Identification of ultra-rare disruptive variants in voltage-gated calcium channel-encoding genes in Japanese samples of schizophrenia and autism spectrum disorder

Chenyao Wang1,10, Shin-ichiro Horigane2,3,10, Minoru Wakamori4,10, Shuhei Ueda2,3, Takeshi Kawabata5,6, Hajime Fujii7, Itaru Kushima8, Hiroki Kimura1, Kanako Ishizuka1, Yukako Nakamura1, Yoshimi Iwayama8, Masashi Ikeda9, Nakao Iwata9, Takashi Okada4,1, Branko Aleksic2,3, Daisuke Mori8, Takashi Yoshida4, Haruhiko Bito7, Takeo Yoshikawa9, Sayaka Takemoto-Kimura8, and Norio Ozaki1

Several large-scale whole-exome sequencing studies in patients with schizophrenia (SCZ) and autism spectrum disorder (ASD) have identified rare variants with modest or strong effect size as genetic risk factors. Dysregulation of cellular calcium homeostasis might be involved in SCZ/ASD pathogenesis, and genes encoding L-type voltage-gated calcium channel (VGCC) subunits Ca1.1 (CACNA1S), Ca1.2 (CACNA1C), Ca1.3 (CACNA1D), and T-type VGCC subunit Ca3.3 (CACNA1I) recently were identified as risk loci for psychiatric disorders. We performed a screening study, using the Ion Torrent Personal Genome Machine (PGM), of exon regions of CACNA1S, CACNA1C, CACNA1D, and CACNA1I in 370 Japanese patients with SCZ and 192 with ASD. Variant filtering was applied to identify biologically relevant mutations that were not registered in the dbSNP database or that have a minor allele frequency of less than 1% in East-Asian samples from databases; and are potentially disruptive, including nonsense, frameshift, canonical splicing site single nucleotide variants (SNVs), and non-synonymous SNVs predicted as damaging by five different in silico analyses. Each of these filtered mutations were confirmed by Sanger sequencing. If parental samples were available, segregation analysis was employed for measuring the inheritance pattern. Using our filter, we discovered one nonsense SNV (p.C1451* in CACNA1D), one de novo SNV (p.A36V in CACNA1C), one rare short deletion (p.E1675del in CACNA1D), and 14 NSstrict SNVs (non-synonymous SNV predicted as damaging by all of five in silico analyses). Neither p.A36V in CACNA1C nor p.C1451* in CACNA1D were found in 1871 SCZ cases, 380 ASD cases, or 1916 healthy controls in the independent sample set, suggesting that these SNVs might be ultra-rare SNVs in the Japanese population. The neuronal splicing isoform of Ca1.2 with the p.A36V mutation, discovered in the present study, showed reduced Ca2+-dependent inhibition, resulting in excessive Ca2+ entry through the mutant channel. These results suggested that this de novo SNV in CACNA1C might predispose to SCZ by affecting Ca2+ homeostasis. Thus, our analysis successfully identified several ultra-rare and potentially disruptive gene variants, lending partial support to the hypothesis that VGCC-encoding genes may contribute to the risk of SCZ/ASD.

Translational Psychiatry (2022) 12:84; https://doi.org/10.1038/s41398-022-01851-y

INTRODUCTION

Schizophrenia (SCZ) is a severe, chronic, and common psychiatric disorder that is characterized by psychotic symptoms such as hallucinations and delusions; the prevalence of SCZ is estimated to be 1% [1]. Autism spectrum disorders (ASDs) are a range of conditions characterized by repetitive patterns of behavior and interests, as well as persistent deficits in social communication and interaction [2]. SCZ/ASD both have been implicated to have a high heritability that is estimated as 60–90% from population-based and twin studies [3, 4]. Recently, genomic studies coupled with large-scale collaborative projects have identified hundreds of common and rare mutations that contribute to SCZ/ASD [5–8].

Voltage-gated calcium channels (VGCCs) are transmembrane proteins that are activated by depolarization, triggering Ca2+ influx into neurons and other excitable cells [9]. VGCCs are composed of a pore-forming α1 subunit and auxiliary αδ and β subunits [10]. The α1 subunit is encoded by CACNA1 genes, including CACNA1C and CACNA1D; both of these genes have been associated with SCZ/ASD, as elucidated by several previous genetic and biological studies [5, 9–12]. Proper VGCC activity...
underlies many essential physiological functions, governing neuronal activity, sensory functions, and muscle contraction [9]. Notably, CACNA1C and CACNA1D are two genes that are expressed predominantly in the nervous system; the encoded Ca²⁺-permeating α₁ subunits (Caₐ1.2 and Ca₀ₑ.₃, respectively) are components of channels that are located at postsynaptic somatodendritic sites [13]. Several mouse studies have provided important insights into the roles of these proteins in the pathophysiology of psychiatric disease. For example, decreased expression of Caₐ1.2 in the forebrain has been shown to cause anxiety-like behavior in mice, suggesting that changes in expression or function of this protein may contribute to neuropyschiatric anxiety [12].

Timothy Syndrome is a rare multi-organ disease caused by a de novo missense mutation (p.G406R) in CACNA1C, and surviving patients may develop syndromic autism [14, 15]. Knock-in mice expressing p.G406R CACNA1C show behavioral traits reminiscent of autistic symptoms in the social domain as well as in the repetitive/restricted behavior domain [16–18].

Recent genetic studies utilizing next-generation sequencing implicated common and rare genetic variations in VGCC-encoding genes in the etiology of psychiatric disorders such as SCZ/ASD [5, 19–26]. For instance, several large-scale genome-wide association studies (GWASs) identified CACNA1C as a critical candidate susceptibility gene in multiple psychiatric disorders, including SCZ, autism, bipolar disorder (BD), and major depression [11]. Whole-exome sequencing studies (WESs) identified recurrent de novo mutations in CACNA1D in patients with ASD [23]. The role of other VGCC-related genes, including CACNA1I and CACNA1S, in SCZ/ASD remains unexplored, in spite of genetic evidence supporting the contribution of these loci to SCZ/ASD in WESs, GWASs, and genetic association studies [5, 27–29]. Furthermore, how these genetic variations associated with SCZ/ASD directly alter biophysical channel properties and causally affect SCZ/ASD pathophysiology remains unknown.

To address these issues, we report here a genetic and biological study that sought to discover possible disease-associated, rare, single-nucleotide variants (SNVs) with potentially damaging effects, in four VGCC-encoding genes, including CACNA1C, CACNA1D, CACNA1I, and CACNA1S. We first sequenced the exonic regions of CACNA1C, CACNA1D, CACNA1I, and CACNA1S in Japanese patients with SCZ/ASD, and then performed association studies for prioritized variants to detect rare, potentially disruptive SNVs. Our analysis identified a novel de novo SNV, p.A36V CACNA1C, in a SCZ patient. We investigated the functional consequence of this mutation on the voltage-gated calcium channel, via both in-silico three-dimensional (3D) structure prediction modeling and electrophysiological patch-clamp recording. The neuronal splicing isoform of Caₐ1.2 with the p. A36V mutation showed reduced Ca²⁺-dependent inhibition, presumably resulting in excessive Ca²⁺ entry through the mutant channel. Thus, our analysis successfully identified an ultra-rare and functionally significant variant in VGCC genes, supporting the hypothesized role of these loci in the risk of SCZ/ASD.

METHODS

Subjects

Three independent sample sets were used in this study. The first set, comprising samples from 370 patients with SCZ (mean ±SD age = 49.73 ± 14.75 years; males = 52.97%) and 192 patients with ASD (mean age = 16.34 ± 8.36 years; males = 77.60%), was sequenced for rare point mutations. The second, larger set, comprising samples from 1871 patients with SCZ (mean age = 48.59 ± 14.12 years; males = 51.84%), 380 patients with ASD (mean age = 18.97 ± 9.89 years; males = 76.48%), and 1916 controls (mean age = 44.95 ± 15.16 years; males = 52.07%), was used for association analysis of selected variants detected in the first set. For further analysis of variants detected in the first and second set, we included an independent sample set composed of 2398 patients with SCZ (mean age = 52.06 ± 13.63 years; males = 58.55%) and 3679 controls (mean age = 40.60 ± 13.42 years; males = 39.14%).

All participants in this study were recruited in the Nagoya University Hospital and its associated Institutes. Patients were included in the study if they met DSM-5 criteria for SCZ or ASD and were physically healthy. Healthy controls were selected from the general population and had no personal or family history of psychiatric disorders (first-degree relatives only, based on an interview with the subject). Selection was based on the following: questionnaire responses from the subjects themselves during the sample inclusion step, or an unstructured diagnostic interview conducted by an experienced psychiatrist during the blood collection step. All subjects were unrelated, lived in the central area of the Honshu island, and self-identified as members of the Japanese population.

The Ethics Committees of the Nagoya University Graduate School of Medicine and Research Institute of Environmental Medicine approved this study. All experiments were performed in accordance with the Committee’s guidelines and regulations. Written informed consent was obtained from all participants. In addition, each patient’s capacity to provide consent was confirmed by a family member when needed. Individuals with a legal measure of reduced capacity were excluded.

Resequencing and data analysis

Genomic DNA was extracted from whole blood or saliva using the QIAGEN QIAamp DNA blood kit or tissue kit (QIAGEN, Hilden, Germany). Custom amplification primers were designed, using Ion AmpliSeq Designer (Thermo Fisher Scientific, Waltham, MA, USA), to cover coding exons and flanking introns of the selected genes. Sample amplification and equalization were achieved using Ion AmpliSeq Library Kits 2.0 and the Ion Library Equalizer Kit, respectively (Thermo Fisher Scientific). Amplified sequences were ligated with Ion Xpress Barcode Adapters (Thermo Fisher Scientific). Emulsion PCR and subsequent enrichment were performed using the Ion OneTouch Template Kit v2.0 on Ion OneTouch 2 and IonOneTouch ES, respectively (Thermo Fisher Scientific). The final product then was sequenced on the Ion PGM sequencing platform (Thermo Fisher Scientific). Raw data output from the sequencer was deposited in the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp) under Accession Number DRAA004490, and uploaded to the Torrent Server (Thermo Fisher Scientific) for variant calling, with NCBI GRCh37 as a reference. The resulting VCF (variant call format) files were analyzed by Ingenuity Variant Analysis (QIAGEN) for annotation and visualization. Combined Annotation Dependent Depletion (http://cadd.gs.washington.edu/) was applied for annotation of genetic variants.

Prioritization and association analysis

Missense mutations, small insertions/deletions, and splicing site variations with a minor allele frequency <1% were selected from the annotated data. The mutation calls then were validated for confidence by Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). Genotyping prioritization was based on whether the mutant was: 1) located in a functional domain or motif of the protein, according to the Human Protein Reference Database (http://www.hprd.org), Pfam (http://pfam.xfam.org/), and existing literature, or functionally important, such as causing a frame shift, stop gain, or cysteine gain/loss; 2) a rare variant with a minor allele frequency <1%, or novel or registered in the NCBI dbSNP database (Build 137) (http://www.ncbi.nlm.nih.gov/snp/), the 1000 Genomes Project (http://www.1000genomes.org/), the Exome Variant Server of NHLBI GO Exome Sequencing Project (ESP650005-V2) (http://evs.gs.washington.edu/evs/), the Human Genetic Variation Database of Japanese genetic variant consortium (http://www.genome.med.kyoto-u.ac.jp/SnpDB), or the Integrative Japanese Genome Variation database (https://igvdb.megabank.tohoku.ac.jp/); and 3) predicted to represent a non synonymous protein-terminating mutation by at least one of five in silico algorithms (PolyPhen2 HumDiv and HumVar [30], LRT [31], MutationTaster [32], and SIFT [33]). Additional in silico analysis, including conservation status, was performed for the prioritized SNVs using HomoloGene (http://www.ncbi.nlm.nih.gov/homologene).

Custom TaqMan SNP (single nucleotide polymorphism) genotyping assays were designed and ordered from Applied Biosystems. Allelic discrimination analysis was performed on an ABI PRISM 7900HT Sequence Detection System (Thermo Fisher Scientific). Allele and genotype frequencies of the mutations were compared between patients with SCZ and controls, or between patients with ASD and controls, using Fisher’s exact test (two-tailed), with a threshold of significance set at p < 0.05.
Identification of de novo SNVs

We examined the inheritance pattern of the prioritized SNVs if parental samples were available. For cases with de novo SNVs, we performed paternity testing using an Identifier plus kit for 15 short tandem repeats (AmpliFSTR Identifier plus kit, Thermofisher Scientific), and each parental genotype was consistent with each of their child’s genotypes. The amplification was performed using an ABI 3130 Genetic Analyzer (Thermo Fisher Scientific) and genotyped automatically using GeneMapper ID v3.2 software (Thermo Fisher Scientific). The probabilities for calculated paternity supported the conclusion that parental samples were collected from the proband’s biological parents. Among parents of cases with de novo SNVs, all were psychiatrically healthy based on self-reporting or questionnaire responses.

Plasmid construction and mutagenesis

Human brain and human cerebral cortex total RNA (Takara Bio, Kusatsu, Japan) or human heart total RNA (Takara Bio) was reverse-transcribed using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific). Human CACNA1C isoforms were amplified by PCR using Platinum SuperFi DNA polymerase (Thermo Fisher Scientific) and the following primer pairs: the short and neuronal CACNA1C isoform was amplified using forward, CGGGCGCTTCACTGGCCATGGGCTGGTTCAG, and reverse, GGCCCGTGCACCTTCAAGGCTGCTGACGTAGA; the long and cardiac CACNA1C isoform was amplified using forward, CTGGAGTCCAGCTGGCAGGCTGCATTG, and reverse, GGCCCGGATCCTCAAGGCTGCTGACGGCTAGA; the long and cardiac CACNA1C isoform was amplified using forward, CTGGAGTCCAGCTGGCAGGCTGCATTG, and reverse, GGCCCGGATCCTCAAGGCTGCTGACGGCTAGA. The resulting PCR fragment was inserted into a pCAG vector [34]. The clones prepared from human brain and cerebral cortex total RNA were identified as CACNA1C isoform 14 in the RefSeq database (NM_00112940.1) and used as neuronal (exon 1-containing short) isoform clones in this study. The clones from human heart total RNA were identified as CACNA1C isoform X30 (XM_006719017.2) and used as cardiac (exon 1a-containing long) isoform in this study. We introduced the point mutation corresponding to p.A36V into CACNA1C-WT or pCAG-CACNA1C-A36V plasmids using T-remarkGENE 9 DNA Transfection Reagent. After 24 hours, transfected cells were fixed for 15 min with PBS containing 4% paraformaldehyde, permeabilized for 10 min with 0.2% Triton X-100, and then 10 min with 5% goat normal serum and 1% bovine serum albumin in PBS to block nonspecific antibody binding. After pretreatment, cells were incubated overnight with rabbit polyclonal anti-Ca,1.2 antibody (1:1000, Alomone Labs) in blocking solution, and then stained for 1 hour with an AlexaFluor 488-conjugated anti-rabbit IgG goat antibody (1:1000, Thermo Fisher Scientific) and Hoechst 33342 (Thermo Fisher Scientific) in PBS. All images were taken using a LSM 710 confocal microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with a 20×/0.8 numerical aperture (NA) objective. To evaluate Ca,1.2 trafficking to the plasma membrane, we defined the regions of interest (ROIs) of the plasma membrane based on membrane-tethering red fluorescent protein (RFP-KrasCT) images using an intensity threshold. Cytoplasmic ROIs were defined as inside regions of the plasma membrane ROIs excluding nuclear regions. For background subtraction, mean Ca,1.2 intensity from each ROI was used to calculate Ca,1.2 intensity ratio (membrane/cytoplasm). Statistical comparison between WT and A36V mutant channels was performed by two-tailed Welch’s t test.

Modelling of the 3D structure of the Ca,1.2 calcium channel and its N-terminus

We performed homology modeling of Ca,1.2 using three-dimensional (3D) structures homologous to Ca,1.2, as obtained from the Protein Data Bank (PDB, version 2021/11/17) using PSI-BLAST [36] with the help of the HOMCOS server [37]. We focused here on three structures: the rabbit Ca,1.1 channel (PDB ID:5gjw; 78% protein sequence identity to Ca,1.2) [38], the cockroach Na, channel (PDB ID:6a95; 32% protein sequence identity to Ca,1.2) [39], and a complex of CaM and the N-terminal spatial Ca”-transforming element (NSCaTE)region of Ca v1.2 (PDB ID:5gjw; 78% protein sequence identity to Ca,1.2) [38], and each parental sample with genotyping automatically using GeneMapper ID c). The N-terminal region (amino acids 1-89) was predicted to form an intrinsically disordered region using DISOPRED 3.16 [41], we hypothesized that the N-terminal region might be able to assume several structures (Fig. S4). Details of the modeling are described in Supplementary Information. The model of the folded N-terminal structure is shown in Fig. S3, and the overall aligned regions are shown graphically in Fig. S1. Because the most similar structure (5gjw) does not cover the mutated residue 36, we employed a chimeric template. We observed that the conformations of these structures were inconsistent in the N-terminal region; notably, the conformation of the NSCaTE region is α-helical in 5gjw, but assumes a β-like structure in 6a95 (see Figs. S2 and S3). Given that the N-terminal region (amino acids 1-89) was predicted to form an intrinsically disordered region using DISOPRED 3.16 [41], we hypothesized that the N-terminal region might be able to assume several different conformations, and therefore decided to build models of two different structures: a folded N-terminal structure and a CaM-binding structure. The template of the folded N-terminal structure was generated by combining the structure 5gjw and the N-terminal structure of 6a95 (Figs. S1, S3b). The template of the CaM-binding structure also was generated by combining the structure 5gjw and the N-terminal structure of 6a95, but in this case the NSCaTE region (47-68) was taken from 2lqc chain B (Figs. S1, S3c). The combination of several structures was performed using MATRAS [42] and an in-house Python script. Using the two hybrid template structures, we successfully built two distinct models of Ca,1.2 using MODELLER 10.2 [43]. To refine the 3D structure of the model, we repeated short molecular dynamics simulations of N-terminal domain (31-111 residues) of the 3D model using AMBER 20 [44] to generate 10 final structures, and chose one representative from these ten structures (Fig. S4). Details of the modeling are described in Supplementary Information. The model of the folded N-terminal structure is shown in Fig. 2a, and that of the CaM-binding structure is shown in Fig. 2b.

Electrophysiology

For electrophysiology, BHK cells stably expressing β2 cells ( Alexis Fluor 488-conjugated anti-rabbit IgG goat antibody, 1:1000, Thermo Fisher Scientific) and Hocke, 33342 (Thermo Fisher Scientific) in PBS. All images were taken using a LSM 710 confocal microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with a 20×/0.8 numerical aperture (NA) objective. To evaluate Ca,1.2 trafficking to the plasma membrane, we defined the regions of interest (ROIs) of the plasma membrane based on membrane-tethering red fluorescent protein (RFP-KrasCT) images using an intensity threshold. Cytoplasmic ROIs were defined as inside regions of the plasma membrane ROIs excluding nuclear regions. For background subtraction, mean Ca,1.2 intensity from each ROI was used to calculate Ca,1.2 intensity ratio (membrane/cytoplasm). Statistical comparison between WT and A36V mutant channels was performed by two-tailed Welch’s t test.
were sampled at 20 kHz after low-pass filtering at 2.9 kHz. The pipette solution contained the following components (in mM concentrations): Cs-aspartate 90, MgCl2 40, ATP-Na2 2, phosphocreatine-Na2 5, EGTA 0.5, and HEPES 10, and was adjusted to pH 7.2 with CsOH. The pipette solution for Ca2+-dependent inactivation (CDI) recording contained (in mM concentrations): Cs-methanesulfonate 130, CsCl 5, MgCl2 5, ATP-Na2 2, phosphocreatine-Na2 5, EGTA 0.5, and HEPES 10, and was adjusted to pH 7.2 with CsOH. The ‘10 mM Ba2+’ external solution contained the following components (in mM concentrations): TEA-Cl 140, BaCl2 10, glucose 10, and HEPES 10, and was adjusted to pH 7.4 with TEA-Cl. The ‘100 mM Ca2+’ external solution contained the following components (in mM concentrations): TEA-Cl 140, CaCl2 10, glucose 10, and HEPES 10, and was adjusted to pH 7.4 with TEA-Cl. Osmolarity of all solutions was approximately 300 mOsm. Rapid exchange of the external solutions surrounding the cell was provided within 1 sec by a modified ‘Y-tube’ method [47].

The current-voltage (I-V) relationship was fitted with the following equation: 

\[
I(V_m) = G \ast (E_{rev} - V_m)/1 + \exp((V_{0.5} - V_m)/\delta),
\]

where \(I(V_m)\) is the peak Ba2+ current at the membrane potential of \(V_m\), \(G\) is the maximum conductance, \(E_{rev}\) is the apparent zero current potential, and \(V_{0.5}\) and \(\delta\) are the slope factor that determines the steepness of the curve. To examine the voltage-dependency of inactivation, currents were evoked by 20-ms test pulses to 20 mV after 10-ms repolarization to −80 mV following 2-s conditioning membrane potential displacement from −80 to 10 mV with 10-mV increments. Currents were recorded every 30 s. The curves were fitted using the Boltzmann’s equation: 

\[
1/(1 + \exp((V_m - V_{0.5})/\delta)),
\]

where \(V_{0.5}\) is the potential to give a half-value of conductance, and \(\delta\) is the slope factor that determines the steepness of the curve. Statistical comparison between WT and A36V mutant channels was performed by a two-tailed non-paired Student’s t test.

RESULTS

Sequencing and association study

After sequencing of the CACNA1C, CACNA1D, CACNAII, and CACNA1S coding regions and the prioritization of sequencing data, we identified several potentially interesting variants, including 1 rare nonsense SNV (p.C1451* in CACNA1D), 1 rare short deletion (p. E1675del in CACNA1D), and 14 rare NSstrict-damaging SNVs (Table 1). All mutations were confirmed using Sanger sequencing, and all of these alleles were heterozygous. Our sequence data are available in the DDBJ (http://www.ddbj.nig.ac.jp) as Accession Number DRA004490DNA. Among the identified mutations, potentially highly disruptive variants (including p.A36V in CACNA1C, p.C1451* in CACNA1D, p.E1675del in CACNA1D, and NSstrict SNV p.G330R in CACNA1D) were genotyped in an independent sample set comprising 1871 SCZ cases, 380 ASD cases, and 1916 controls (Table 2). Neither p.A36V in CACNA1C nor p.C1451* in CACNA1D were found in SCZ/ASD cases or healthy controls in the independent sample set, suggesting that these SNVs might be ultra-rare SNVs in the Japanese population. p.E1675del CACNA1D was found in both SCZ/ASD cases and healthy controls, but frequencies in psychiatric patients and healthy controls did not exhibit a statistically significant difference. p. G330R CACNA1D was found in SCZ/ASD cases, but not in healthy controls, suggesting that this mutation might constitute a significant SNV that is enriched exclusively in patients with psychiatric disorders. Therefore, an additional association study was performed in a much larger independent sample set comprising 4255 SCZ cases and 5565 controls. This analysis revealed that p.G330R CACNA1D was found in both SCZ cases and healthy controls, but frequencies in psychiatric patients and healthy controls did not exhibit a statistically significant difference (Table 3).

The A36V mutation does not show apparent deficits in membrane trafficking by Ca1,2 channels

To assess the biological significance of the A36V mutation in Ca,1,2 channels expressed in the brain, we constructed an A36V Ca,1,2-encoding plasmid vector. The CACNA1C gene includes alternative exons designated exon 1 (short and neuronal) and exon 1a (long and cardiac); we cloned a short isoform cDNA containing the alternative exon 1 from adult human brain total RNA. Six clones were obtained in total, and all clones were identified as transcription variant 14 in the RefSeq database (NM_001129840.1). We introduced the mutation encoding the A36V substitution into the plasmid encoding this neuronal isoform, which typically would be expressed predominantly in adult human brain (Fig. 1c). An A39V mutation, located in the vicinity of A36V (Fig. 1a, b) in Ca,1,2’ channels, previously was linked to Brugada syndrome, which presents with atrial fibrillation and abbreviated QT interval. A cardiac ‘long’ isoform of Ca,1,2 containing both the alternative exon 1a and the A39V mutation has been shown to induce a membrane trafficking defect and to exhibit drastically decreased Ca2+ currents [48]. Therefore, we examined whether membrane trafficking of neuronal Ca,1,2 channels harboring the A36V mutation is defective. When WT and A36V Ca,1,2 neuronal channels were expressed with \(\delta\) and \(\beta\) auxiliary subunits in BHK cells, we found that the two forms accumulated to similar levels at the plasma membrane regions (Fig. 1d–f). This result is consistent with a prior observation that A39V Brugada mutation in the neuronal Ca,1,2 isoform did not exhibit apparent membrane trafficking deficits [49]. These data suggested that a general loss of membrane expression is unlikely to account for the neuronal phenotype of the de novo A36V mutation in Ca,1,2.

The A36V mutation may alter conformational equilibrium between folded and CaM-binding structures at the Ca,1,2 N-terminus, thereby modulating CDI

We next tested whether the A36V mutation modulated the biophysical properties of the Ca,1,2 protein via alteration of structural dynamics of the alpha1 subunit conformations. Ca,1,2...
| Type          | Chromosome | Position b | Reference allele | Sample Allele | Gene     | Protein Variant | dbSNPc | Phenotypea | SIFT | PolyPhen-2 HumanVar | PolyPhen-2 HumanDiv | Mutation Taster | LRT | Primate PhastConsb |
|--------------|------------|------------|-------------------|---------------|----------|----------------|--------|-------------|------|---------------------|---------------------|------------------|-----|-------------------|
| Rare disruptive | 3          | 53810063   | T                 | A             | CACNA1D  | p.C1451*       |        | ASD         |      |                     |                     |                  |     |                   |
| Rare indel   | 3          | 53834368   | AGA               | CACNA1D       | p.E1675del| n778776240    | SCZ/ASD|             |      |                     |                     |                  |     |                   |
| Rare NSstrict | 1          | 201028331  | C                 | T             | CAONA15  | p.A1171T       | SCZ    | Damaging    | Probably | Damaging              |                     | Damaging         |     | 0.997             |
|              | 1          | 201031171  | A                 | G             | CAONA15  | p.L985P        | SCZ    | Damaging    | Probably | Damaging              |                     | Damaging         |     | 0.034             |
|              | 1          | 201034980  | C                 | T             | CAONA15  | p.V947I       | n76460090 | SCZ/ASD    | Damaging | Probably              |                     | Damaging         |     | 0.923             |
|              | 1          | 201036037  | A                 | G             | CAONA15  | p.S879P       | n73397311 | SCZ/ASD    | Damaging | Probably              |                     | Damaging         |     | 0.997             |
|              | 1          | 201036040  | C                 | T             | CAONA15  | p.V878M       | n20131129 | SCZ        | Damaging | Probably              |                     | Damaging         |     | 0.998             |
|              | 1          | 201043740  | G                 | T             | CAONA15  | p.L653I       | SCZ    | Damaging    | Probably | Damaging              |                     | Damaging         |     | 0.957             |
|              | 1          | 201046194  | A                 | G             | CAONA15  | p.S561P       | SCZ    | Damaging    | Probably | Damaging              |                     | Damaging         |     | 0.998             |
|              | 1          | 201047034  | C                 | T             | CAONA15  | p.R531H       | n748711395 | SCZ        | Damaging | Probably              |                     | Damaging         |     | 0.998             |
|              | 1          | 201058429  | T                 | C             | CAONA15  | p.Y286C       | SCZ    | Damaging    | Probably | Damaging              |                     | Damaging         |     | 0.998             |
|              | 3          | 53700434   | G                 | C             | CAONA1D  | p.G330R       | SCZ    | Damaging    | Possibly | Damaging              |                     | Damaging         |     | 0.926             |
|              | 3          | 53760951   | A                 | G             | CAONA1D  | p.M736V       | n773056182 | SCZ        | Damaging | Possibly              |                     | Damaging         |     | 0.993             |
|              | 3          | 53769587   | G                 | A             | CAONA1D  | p.Y748I       | n184573217 | SCZ/ASD    | Damaging | Possibly              |                     | Damaging         |     | 0.989             |
|              | 12         | 2775874    | G                 | T             | CAONA1C  | p.V1545L      | SCZ    | Damaging    | Possibly | Damaging              |                     | Damaging         |     | 0.981             |
|              | 22         | 40066215   | G                 | T             | CAONA11  | p.R1456L      | n766713729 | SCZ        | Damaging | Possibly              |                     | Damaging         |     | 0.998             |
| De novo      | 12         | 2224447    | C                 | T             | CAONA1C  | p.A36V        | n755028000 | SCZ        | Tolerated | Benign                | Possibly             | Damaging         | Neutral | 0.06             |

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*aRare NSstrict: Rare nonsynonymous strict-damaging SNVs*

*bGenomic position is based on GRCh37/hg19*

cdbSNP: dbSNP build 153 ([https://www.ncbi.nlm.nih.gov/snp/](https://www.ncbi.nlm.nih.gov/snp/))

dASD = discovered in autism patients; SCZ = discovered in schizophrenia patients; SCZ/ASD = discovered in schizophrenia and autism patients

ePrimate PhastCons: Primate PhastCons conservation score ([http://compgen.cshl.edu/phast/phastCons-HOWTO.html](http://compgen.cshl.edu/phast/phastCons-HOWTO.html))

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Table 2. Association study of selected variants.

| Type               | Chromosome | Position | Gene   | Schizophrenia | Autism spectrum disorder | Healthy control |
|--------------------|------------|----------|--------|---------------|--------------------------|-----------------|
| Rare disruptive    | 3          | 53810063 | CACNA1D| 0/0/1916      | 0/0/1916                 | 0/0/1916        |
| De novo            | 12         | 2224447  | CACNA1C| 0/0/1871      | 0/0/1871                 | 0/0/1871        |
| Rare indel         | 3          | 53834368 | CACNA1D| 0/3/1868      | 0.6452                   | 0.2196          |
| Rare NSstrict      | 3          | 53700434 | CACNA1D| 0/0/1871      | 0/0/1871                 | 0/0/1871        |

Table 3. Association study of selected variant in the third and largest independent sample set.

| Type               | Chromosome | Position | Gene               | Schizophrenia | Autism spectrum disorder | Healthy control |
|--------------------|------------|----------|--------------------|---------------|--------------------------|-----------------|
| Rare NSstrict      | 3          | 53700024 | CACNA1D            | 0/3/4252      | 0/3/4252                 | 0/3/4252        |

DISCUSSION

Recent large-scale genetic studies have reported that ultra-rare disruptive SNVs are highly enriched in patients with SCZ/ASD, especially SNVs in sets of VGCC genes, providing an opportunity to discover the cellular processes that are relevant to the pathogenesis of SCZ/ASD [5, 11, 22, 23, 55]. However, the cellular processes whereby these rare disruptive SNVs give rise to psychiatric disorders remain unknown. To address this question, we performed a comprehensive search, including a genetic and

has multiple CaM-binding sites in its N-terminus and C-terminus. Ca\(^{2+}\) ions entering through Ca\(_{1.2}\) pore bind to CaM, and consequently cause CDI [50–53]. We noticed that the A36V mutation is located close to the NSCaTE region, a CaM interaction site that has been shown to be necessary for N-lobe dependent CDI [50, 53]. Using homologous 3D-structures, we modeled two different structures of Ca\(_{1.2}\) (a folded N-terminal structure and an open CaM-binding N-terminal structure), and assumed the conformational ensemble of Ca\(_{1.2}\) is in an equilibrium among various structures including these two structures (Fig. 2). The 36\(^{th}\) residue of Ca\(_{1.2}\) (alanine in the WT) have hydrophobic contacts with other nonpolar residues (A39 and A97) in the folded N-terminal structure (Fig. 2a, c), whereas this residue is more exposed to solvent in the CaM-binding structure (Fig. 2b). Because valine is more hydrophobic than alanine, the models suggested that the A36V substitution may shift the conformational equilibrium toward the folded N-terminal structure, thereby decreasing the frequency of formation of the CaM-binding structure at the NSCaTE region of Ca\(_{1.2}\) (Fig. 2d).

To determine the electrophysiological characteristics of the A36V Ca\(_{1.2}\) channels, we recorded whole-cell Ba\(^{2+}\) currents through recombinant Ca\(_{1.2}\) channels transiently expressed in BHK-\(\alpha_{2}\)\(\beta_{2}\) cells [45]. The current–voltage (I–V) relationship for the neuronal A36V Ca\(_{1.2}\) channel was similar to that for the WT channel. The peak current density (peak current amplitude divided by cell capacitance) for the neuronal A36V Ca\(_{1.2}\) channel (15.8 ± 2.0 pA/pF, \(n = 18\)) did not differ significantly from that of the neuronal WT Ca\(_{1.2}\) channel (19.8 ± 2.9 pA/pF, \(n = 12\)) (Fig. 3a, b), consistent with a lack of a mutation effect on channel membrane trafficking (Fig. 1d–f). The steady-state inactivation curves for both channels were very similar (Fig. 3c). These results suggest that the A36V mutation does not change the voltage dependency of activation and inactivation of the neuronal Ca\(_{1.2}\) channel.

Finally, we examined the effect of A36V on global CDI. This parameter was tested with the use of the pipette solution containing 0.5 mM EGTA. We recorded Ba\(^{2+}\) and Ca\(^{2+}\) currents at 30 mV from the same BHK cell expressing neuronal Ca\(_{1.2}\) (Fig. 3d–f). Ba\(^{2+}\) currents decayed slowly, but Ca\(^{2+}\) currents decayed completely within 350 msec (Fig. 3d). The ratio of remaining current amplitude to the peak current amplitude was calculated at different time points after depolarization to 30 mV (Fig. 3e, f). Ca\(^{2+}\) currents (Fig. 3f), but not Ba\(^{2+}\) currents (Fig. 3e) of neuronal A36V Ca\(_{1.2}\), were reduced mildly, but significantly more slowly, than Ca\(^{2+}\) currents of the neuronal WT Ca\(_{1.2}\), suggesting that the A36V mutation affects global CDI in neurons. We further examined the impact of A36V in the cardiac isoform that contain the longer C-terminus (Fig. 3h) of cardiac A36V Ca\(_{1.2}\). The reported parameter was tested with the use of the pipette solution containing 0.5 mM EGTA. We recorded Ba\(^{2+}\) currents at 30 mV from the same BHK cell expressing neuronal Ca\(_{1.2}\) (Fig. 3i). Ba\(^{2+}\) currents decayed slowly, but Ca\(^{2+}\) currents decayed completely within 350 msec (Fig. 3i). The ratio of remaining current amplitude to the peak current amplitude was calculated at different time points after depolarization to 30 mV (Fig. 3j, f). Ca\(^{2+}\) currents (Fig. 3f), but not Ba\(^{2+}\) currents (Fig. 3e) of neuronal A36V Ca\(_{1.2}\), were reduced mildly, but significantly more slowly, than Ca\(^{2+}\) currents of the neuronal WT Ca\(_{1.2}\), suggesting that the A36V mutation affects global CDI in neurons. We further examined the impact of A36V in the cardiac isoform that contain the longer N-terminal region encoded by exon 1a (Fig. 3g–i). In contrast, Ca\(^{2+}\) currents (Fig. 3i) and Ba\(^{2+}\) currents (Fig. 3h) of cardiac A36V Ca\(_{1.2}\) decayed similarly to those of cardiac WT Ca\(_{1.2}\). The reported electrophysiological properties of the A39V Brugada mutation in a neuronal isoform is more complex [54], suggesting co-existing overlapping pathophysiology of the A39V mutation, especially in cardiac mutant channels. Taken together, these data indicated that A36V neuronal Ca\(_{1.2}\) is a mild gain-of-function mutant channel, while the A36V cardiac Ca\(_{1.2}\) behaves similarly to the WT channel, consistent with the relatively late onset of SCZ symptoms and normal cardiac functions.
functional analysis of VGCC genes. Our analysis identified 1 nonsense SNV (p.C1451* in CACNA1D), 1 de novo SNV (p.A36V in CACNA1C), 1 rare short deletion (p.E1675del in CACNA1D), and 14 NSstrict SNVs (Non-synonymous SNV predicted as damaging by all five in silico analyses). Neither p.A36V CACNA1C nor p.C1451* CACNA1D were found in 1871 SCZ cases, 380 ASD cases, or 1916 healthy controls in the independent sample set, suggesting that these SNVs might be ultra-rare SNVs in the Japanese population. p.G330R CACNA1D was detected in both SCZ and healthy controls, at frequencies that did not show statistically significant differences between the two groups. However, it should be noted that the sample size was too small to completely rule out the possibility of association. The p.G330R mutation is located in the S5-S6 linker of repeat I of the encoded protein; this region previously was shown...
to be a critical determinant of L-type VGCC conductance [56], suggesting that this mutation may have biological significance. To assess the effect of the A36V mutation on Ca\textsubscript{v}1.2 channel properties, we performed in silico 3D structural prediction modeling and electrophysiological studies. The modeling analysis led to a previously unexplored (to our knowledge) hypothesis, that the N-terminal region of Ca\textsubscript{v}1.2 exists in an equilibrium between two alternative conformations, a folded N-terminal structure and an open CaM-binding structure. We postulated that the A36V mutation of neuronal Ca\textsubscript{v}1.2 may shift the conformational equilibrium toward the folded N-terminal structure, thereby modulating the CDI of neuronal Ca\textsubscript{v}1.2 channels. Consistent with this hypothesis, we observed a decrease in the CDI of A36V neuronal Ca\textsubscript{v}1.2 channels, with little change in voltage-dependent activation and inactivation. CDI is a negative feedback mechanism limiting excessive Ca\textsuperscript{2+} entry [51, 57]; therefore, A36V can be regarded as a gain-of-function mutation in neuronal Ca\textsubscript{v}1.2. Thus, our analysis successfully identified an ultra-rare and functionally significant variant in VGCC-encoding genes, supporting the hypothesized role of these loci in the risk of SCZ/ASD, and indicating that a mild gain-of-function in Ca\textsubscript{v}1.2 may play a role in the etiology of SCZ.

A series of GWAS and WES studies have suggested causal links between common and rare variants of \textit{CACNA1C} and psychiatric disorders including SCZ, BD, and ASD [19]. Growing evidence has shown that gain-of-function mutations in Ca\textsubscript{v}1.2 are associated with psychiatric disorders. For instance, a \textit{CACNA1C} lesion causing a G406R substitution in the encoded protein was identified in patients with Timothy syndrome; multiple studies have shown that this gain-of-function mutation is associated with prolonged QT intervals, syndactyly, and ASD [14, 15]. Furthermore, the \textit{CACNA1C} gene polymorphism rs1006737, known to be associated with SCZ and BD, has been shown to augment Ca\textsubscript{v}1.2 channel activity and \textit{CACNA1C} mRNA expression in induced human neurons and in human brain.
Intriguingly, the Brugada syndrome A39V Ca_{1.2} mutation, when encoded in a long splicing isoform (exon 1a-containing cardiac type), was shown to function as a loss-of-function mutation with impaired membrane trafficking [48]; when encoded in a short splicing isoform (exon 1-containing neuronal type), this change possibly functions as a gain-of-function mutation by modulating CDI [54]. These splicing isoform-specific observations suggest that the presence of the same Ca_{1.2} mutation in separate isoforms leads to distinct outcomes.

The present study showed that the A36V mutation, when carried by the short neuronal isoform, inhibits CDI, suggesting that A36V and A39V, which are located near the NSCaTE domain in
Cav1.2, both serve as mild gain-of-function mutations in the neuronal isoform. In silico 3D structural modeling indicated that these residues form part of a hydrophobic core in the N-terminal folded structure, but are exposed in the CaM-binding structure (Fig. 2). These substitutions with more hydrophobic residues may stabilize the N-terminal folded structure while destabilizing the CaM-binding structure, which in turn could affect the CDI. Given that the A36V mutation was identified from an individual with SCZ without cardiac symptoms, this mutation may not influence the activity of cardiac channels. Consistent with this notion, it was found that A36V mutation did not affect electrophysiological properties when we tested the mutation in the cardiac isoform. Cav1.2 has multiple isoforms with different functional properties and tissue-selective expression [60, 61]. To understand the complexity of the contribution of A36V and other mutations to psychiatric disorders and cardiac arrhythmia, future studies will need to consider cell type (neuronal or cardiac), Cav1.2 splicing isoform, and co-expressed auxiliary subunits that contribute to the functional diversity of Cav1.2 channels [62].

Trios-based exome sequencing studies have supported the hypothesis that de novo SNVs, especially de novo SNVs in protein-coding sequences, are related to increased risk for psychiatric disorders such as SCZ/ASD and BD [63–66]. Furthermore, a relationship between advanced paternal age and increased ASD risk has been established in different studies [67], and de novo SNVs were indicated as potential risk factors [68]. Notably, a sibling of the A36V carrier identified in the present study also was diagnosed with SCZ; therefore, one or more additional factors (genetic or environmental) may contribute to increased susceptibility to SCZ in this particular family, a conjecture that may be explained, in part, by a two-hit model [69].

Given the potentially important role of de novo SNVs in SCZ/ASD susceptibility, we tested for the presence of candidate SNVs in the parents of the carrier patients, if parental DNA samples were available. We discovered one de novo missense SNV (p.A36V in CACNA1C) in a patient with SCZ, and showed (by electrophysiological recording) that cells expressing the A36V neuronal Cav1.2 channel exhibit slower decreases in Ca²⁺ current than do cells expressing the WT channel. This finding supports the hypothesis that this de novo missense SNV is an ultra-rare disruptive SNV affecting CDI, possibly contributing to susceptibility to psychiatric disorders. The Ca²⁺-dependent signaling pathway is a key component in neural circuit formation, gene expression, and neuronal plasticity [70, 71]. In addition to the excessive Ca²⁺ entry per se, disturbance of intracellular Ca²⁺ homeostasis dynamics may lead to neural dysfunction by the dis-regulation of downstream signaling pathways that also are associated with SCZ/ASD [72].

Several limitations should be considered when interpreting the results of our study. First, the lack of statistical significance in our association study may reflect the small sample size; a new study in a larger cohort is needed. Nonetheless, our data revealed that these SNVs are ultra-rare in the Japanese population. These genetic data may be incorporated into future large-scale WESs, potentially supporting the hypothesis that an increased burden of ultra-rare deleterious mutations is observed in patients with SCZ/ASD. Second, due to the lack of enough parental DNA samples, we were able to perform segregation analysis for only a few of the prioritized SNVs. To identify more de novo mutations associated with susceptibility to SCZ/ASD, we will need to collect more DNA samples of family members and perform trios-based exome-sequencing studies. Finally, our sequence analysis did not cover the promoters, untranslated regions, or intronic regions of the target genes, which may contain important mutations at regulatory sites.

In conclusion, we identified 1 nonsense SNV (p.1451* in CACNA1D), 1 de novo SNV (p.A36V in CACNA1C), 1 rare short deletion (p.E1675del in CACNA1D), and 14 N-strict SNVs by genetic analysis of the CACNA1C, CACNA1D, CACNA1H, and CACNA1S genes in humans. Our genetic and biological data imply that the de novo SNV (p.A36V in CACNA1C) may increase susceptibility to SCZ pathogenesis by perturbing CDI. Further, our data provide evidence in support of the potential role of VGCC-encoding genes in psychiatric disorders.

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ACKNOWLEDGEMENTS
We thank Dr. Yasuo Mori for providing the BHK-αδ+β2 cells. This work was supported by JSPS KAKENHI Grant Numbers JP20K16490 (S.H.), JP20H03339, JP21H05091 (S.T.-K.), JP19H01007 (H.B.), and JP16H06276 (AdAMS to H.B. and S.T.-K.); the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Ministry of Health, Labour and Welfare of Japan; the Strategic Research Program for Brain Sciences from the Japan Agency for Medical Research and Development (AMED) under Grant Numbers JP20dm0107131 (M.W.), JP20dm0107132 (H.F.), JP20dm0107130 (B.A. and S.T.-K.), JP20dm0107087, JP20dm0207075, and JP20ak0101113 (N.O.); the Brain Mapping by Integrated Neurotechnologies for Disease Studies (Brain/MINDS) from AMED; the Innovative Areas “Glia assembly: a new regulatory machinery of brain function and disorders” program; the Innovative Areas “Comprehensive Brain Science Network” program; the Platform Project for Supporting Drug Discovery and Life Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)) from AMED under Grant Number JP21am0101066 (Support Number 0305) (T.K.); the Takeda Science Foundation (S.H.); the Toray Science Foundation (S.T.-K.); JST-Mirai Program Grant Number JPMJMI21G6 (S.T.-K.); and the Toyoaki Scholarship Foundation (S.H., S.T.-K.). We also thank the patients and their families for participating in this study. The model structures have been submitted to the Biological Structure Model Archive (BSM-Arc) under BSM-ID BSM00025 [https://bsma.pdbj.org/entry/25] [73].

AUTHOR CONTRIBUTIONS
CW, SIH, MW, SU, TK, HF, BA, HB, and STK contributed to conception and design. CW, SIH, MW, SU, TK, HF, BA, HB, TD, and STK contributed to data acquisition and pre-processing. CW, SIH, MW, SU, TK, HF and BA analyzed the data and drafted the manuscript. All authors participated in revising it critically for important intellectual content.

COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41398-022-01851-y.

Correspondence and requests for materials should be addressed to Branko Aleksic or Sayaka Takemoto-Kimura.

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