimate that 0.0567% (s.e. 0.0391%), 0.5051% (s.e. 0.1172%), 0.2453% (s.e. 0.0946%), 0.1788% (s.e. 0.0708%), 0.2578% (s.e. 0.0929%), 0.176% (s.e. 0.0658%), 0.0272% (s.e. 0.0316%), and 0.0569% (s.e. 0.0464%) of the variance in schizophrenia can be explained by $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, $Ca_v2.1$, $Ca_v2.2$, $Ca_v2.3$, $Ca_v3.1$, and $Ca_v3.3$ SNPs, respectively (Figure 2a). The $Ca_v1.2$ account for the largest amount of chip heritability (0.5051%, s.e. 0.1172%) and the $Ca_v3.1$ account for the least (0.0272%, s.e. 0.0316%). However, after accounting for the number of SNPs included in each Ca_v subtype, $Ca_v3.1$ and $Ca_v3.3$ show largest fold enrichment (39.83 and 36.51, respectively) (Figure 2b). All tested subtypes of Ca_v channels show more than sixfold enrichment. The variance explained by each subtype of Ca_v channels is proportional to its number of SNPs (Supporting Information Figure S1). This is in line with the previous discovery that the larger the genomic region, the higher the proportion of chip heritability that can be accounted for (Yang et al., 2011).

3.4 | Robustness of the channel-based association

Ca_v channels that are significantly associated with schizophrenia reported by SKAT were also identified by another program MAGMA (de Leeuw, Mooij, Heskes, & Posthuma, 2015) (Supporting Information Tables S4 and S5). However, MAGMA identified fewer channels at the discovery stage compared with SKAT (Table 2; Supporting Information Table S5). But for the largest European dataset (49 substudies), MAGMA reports similar results with SKAT.

4 | DISCUSSION

In the current study, we applied a macromolecule approach to a subsample of published schizophrenia GWAS ($N = 56,605$) and identified eight subtypes of Ca_v channels associated with schizophrenia, including the L-type Ca_v channels ($Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$), P-/Q-type $Ca_v2.1$,

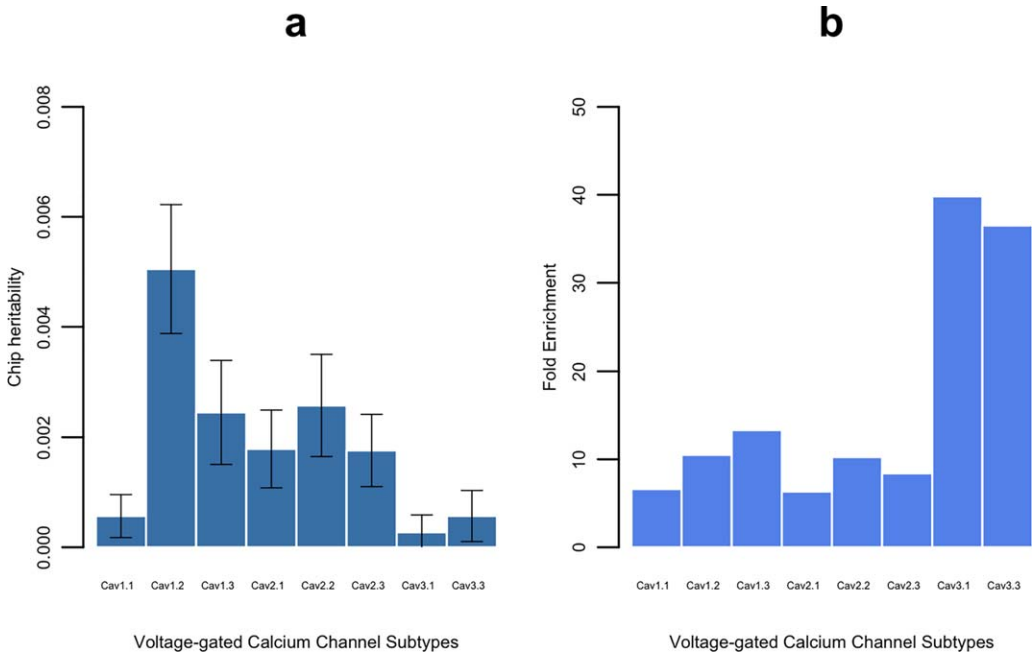

FIGURE 2 Estimates of the schizophrenia variance explained by SNPs from each subtype of Ca_v channels. (a) Chip heritability of each significant subtype of Ca_v channel, (b) fold enrichment of each significant subtype of Ca_v channel in schizophrenia. The fold enrichment is the ratio of the proportion of chip heritability (from each significant subtype of channel) in total heritability (33%) to the proportion of their SNPs in all SNPs (9,423,850 variants, minor allele frequency >0.05) from 1000 Genomes Projects [Color figure can be viewed at wileyonlinelibrary.com]

N-type $Ca_v2.2$, R-type $Ca_v2.3$, T-type channels ($Ca_v3.1$, $Ca_v3.3$). Only genes (*CACNA1C*, *CACNB2*, and *CACNA1I*) from $Ca_v1.2$ and $Ca_v3.3$ were implicated in the primary PGC analysis, which was based on a larger sample ($N = 82{,}315$) (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). In addition, we used another published statistical tool MAGMA to confirm our analysis. The results are highly consistent, although the two programs are based on different assumptions and statistical models. It demonstrates that analyzing macromolecule subunit genes in aggregate is a complementary way to identify more genetic variants of schizophrenia compare to the traditional GWAS that treating each SNP separately.

The macromolecule subunits physically bind together to achieve their cellular functions, thus perturbations of any of their subunits may contribute to disease pathogenesis. In previous, GWAS of schizophrenia, only a handful of channel subunits were implicated, perhaps due to the limited power of the massive univariate tests (Lichtenstein et al., 2009; Ripke et al., 2013; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). To the best of our knowledge, only Askland et al. (2012) have performed an association analysis of ion channels with schizophrenia, but the gene sets defined in their study is a mixture of subunit-encoding genes from many ionic species and does not therefore correspond to a macromolecule existing in nature. In addition, it was tested in a much smaller sample. To test whether each functional Ca_v channel is associated with schizophrenia or not, we composed specific gene set based on molecular structures of Ca_v channels (Buraei & Yang, 2010; Catterall, 1996; Davies et al., 2010; Schlick et al., 2010; Simms & Zamponi, 2014). For each channel (macromolecule-based analysis), although the containing genes locate far away or even on different chromosomes, the encoding subunits are physically binding together in one functional unit to deal with flow of calcium ions. This macromolecule-based approach is different from grouping genes based on their functional catalogs or pathways since their products (proteins) interact directly or indirectly and they could not form a unique functional macromolecule. Our approach combining biological priors with GWAS data identified eight subtypes of Ca_v channels associated with the risk of schizophrenia. It is possible that the associations of whole channels with schizophrenia may be due to a highly associated component gene. This is likely the case for $Ca_v1.2$, where a few possible subunit combinations (e.g., $Ca_v1.2$: α_{1C} β_1 $\alpha_2\delta_2$ that encoded by genes *CACNA1C*, *CACNB1*, and *CACNA2D2*) show their significance thanks to the α_1 subunit gene *CACNA1C* (Table 1; Supporting Information Table S7), although most of the others are not. The significant associations of the other heteromultimeric channels may be not due to a single significant gene. For example, during the discovery and replication stages, the $Ca_v2.3$ channel (subunits encoded by *CACNA1E*, *CACNB2*, and *CACNA2D1*) was discovered and replicated by SKAT but none of their composing genes was identified at the gene-level test. The univariate analysis (min*P* SNP represents channel) could not identify this channel in small samples (discovery and replication stages), but the combined sample could confirm this finding when applying a macromolecule-based approach (Supporting Information Table S3). None of the channels $Ca_v1.1$, $Ca_v1.3$, $Ca_v2.1$, and $Ca_v2.2$ subunit genes was identified in gene-level testing, but the channels show significant association with schizophrenia in the combined sample. These results indicate that subunit genes can collectively associate with disease susceptibility, even if individual genes do not exhibit significant

association. It seems that analyzing channel SNPs as a set can capture the joint effect of multiple variants located on different chromosomes. Thus, genetic variants with weak or moderate effects could be identified when we combined them together based on biological knowledge of the macromolecule.

We also observed enrichment of heritability in significant Ca_v channels SNPs for schizophrenia and it may point to a major role of the inherited genetic variants in the risk of schizophrenia. These eight subtypes of Ca_v channels may provide more knowledge about the pathology of schizophrenia. Ca_v channels are the primary mediators of depolarization-induced calcium entry into neurons (Simms & Zamponi, 2014). Calcium-dependent processes such as neurotransmitter release, neuronal gene transcription, and activation of calcium-dependent enzymes are of critical importance to brain function (Clapham, 2007; Simms & Zamponi, 2014). L-type Ca_v channels ($Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$) are involved in learning, memory, and synaptic plasticity (Moosmang et al., 2005; White et al., 2008; Woodside, Borroni, Hammonds, & Teyler, 2004). Mutations in *CACNA1C*, the gene encoding the α_1 subunit of $Ca_v1.2$, are responsible for Timothy syndrome, a multisystem disorder including cognitive impairment and autism spectrum disorder (Splawski et al., 2004, 2005). SNPs located in *CACNA1C* are linked to development of schizophrenia, bipolar disorder and depression (Dao et al., 2010; Green et al., 2010; He et al., 2014). Data from mice and humans suggest an involvement of $Ca_v1.3$ channels in neurophysiological functions, in particular in the dopaminergic system (Simms & Zamponi, 2014), which is involved in the pathology of schizophrenia (Brisch et al., 2014). Although, in humans, mutations in $Ca_v1.1$ have been linked to hypokalemic periodic paralysis (Ptáček et al., 1994) and malignant hyperthermia (Monnier, Procaccio, Stieglitz, & Lunardi, 1997), a pathway analysis for a set of calcium channel genes implicated *CACNA1S* ($Ca_v1.1$ channel α_1 subunit gene) as one of the 20 gene regions associated in the five psychiatric disorder meta-analysis (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013). P-/Q-type channel $Ca_v2.1$ and N-type channel $Ca_v2.2$ play a role in neurotransmitter release at the presynaptic terminal and in neuronal integration in many neuronal types (Williams et al., 1992). R-type channel $Ca_v2.3$ is strongly expressed in cortex, hippocampus, striatum, amygdala, and interpeduncular nucleus (Parajuli et al., 2012). The T-type channels ($Ca_v3.1$, $Ca_v3.3$) appear to play important roles in regulating neuronal excitability (Simms & Zamponi, 2014). Although, there is no direct evidence associating $Ca_v2.1$, $Ca_v2.2$, $Ca_v2.3$, and $Ca_v3.1$ with schizophrenia, due to their strong expression and wide distribution in the human brain, these four subtypes of Ca_v channels are likely involved in some aspects of schizophrenia pathology. A recent study of rare variants in schizophrenia demonstrated that a gene set containing 26 Ca_v genes yielded a large odds ratio of 8.4 (Purcell et al., 2014). Given the central role of Ca_v channels in regulating neurotransmitter release and neuronal gene transcription, the identified channels may represent convenient drug targets for novel therapeutics. Designing drugs for specific channels by targeting α_1 subunit, or designing more universal drugs for some channels by targeting shared ancillary subunits can improve efficiency of treatments. There are some Ca_v channels blockers in clinical use. A few L-type Ca_v channel antagonists such as verapamil and nifedipine, which are used for hypertension, have been examined in clinical trials in schizophrenia (Lencz & Malhotra, 2015). Revisiting the effect of existing agents on Ca_v channels or designing new drugs could be a high priority for new schizophrenia treatment development.

The genetic association test of macromolecules may also suggest candidates for nonadditive interactions (epistasis) and improve polygenic predictions. In addition, while we only considered Ca_v channels, future work could consider other types of channels, such as potassium channels, sodium channels, and proton channels as interesting susceptibility candidates for schizophrenia and other psychiatric disorders.

The present findings illustrate the power of the macromolecule-based approach applied to schizophrenia, which identified eight subtypes of Ca_v channels associated with the disorder. The results highlight the combined role of different aspects of calcium signaling in schizophrenia pathophysiology, and suggest several new potential drug targets for development of novel therapeutics.

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COMPLIANCE WITH ETHICAL STANDARDS

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Data availability

Schizophrenia genotype data from the Psychiatric Genomics Consortium can be accessed by following the consortium's data policies: https://www.med.unc.edu/pgc/shared-methods

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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