Research Paper

RGS3 and *IL1RAPL1* missense variants implicate defective neurotransmission in early-onset inherited schizophrenias

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Background: Schizophrenia is characterized by hallucinations, delusions and disorganized behaviour. Recessive or X-linked transmissions are rarely described for common psychiatric disorders. We examined the genetics of psychosis to identify rare large-effect variants in patients with extreme schizophrenia. **Methods:** We recruited 2 consanguineous families, each with patients affected by early-onset, severe, treatment-resistant schizophrenia. We performed exome sequencing for all participants. We checked variant rarity in public databases and with ethnically matched controls. We performed in silico analyses to assess the effects of the variants on proteins. **Results:** Structured clinical evaluations supported diagnoses of schizophrenia in all patients and phenotypic absence in the unaffected individuals. Data analyses identified multiple variants. Only 1 variant per family was predicted as pathogenic by prediction tools. A homozygous c.649C > T:p.(Arg217Cys) variant in *RGS3* and a hemizygous c.700A > G:p.(Thr234Ala) variant in *IL1RAPL1* affected evolutionary conserved amino acid residues and were the most likely causes of phenotype in the patients of each family. Variants were ultra-rare in publicly available databases and absent from the DNA of 400 ethnically matched controls. *RGS3* is implicated in modulating sensory behaviour in *Caenorhabditis elegans*. Variants of *IL1RAPL1* are known to cause nonsyndromic X-linked intellectual disability with or without human behavioural dysfunction. **Limitations:** Each variant is unique to a particular family's patients, and findings may not be replicated. **Conclusion:** Our work suggests that some rare variants may be involved in causing inherited psychosis or schizophrenia. Variant-specific functional studies will elucidate the pathophysiology relevant to schizophrenias and motivate translation to personalized therapeutics.

Introduction

Psychosis affects 3% of the world's population.[1] The most common diagnosis associated with psychosis is schizophrenia, with a worldwide prevalence of 1%. Schizophrenia is a chronic, frequently disabling, lifelong illness. Patients may experience hallucinations, delusions, paranoia, catatonia, disorganized thinking, functional impairment, poor planning, flattening behaviour and social withdrawal.

Schizophrenia has a strong genetic component, with about 80% to 85% heritability as estimated through family, twin and adoption studies.[2] In the past 2 decades, worldwide collaborations have enabled genome-wide association studies with large cohorts, in search of genetic risk loci associated with schizophrenia. More than 176 loci have been associated with schizophrenia through case–control association studies of 56 000 patients with schizophrenia and 78 000 controls.[3] Many copy-number variants have also been implicated as causes of schizophrenia.[4]

Although schizophrenia is considered to be a complex disorder with common variants of multiple genes involved in causing the phenotype, rare high-impact variants also contribute to the disorder. In a large cohort study, whole-genome sequencing was performed in 112 patients affected with extreme early-onset, treatment-resistant schizophrenia and whole-exome sequencing was completed for 4185 controls.[5] The research revealed several rare, damaging missense variants in multiple genes, of which *ACACA*:p.(Asp251Gly), *CACNA1C*:p.(Ile1153Arg) and *GABRA*:p.(Thr234Ala) had been previously associated with schizophrenia. A total of 48.2% of patients with treatment-resistant schizophrenia had at least 1 damaging missense or loss-of-function variant in a variant-intolerant gene, compared to 25.4% in controls. Interestingly, loss-of-function variants in genes were significantly enhanced for Mendelian syndromes in which some patients also exhibit aggressive behaviours or hallucinations. Moreover, many genes previously associated with schizophrenia had higher numbers of both missense and loss-of-function variants compared to controls.[5]

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Multiple studies involving inherited psychiatric disorders have identified a large number of likely pathogenic variants in each patient from an individual family.[6] However, monogenic loci and single-gene variants with large effects have also been implicated as causes of inherited schizophrenia. Loci at chromosomes 2p16.3,[7] 22q13.1[8] and 13q22–31[9] (among others) have been mapped in familial cases of schizophrenia, but gene variants have not been specifically identified.

Exome sequencing studies have identified heterozygous variants in genes that may cause schizophrenia in families with multiple affected individuals. These variants include the following: *LRP1B*:p.(Gly3458Lys), *GRM5*:p.(Gly369Val), *PPEF2*:p.(Arg86His), *LRP1B*:p.(Ala924Gly) and *LRP1B*:p.(Gly4525Glu).[10] An autosomal-dominant heterozygous frameshift variant in *GRIN3B*:p.(Gly466AlafsTer482) has also been identified as a possible cause of schizophrenia in 5 patients.[11] In a separate study involving 4 patients with schizophrenia, a heterozygous missense variant in *ITGB4*:p.(Ala1689Val) was found segregating with the disorder.[12] Apart from exome sequencing, whole-genome sequencing has been used to study psychoses in 2 multiplex families. This work revealed hemizygous *SMARCA1*:p.(Val384Met) and heterozygous *SHANK2*:p.(Ala578Val) variants as potential candidates segregating with psychoses.[13]

Research in the Pakistani population also supports the involvement of common gene variants or a few rare alleles predicted to be pathogenic in psychiatric disorders. Multiple rare heterozygous changes were identified with high penetrance in 2 multiplex Pakistani families that had members with schizophrenia.[14] One of these was a microduplication at 5q14.1–14.2 involving multiple genes, including *HOMER*, *RAS-GRF2* and *CMYA5* in participants of 1 family. In the second family, heterozygous missense variants in *GRIN2A*:p.(Arg1169Trp) and *NRG3*:p.(Glu651Lys) segregated with the disorder in a digenic model. This study implicated gene variants that affected glutamatergic neurotransmission as pathogenic for schizophrenia.

The above studies indicate that high-penetrance causative loci for schizophrenia, although rare, are still present and may cause the disorder in individual families. The likelihood of finding such loci in consanguineous populations is enhanced,[15] but so far only 2 loci on chromosomes 22q12.3-q13.3 and 13q22–31 have been mapped for autosomal, recessively inherited schizophrenia in families of Pakistani origin.[8,9] The Pakistani population is ideal for the study of autosomal recessive variants in disorders such as schizophrenia because about 60% of marriages are consanguineous and three-quarters of these involve first cousins.[16] The incidence of schizophrenia in the Sindh and Punjab provinces of Pakistan is also higher (2.5%), especially in rural areas.[17] We investigated 2 consanguineous Pakistani families, each including 2 individuals with schizophrenia, and identified rare missense variants segregating with the disorder.

Methods

Family identification and sample collection

We identified families PSYAK2 and PSYAK3 from Lahore, Punjab, with the help of doctors at the Punjab Institute of Mental Health. We obtained ethical approval from institutional review boards at the School of Biological Sciences, University of the Punjab, Lahore, Pakistan (IRB# 00005281, FWA 00010252) and the University of Minnesota, Minneapolis, United States (FWA00000312). We obtained written informed consent from all participants (or legal guardians for patients with severe ongoing symptoms). Patient anonymity was preserved. The aunt III:1 and the uncle III:4 in families PSYAK2 and PSYAK3 refused to participate in the study because of personal beliefs associated with COVID-19.

We acquired medical records and kinship and family histories; these are described in detail elsewhere (doi:10.22541/au.162626101.13669766/v1), along with information from an additional 6 families, which are not presented here because their exome data yielded ambiguous results (data not shown). We also acquired information about age of onset of schizophrenia, symptoms, triggering factors and medications. We obtained illness histories from parents and hospital records; the patients themselves had limited ability to detail their history and characterize their symptoms. Patients had been under hospital care for 10 to 13 years and had been admitted multiple times during this period. Between hospitalizations, they were seen approximately monthly as outpatients in the psychiatry department. Board-certified psychiatrists assessed the patients and prescribed medications to manage their symptoms.

We determined the parents' consanguinity by questioning. We also accessed the health records of all unaffected siblings and the parents to find previous psychiatric evaluations. Blood samples were drawn from patients and available family members by a trained phlebotomist. DNA was extracted from the samples using a standard protocol.

Exome sequencing and data analysis

We completed exome sequencing using Agilent SureSelect V7-postcapture kit (Agilent Technologies) at 100× depth using standard procedures for the parents, affected individuals and unaffected siblings in families PSYAK2 and PSYAK3 (Appendix 1, Methods, available at www.jpn.ca/lookup/doi/10.1503/jpn.220070/tab-related-content). Data were analyzed and variants were filtered using Franklin software (https://franklin.genoox.com/).

We examined heterozygous and homozygous variants detected in the exome data, as well as hemizygous variants on the X-chromosome for the 2 affected brothers. We retained exonic and splice-site variants with allele frequencies of less than 1% (0.01) in public databases. We further prioritized variants based on their high probability of deleteriousness, as assessed by 7 prediction tools. Conservation was scored using Genomic Evolutionary Rate Profiling (GERP).

To detect local polymorphisms, we examined allele frequencies in the in-house exome data of 300 unrelated individuals. We also identified shared regions of homozygosity from exome data using AgileVCFMapper (www.dna-leeds.co.uk/agile/AgileVCFMapper/).[18] We checked the conservation of affected amino acids in different species; highly conserved amino acids were prioritized.

Sanger sequencing and allele-specific PCR

We confirmed segregation with the phenotype of the likely pathogenic variants by amplifying the respective regions using polymerase chain reaction (PCR) in all available family members. We designed primers (Appendix 1, Table S1) based on the genomic sequences of *RGS3* (NM_144488.8) and *IL1RAPL1* (NM_014271.4), followed by PCR. We completed Sanger sequencing using Big Dye Terminator V3.1 on an ABI 3730 genetic analyzer (ABI Thermo Fisher). We checked the allele frequency of the *RGS3* variant in 100 ethnically matched controls using Sanger sequencing, as well as by accessing the exome data of 300 in-house unrelated controls.

For *IL1RAPL1*, we designed primers to amplify the wild-type and mutant alleles for allele-specific PCR (Appendix 1, Table S1).[19] We checked the allele frequency of the *IL1RAPL1* variant in 230 ethnically matched controls using allele-specific PCR and in 300 unrelated ethnically matched controls by examining in-house exome data.

Protein alignment and modelling

To compare the human protein sequences of RGS3 and IL1RAPL1 with the most diverse vertebrate species, we obtained protein sequences from UniProt (www.uniprot.org/) and aligned them using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/). We identified conserved domains using a National Center for Biotechnology Information conserved domain search (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

We generated 3-dimensional structures of the proteins with an automated I-TASSER modeller (https://zhanglab.dcmb.med.umich.edu/I-TASSER/) and using RGS3 and IL1RAPL1 wild-type protein sequences. We prepared structural representations using the PyMoL program (https://pymol.org/2/) for both wild-type and mutated protein sequences. We analyzed the stability of the proteins with single-point mutations using I-Mutant Suite (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi). We used the Protein Data Bank (PDB; www.rcsb.org/) to access the structures of RGS3 (PDB ID: 2F5Y) and IL1RAPL1 (PDB ID: 4YH6).

We used computational tools PredictProtein (https://predictprotein.org/), SNAP2 (https://rostlab.org/services/snap/) and CUPSAT (http://cupsat.tu-bs.de) to determine the predicted localization of wild-type proteins and the possible effects of variants on the conformation of the mutant proteins.

Results

Clinical manifestations

Detailed histories, symptoms and prescribed medications for patients in families PSYAK2 and PSYAK3 are described in detail elsewhere (doi:10.22541/au.162626101.13669766/v1). In brief, 2 sisters in family PSYAK2 (IV:2 and IV:4; Figure 1A) and 2 brothers in family PSYAK3 (IV:3 and IV:4; Figure 1B) were diagnosed with schizophrenia at the ages of 12, 14, 18 and 26 years, respectively, by board-certified psychiatrists. On average, the affected individuals had been living with schizophrenia for approximately 10 years and had required multiple hospital admissions.

Clinical manifestations included positive symptoms of schizophrenia and other psychotic symptoms in the affected individuals from family PSYAK2 and in patient IV:3 from family PSYAK3; patient IV:4 from family PSYAK3 had mostly negative symptoms. Positive symptoms included auditory or visual hallucinations, self-talking and self-smiling; negative symptoms included lack of emotional expression, social withdrawal and flat affect. Aggressive behaviours and feelings of paranoia were present in all affected individuals. The parents of IV:2 from PSYAK2 reported that she was unable to take care of herself or her 3-year-old son. Patients from both families were taking antipsychotics, antidepressants, anticonvulsants and mood stabilizers. In all affected individuals, their schizophrenia had worsened with age and had become unresponsive to medications. Parents also reported that their children's schizophrenia was manageable in the initial stages but gradually became less responsive to medications, and that their children became more difficult to care for.

RGS3 *and* IL1RAPL1 *missense variants*

In family PSYAK2, a total of 574 homozygous variants with allele frequencies ranging from 0 to 0.99 were common between the 2 affected individuals, including 203 exonic and splicing-region (± 10) variants (data not shown). Of these, we found 37 variants in regions of homozygosity (homozygous in affected individuals and heterozygous in unaffected relatives; Appendix 1, Figure S1, Table S2, and data not shown). After applying the filtering criteria of allele frequency less than 1% in different populations, only 9 homozygous variants were found segregating with the phenotype, as expected for a Mendelian recessive disorder (Table 1). Four homozygous variants were nonsynonymous substitutions, and 5 were synonymous changes. None of the filtered, shared compound heterozygous variants was predicted to be pathogenic (Appendix 1, Table S3). Only a missense variant of *RGS3* (NM_144488.5) — c.649 C > T, p.(Arg217Cys) — was predicted to be pathogenic by multiple prediction tools, affected an amino acid absolutely conserved in evolution, and had high pathogenic scores.

Sanger sequencing confirmed that the affected individuals were homozygous for the variant, obligate carriers were heterozygous for the mutant allele and the unaffected sibling was homozygous for the wild-type allele (Figure 1C). The aggregated allele frequency for *RGS3* variant rs759123934 was 0.00004692 in gnomAD; none were homozygous. Sanger sequencing of 100 ethnically matched controls and of the in-house exome data of 300 controls revealed no carriers for the variant allele.

In family PSYAK3, 804 homozygous or hemizygous variants were common between the 2 affected individuals, including 228 exonic and splicing region (± 10) variants (data not shown). Among these, 55 variants were found in regions of homozygosity or on chromosome X (Appendix 1, Figure S1, Table S4,

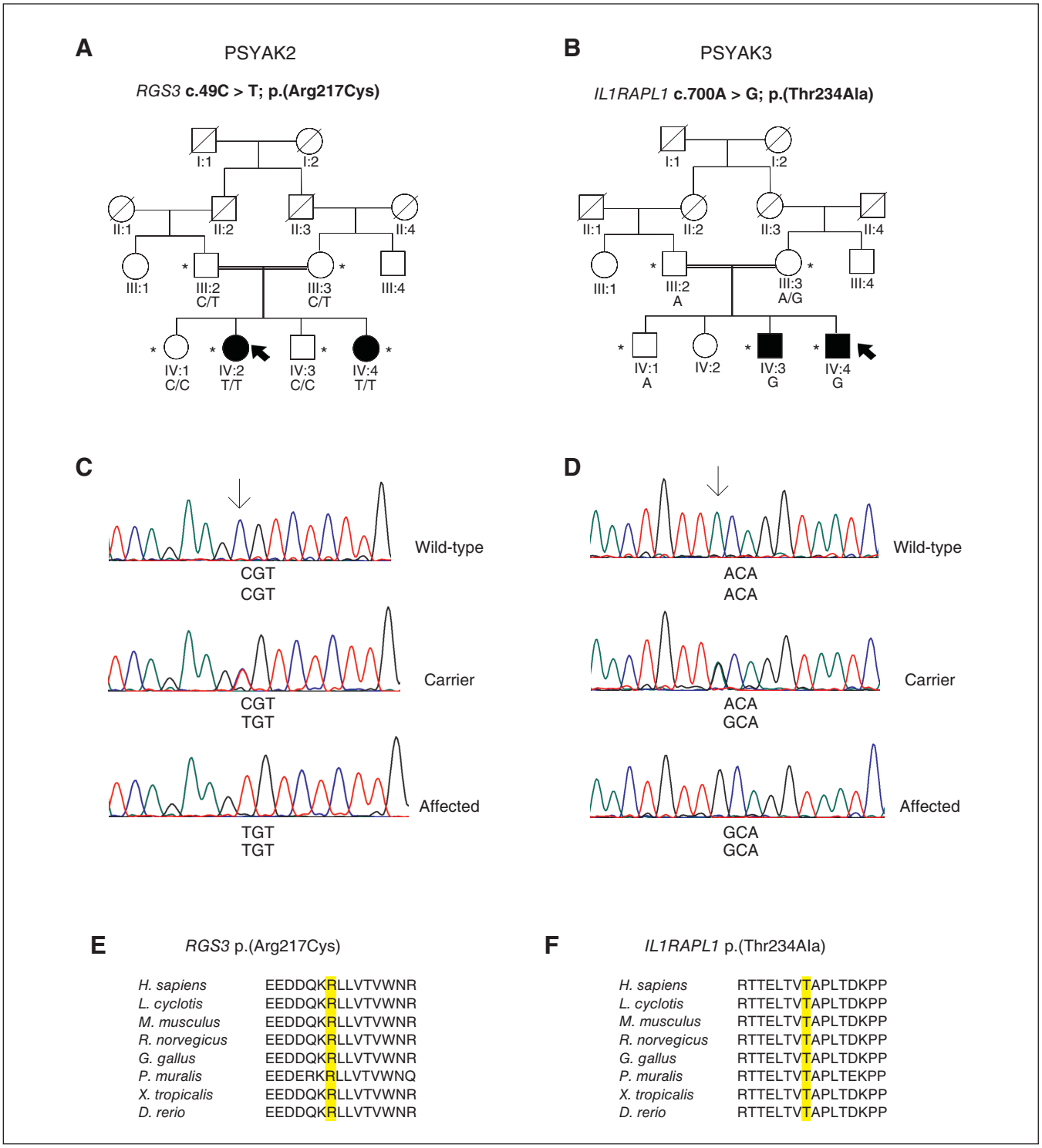

Figure 1: Pedigrees of families PSYAK2 and PSYAK3 with variant segregation and conservation. Asterisks depict individuals who participated in the study. Filled symbols indicate affected individuals. Double lines show consanguineous marriages. (A) Family PSYAK2. Six individuals participated in the study. (B) Family PSYAK3. Five individuals participated in the study. (C) Partial chromatograms of *RGS3* for an unaffected wild-type individual, a heterozygous carrier and a homozygous affected individual. The arrow indicates the point of the variant. (D) Partial chromatograms of *IL1RAPL1*. Affected individuals were hemizygous, whereas their mother was a heterozygous carrier. The unaffected brother and the father were hemizygous for the wild-type allele. (E) Multiple sequence alignment of RGS3 from diverse vertebrates showing p.Arg217 conservation (highlighted). (F) Multiple sequence alignment of IL1RAPL1 from diverse vertebrates showing p.Thr234 conservation (highlighted).

Table 1: Filtered variants for families PSYAK2 and PSYAK3 after exome sequencing

					Aggregated allele frequency, %			Predictions						
Family ID	Gene	gDNA change*	RefSeq ID	cDNA change and amino acid change	gnomAD	From 300 ethnically matched controls†	Conservation GERP‡	CADD	SIFT	PolyPhen2	REVEL	MT	FATHMM	SpliceAI
PSYAK2	*OR13C2*	Chr9: 107367821 G > C	NM_001004481.1	c.88C > G p.Leu30Val, missense	0.0002781 (0 homozygous)	0.00167	1.45	8.129	0.09 T	0.03 B	0 B	0.03 B	2.32 T	0.03 B
	RGS3	Chr9: 116247933 C > T	NM_144488.5	c.649C > T p.Arg217Cys, missense	0.00004903 (0 homozygous)	0	4.66	32	0 D	1 D	0.62 D	0.99 D	−1.07 B	0.13 U
	NR6A1	Chr9: 127287141 G > A	NM_033334.4	c.1213C > T p.Leu405Leu, synonymous	0.0006331 (0 homozygous)	0	NA	11.49	NA	NA	NA	NA	NA	NA
	GAPVD1	Chr9: 128117939 C > T	NM_001282680.2	c.3774C > T p.Thr1258Thr, synonymous	0.0001516 (0 homozygous)	0.00167	NA	8.623	NA	NA	NA	NA	NA	NA
	HMCN2	Chr9: 133224403 C > T	NM_001291815.2	c.2514C > T p.Asp838Asp, synonymous	0.0014 (1 homozygous)	0.01	NA	0.231	NA	NA	NA	NA	NA	NA
	MED27	Chr9: 134735938 G > A	NM_004269.4	c.923C > T p.Thr308Ile, missense	0.0002064 (1 homozygous)	0	5.3	23.3	0.06 T	0.11 B	0.24 B	1 D	NA	0 B
	SETX	Chr9: 135202835 A > T	NM_015046.7	c.4150T > A p.Ser1384Thr, missense	0.0001352 (0 homozygous)	0.00167	−0.18	6.714	0.02 D	0.09 B	0.27 B	0 B	−2.19 D	0 B
	FLI1§	Chr11: 128651917 A > G	NM_002017.5	c.654 A > G p.Glu218Glu, synonymous	0.0002607 (1 homozygous)	0.002	NA	21.1	NA	NA	NA	NA	NA	0.01 D
	SNX19	Chr11: 130749601 C > T	NM_014758.2	c.2764G > A p.Val922Ile, missense	0.00001593 (0 homozygous)	0	−2.38	0.127	0.46 T	0.05 B	0.02 B	0 B	1.55 T	0 B
PSYAK3	*WDR33*	Chr2: 128477570 T > C	NM_018383.5	c.2029A > G p.Met677Val, missense	0.0001316 (0 homozygous)	0.0067	−0.14	18.22	0.06 T	NA	0.1 B	0.66 D	2.55 T	0 B
	CD44	Chr11: 35160895 G > C	NM_000610.3	c.45G > C p.Val15Val, synonymous	0.0008269 (2 homozygous)	0.01	NA	14.61	NA	NA	NA	NA	NA	NA
	STX3	Chr 11: 59557993 C > T	NM_004177.5	c.291C > T p.Ser97Ser, synonymous	0.001515 (2 homozygous)	0.015	NA	14.77	NA	NA	NA	NA	NA	0 B
	ROM1	Chr11: 62380834 G > T	NM_000327.3	c.81G > T p.Leu27Leu, synonymous	0.001259 (0 homozygous)	0.0033	NA	0.699	NA	NA	NA	NA	NA	NA
	C11ORF95	Chr11: 63531586 G > A	NM_001144936.2	c.1509C > T p.Pro503Pro, synonymous	0.0008351 (0 homozygous)	0.005	NA	5.782	NA	NA	NA	NA	NA	NA
	TRIM64B	Chr11: 89608155 A > G	NM_001164397.2	c.495T > C p.His165His, synonymous	0.0002230 (0 homozygous)	0.00167	NA	0.325	NA	NA	NA	NA	NA	NA
	IL1RAPL1	ChrX: 29417422 A > G	NM_014271.3	c.700A > G p.Thr234Ala, missense	0.000005491 (1 hemizygous)	0	5.75	23.8	0.08 T	NA	0.52 D	1 D	−1.07 T	0 B

B = benign; CADD = Combined Annotation Dependent Depletion (higher scores are more damaging); D = damaging or deleterious; GERP = Genomic Evolutionary Rate Prediction; gnomAD = Genome Aggregation Database; FATHMM = Functional Annotation Through Hidden Markov Models; MT = Mutation Taster; NA = not applicable; PolyPhen2 = Polymorphism Phenotyping v2; RefSeq = Reference Sequence; REVEL = Rare Exome Variant Ensemble Learner; SIFT = Sorting Intolerant from Tolerant algorithm; SpliceAI = Splice Altering algorithm; T = tolerated; U = uncertain.

*Variant position according to Human Feb.2009 (GRCh37/hg19) Assembly.

†*RGS3* and *IL1RAPL1* variants were absent from the in-house exome data of at least 300 ethnically matched controls and from the DNA of a further 100 individuals analyzed.

‡Negative and low scores indicate no or low conservation.

§In family PSYAK2, the *FLI1* synonymous variant was predicted to affect splicing. However, the involved nucleotide was not conserved in evolution and had an allele frequency of 0.002 in South Asians, making it unlikely that it would be pathogenic.

and data not shown). After applying the filtering criteria of allele frequency less than 1%, only 7 homozygous or hemizygous variants were obtained (Table 1). Considering only the hemizygous variants on chromosome X, all variants except the one in *IL1RAPL1* were either polymorphisms or also present as hemizygous in unaffected siblings or the unaffected father (Appendix 1, Tables S4 and S5). Comparison of the filtered exome data did not reveal shared likely pathogenic compound heterozygous variants (Appendix 1, Table S6). The 7 homozogyous or hemizygous variants segregated with the phenotype according to a recessive or an X-linked recessive model. However, 5 synonymous variants were predicted to

be nondamaging to splicing, and 1 missense variant was predicted to be benign by multiple prediction tools. The missense variant in *IL1RAPL1* (NM_014271.3) — c.700A > G, p.(Thr234Ala) — was the only clear likely pathogenic variant, because it alone had high pathogenicity scores.

Sanger sequencing confirmed that both affected individuals were hemizygous for the mutant allele (Figure 1D). The mother was a carrier, and the unaffected father and unaffected brother were hemizygous for the wild-type allele. The aggregated allele frequency for *IL1RAPL1* variant rs1273263334 was 0.0000055 in gnomAD, with 1 hemizygous individual. However, analysis of 230 ethnically matched controls with the help of allele-specific PCR and in-house exome data of 300 individuals (total of 1060 chromosomes) detected no individuals who were hemizygous or carriers for this mutant allele.

Clustal Omega analyses revealed that the arginine and threonine residues substituted by the variants in families PSYAK2 and PSYAK3 were conserved among different orthologues (Figure 1E and F).

Regulator of G-protein signalling 3

The longest isoform of *RGS3* (NM_144488.8) consists of 26 exons, and the variant detected in family PSYAK2 was in exon 7 (Figure 2A). Fourteen isoforms of the gene exist, arising from alternative splicing. Two other isoforms (NM_001282923.2 and NM_017790.6) also had the exon containing the detected variant (Figure 2B and C). Eleven isoforms were N-terminally truncated, with alternate 5′ exons and 5′ UTRs (Figure 2D, E and F; https://genome.ucsc.edu/). The full-length RGS3 protein has 3 evolutionary conserved domains: C2, PDZ and regulator of G-protein signalling (RGS). The variant *RGS3* p.(Arg217Cys) identified in family PSYAK2 affects the C2 domain (Figure 2G); this domain contains a calcium binding region and is responsible for the translocation of RGS3 from the cytosol to the plasma membrane (Figure 2H).[20,21]

Interleukin 1 receptor accessory protein-like 1

IL1RAPL1 consists of 10 exons (NM_014271.4), and the variant identified in family PSYAK3 was in exon 5 (Figure 3A; https://genome.ucsc.edu/). The protein IL1RAPL1 has multiple conserved domains, including 3 immunoglobulin (Ig) domains (Ig super family domain), a transmembrane (TM) domain, a Toll/IL-1R domain and a long PDZ domain at the C-terminus (Figure 3B). The variant *IL1RAPL1* p.(Thr234Ala), identified in affected individuals from family PSYAK3, was located in the second Ig domain. This variant may disturb phosphorylation of PSD-95 by c-Jun terminal kinase (Figure 3C).[22]

RGS3 p.(Arg217Cys) variant

The automated I-TASSER modeller predicted the 3-dimensional structure of the wild-type RGS3 protein and suggested that p.Arg217 resides on a loop region (Figure 4A). PredictProtein indicated that the residue p.Arg217 is present on an exposed region with high solvent accessibility and intermediate flexibility (B = 31–70). I-Mutant Suite predicted a large decrease in protein stability because of the p.(Arg217Cys) variant. SNAP2 suggested that the p.(Arg217Cys) variant was strongly unfavourable to the function of the protein (score = 51), with an accuracy of 75%. CUPSAT indicated that the torsion angle created because of this variant was unfavourable with a $\Delta\Delta G$ value of 0.38 kcal/mol. CUPSAT also predicted that the polar side chain of the basic amino acid Arg217 is replaced with a very reactive sulfhydryl group in RGS3 from the p.(Arg217Cys) variant.

IL1RAPL1 p.(Thr234Ala) variant

Structural modelling of the wild-type protein revealed that the residue p.Thr234 resides at the junction between a β-sheet and a turn (Figure 4B). However, PredictProtein indicated that p.Thr234 is buried inside the IL1RAPL1 wild-type protein with limited solvent accessibility and intermediate flexibility (B = 31–70). I-Mutant Suite indicated a large decrease of stability for IL1RAPL1 because of the p.(Thr234Ala) point mutation. SNAP2 functional prediction showed that the p.(Thr234Ala) variant was slightly unfavourable (score = 7), with 53% accuracy. CUPSAT suggested that the torsion angle created by the p.(Thr234Ala) variant was largely unfavourable to the protein structure, with a $\Delta\Delta G$ value of −1.99 kcal/mol. CUPSAT further emphasized that the polar hydroxyl group of threonine is substituted with a nonpolar methyl group in IL1RAPL1 because of the variant p.(Thr234Ala). This substitution could affect some interactions or the accessibility of this residue in the protein.

Discussion

Our research confirmed that ultra-rare variants can cause recessive or X-linked inherited schizophrenias. Previously, linkage analyses in consanguineous families have supported the existence of recessively inherited loci for psychotic disorders including schizophrenia,[5,9] but they did not identify gene variants. In contrast, X-linked inheritance has been previously described, with the identification of a candidate variant.[13] Our work implicates 1 of a few recessively inherited variants in schizophrenia and extends the number of X-linked recessive genes that may be associated with this disorder.

The rare missense alleles — a homozygous variant c.649C > T, p.(Arg217Cys) in *RGS3* and a hemizygous variant c.700A > G, p.(Thr234Ala) in *IL1RAPL1* — are associated with mostly similar phenotypes in the affected individuals; 3 of the 4 had positive symptoms of schizophrenia (self-smiling, self-talking, auditory or visual hallucinations, random thoughts and delusions), whereas the fourth experienced mostly negative symptoms (anhedonia, an inability to concentrate, social withdrawal and flat affect).

There is evidence to support an important role for *RGS3* in the brain. *RGS3* (OMIM: 602189) is located on chromosome 9q32 and encodes a protein of 1198 amino acids. *Rgs3* knockout mice, with an engineered deletion targeting the PDZ protein domain, have developmental abnormalities, small brain

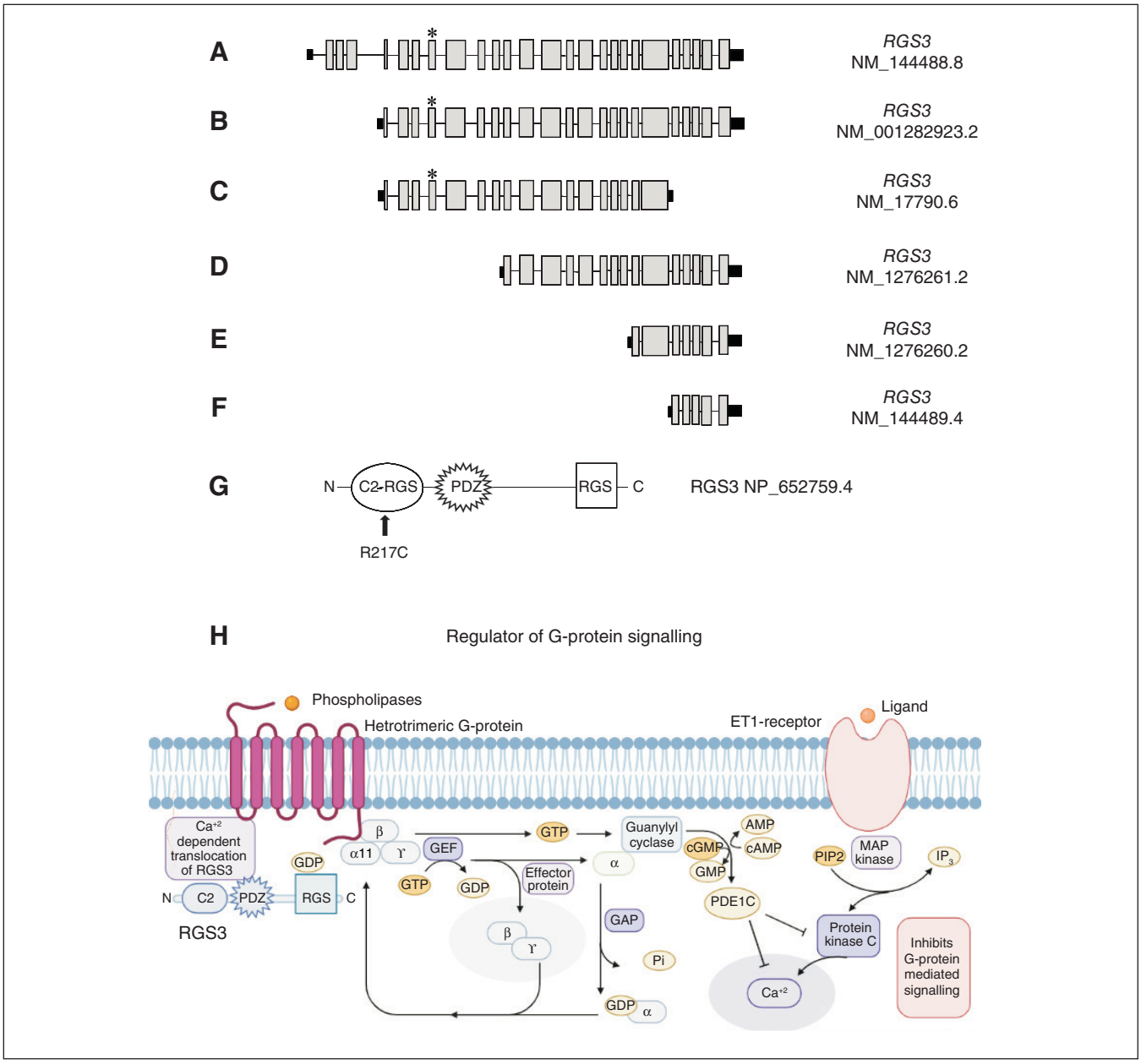

Figure 2: Schematic representation of *RGS3* isoforms, domains and encoded protein. Asterisks depict the position of the variant detected in family PSYAK2. Longer mRNA transcripts encode proteins that are predicted to localize to the cytosol or associate with the plasma membrane. Shorter, N-terminally truncated forms may be nuclear. Black boxes indicate noncoding exons. (A) *RGS3* isoform NM_144488.8 encodes the protein with the highest molecular weight, consisting of 1198 amino acids. (B) *RGS3* isoform NM_001282923.2 contains 22 exons with the variant c.649C > T within exon 4. (C) *RGS3* isoform NM_17790.6 encodes a truncated protein. (D) *RGS3* isoform NM_1276261.2 lacks exons encoding plasma membrane or cytoplasmic domains. (E) *RGS3* isoform NM_1276260.2 encodes a protein that is N-terminally truncated. (F) *RGS3* isoform NM_144490.4 is the shortest isoform. (G) Schematic representation of RGS3 isoform NP_652759.4 showing different domains. The c.649C > T, p.(Arg217Cys) variant is within the C2 domain, which is involved in Ca^{+2}-dependent translocation of the RGS3 protein. (H) RGS3 inhibits the G-protein-mediated postreceptor signalling pathway. The C2 domain at the N-terminal translocates RGS3 from the cytosol to the plasma membrane, whereas the PDZ domain interacts with neuroligin and AMPAR GluR2, which further binds with EphrinB2. After translocation to the plasma membrane, the RGS domain of RGS3 interacts with the heterotrimeric G-proteins, which are composed of α, β and γ subunits. G-protein-coupled receptors activate guanylyl cyclase, which converts GMP to cGMP. It activates adenylyl cyclase to convert cAMP to AMP and activates multiple cytoplasmic proteins, including PED1C. The ligand binds with the ET-1 receptor and activates MAP kinase via cleavage of PIP2 to IP_3 (left side). IP_3 induces the uptake of Ca^{+2} through protein kinase C. PED1C inhibits protein kinase C and reduces intracellular Ca^{+2} by deterring the ET-1 signalling pathway. AMP = adenosine monophosphate; AMPAR = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; cAMP = cyclic AMP; cGMP = cyclic GMP; ET-1 = endothelin-1; GAP = GTPase activating protein; GDP = guanosine diphosphate; GEF = guanidine exchange factor; GMP = guanosine monophosphate; GTP = guanosine triphosphate; IP_3 = inositol triphosphate; MAP = mitogen-activated protein; PDE1C = calmodulin dependent cyclic nucleotide phosphodiesterase; PIP2 = phosphatidylinositol 4,5-bisphosphate.

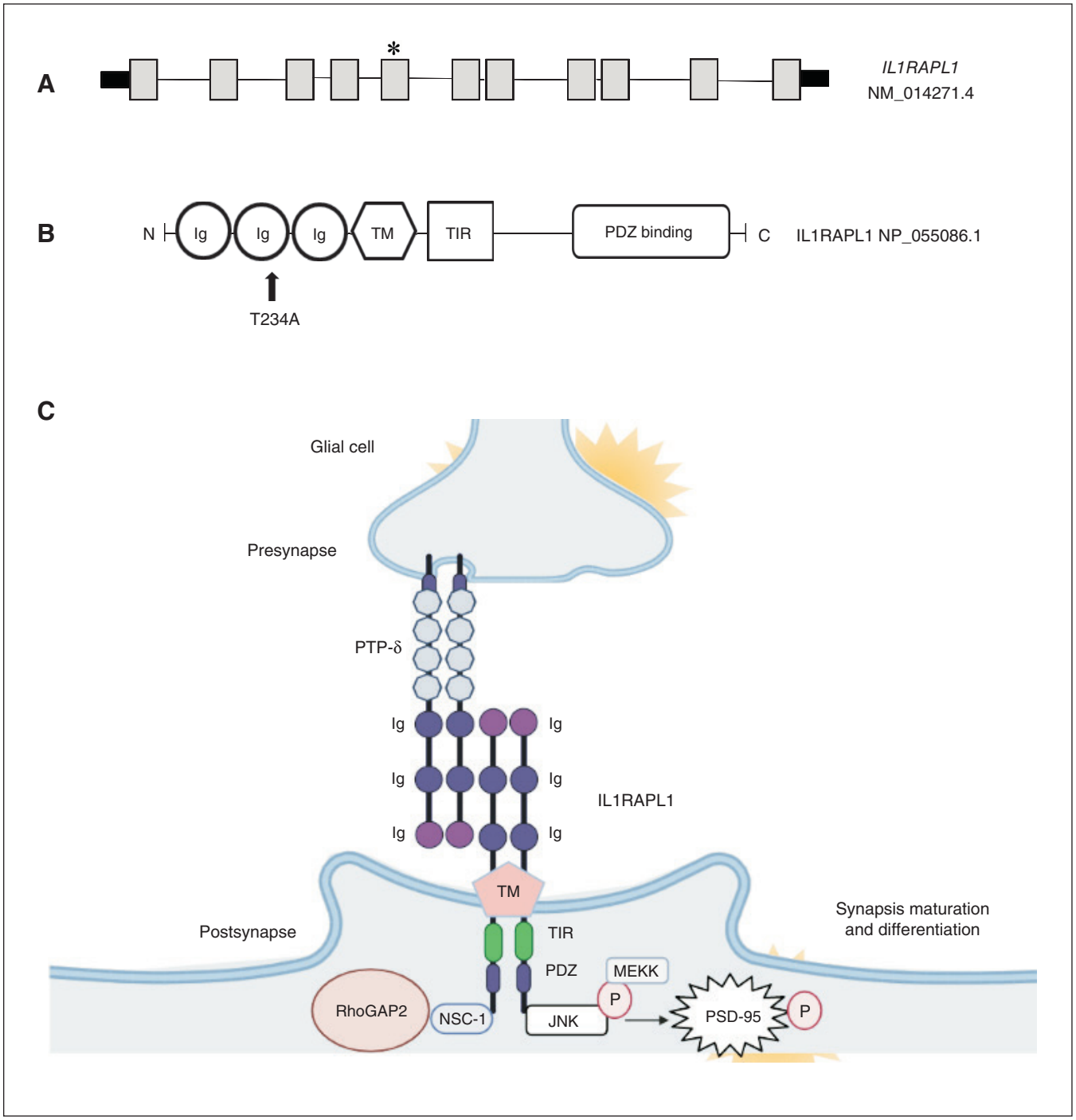

Figure 3: Schematic representation of IL1RAPL1 isoforms, domains and encoded protein. (A) *IL1RAPL1* isoform NM_014271.4 has 11 exons encoding a 696 amino acid protein. An asterisk marks the position of the variant c.700A > G, p.(Thr234Ala), detected in family PSYAK3. (B) Schematic representation of multiple domains in IL1RAPL1, including the N-terminal signal peptide, 3 extracellular Ig-like domains, a TM region, a TIR domain and a C-terminal tail with a PDZ binding domain. (C) At the presynaptic terminal, Ig domains of PTP-δ interact with Ig domains of IL1RAPL1 and increase excitation across the synaptic cleft. The TIR domain interacts with RhoGAP2, with the help of NCS-1 calcium channels. RhoGAP2 is involved in GTPase intrinsic activity and regulates a number of neuronal signalling pathways. The PDZ binding motif of IL1RAPL1 interacts with the PDZ domain of PSD-95 by Ser-295 and regulates the synaptic localization of PSD-95. The C-terminal PDZ binding domain of IL1RAPL1 phosphorylates JNK by MEKK. JNK phosphorylation activates PSD-95 and induces maturation of the synapse. GTPase = guanosine triphosphatase; Ig = immunoglobulin; JNK = C-Jun terminal kinase; MAP = mitogen-activated protein; MEKK = MAP kinase kinase; NCS-1 = neuronal calcium sensor-1; PSD-95 = postsynaptic scaffolding protein-95; PTP-δ = protein tyrosine phosphatase δ; RhoGAP2 = Rho GTPase-activating protein 2; TIR = toll/interleukin-1R; TM = transmembrane.

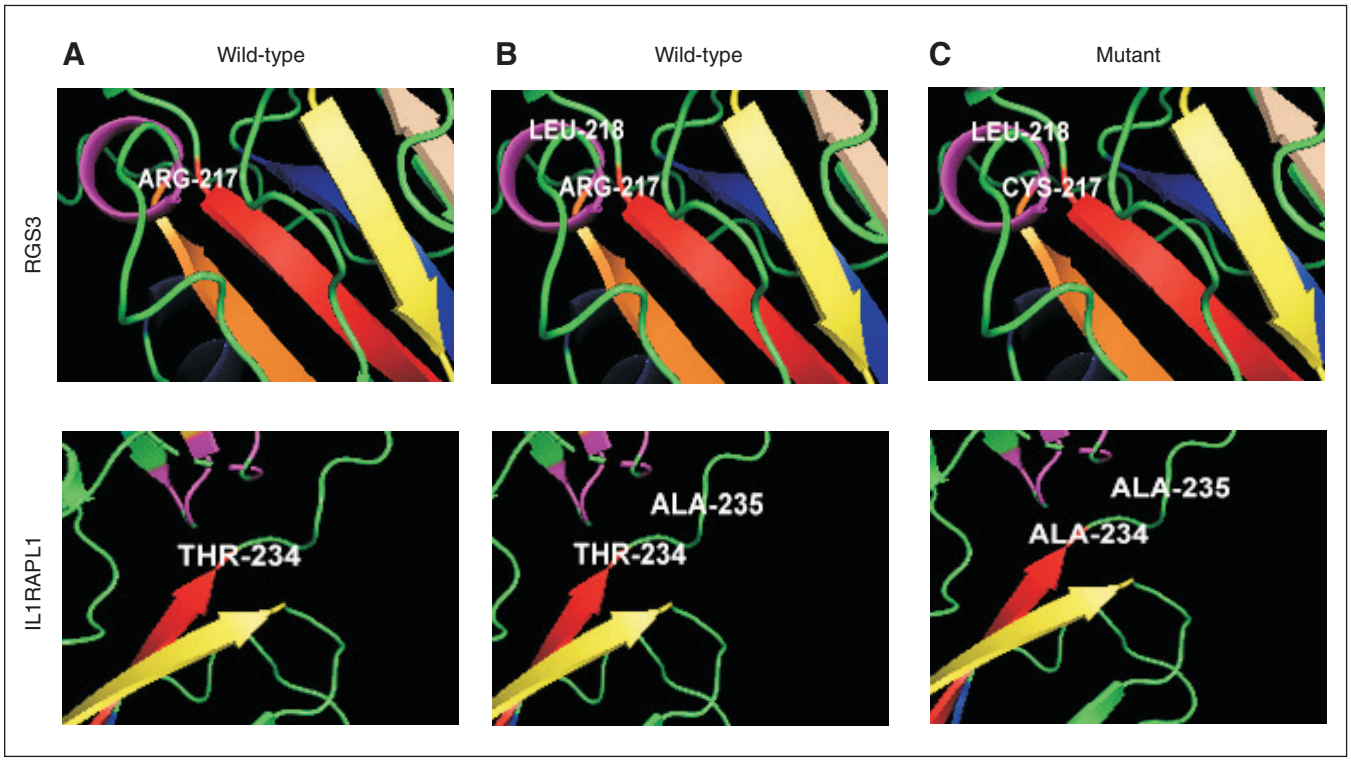

Figure 4: Structural modelling of wild-type and mutant RGS3 and IL1RAPL1 proteins. (A) Structural models of wild-type RGS3 protein (upper panel) and IL1RAPL1 protein (lower panel). (B) Interacting bonds of wild-type RGS3 p.Arg217 and IL1RAPL1 p.Thr234 proteins. (C) RGS3 p.(Arg217Cys) and IL1RAPL1 p.(Thr234Ala) variants. The RGS3 variant cysteine is in the exposed region of the protein, making it highly accessible to solvent molecules. The IL1RAPL1 variant alanine is in a buried region of the protein and may not directly affect the interaction of the protein.

sizes and brain structural abnormalities.[23] That study demonstrated that RGS3 was vital to maintaining neuronal progenitor cells. In a second mouse model of *Rgs3*$^{\Delta RGS}$ with a deleted RGS domain, constructed as a model to study inflammation, no immune defects were observed.[24] Behavioural analyses were not completed for these mice.

Studies in rats also support the function of RGS3 in the brain. Expression of *Rgs3* transcripts in rats is higher in the dorsal root ganglion of neurons. *Rgs3* expression becomes downregulated in injured neurons after peripheral nerve transection, disabling rats from responding to external stimuli.[25] Downregulation of the RGS3 protein in the dorsal root ganglion sensory neurons of rats may introduce new responses to G-protein-coupled receptors (GPCRs) or may alter their interaction with ligands, including neuropeptide Y and cholecystokinin. In axotomized rats (with damaged sensory neurons), RGS3 protein levels were reduced, altering chemosensation from de novo or increased responses to chemokines.[25]

Studies into *rgs-3* loss of function in *Caenorhabditis elegans* have revealed that wild-type RGS3 interacts with GPCRs through heterotrimeric G-protein; its loss leads to abnormally high G-protein-coupled signalling across the neurons.[26] Mutant *C. elegans* were defective in responding to intense attractive and aversive stimuli, analogous to behavioural defects.[27] However, the mutant worms were better able to respond to a weak stimulus of touch compared to wild-type worms. This suggested that in the case of a strong stimulus, oversignalling rendered mutant *C. elegans* unable to respond properly. These defects seen in the mutants in response to strong external stimuli were restored after decreasing G-protein-coupled signalling with the help of chameleon proteins, or after increasing the expression of calcium-binding proteins in the neurons.[27]

RGS is highly expressed in the brain. The missense variant c.649C > T, p.(Arg217Cys) in *RGS3* observed in the patients of family PSYAK2 might disturb the GPCR-mediated signalling pathway affecting neuronal signal transduction and synaptic modulation, perhaps causing behavioural manifestations in humans. A substitution of an arginine variant for the cysteine variant can dramatically change the structural folding and conformation of protein because of the reactive –SH group of cysteine,[28] an effect supported by in silico analyses.

RGS proteins play important roles in GPCR signal transduction, and many are highly expressed in the brain. The RGS group of proteins are considered to be potential druggable targets for the treatment of central nervous system disorders,[29] hypertension[30] and addiction[31] by regulating G-protein-dependent calcium signalling across neurons. Chromosomal region 1q21–22 encompassing *RGS4* has been associated with schizophrenia through linkage studies in 22 families.[32] *RGS4* is considered a susceptibility gene for schizophrenia, but its pattern of association was diverse in different individuals.[33] Furthermore, *RGS4* alleles rs10759 and rs2661319 were associated with a risk of

schizophrenia in patients from 13 different countries.[34] In addition, *RGS2* polymorphisms have been associated with schizophrenia,[35] depression,[36] anxiety and aggression.[37] Another interesting instance is provided by a de novo missense variant of *RGS12*, p.(Arg702Lys), detected in 2 different patients with schizophrenia.[38] Both RGS4 and RGS2 proteins have been shown to interact with dopamine receptors (DRD2), which play a crucial role in neural signalling and behaviour,[39] whereas RGS12 plays a role in the RAS signalling pathway, which is disturbed in patients with schizophrenia[40] and might explain the association of these genes with this disorder.

Our work indicates that the *IL1RAPL1* variant may be a possible cause of schizophrenia in the affected individuals from family PSYAK3. Previously, 49 X-linked recessive variants in *IL1RAPL1* have been reported to cause nonsyndromic intellectual disability (Human Gene Mutation Database, www.hgmd.cf.ac.uk/ac/index.php, accessed May 2022). Apart from intellectual disability, pathogenic variants of this gene have been implicated in anxiety disorders,[41] hypotonia, language delay,[42] infantile-onset seizures,[43] developmental delay[44] and autism with behavioural problems.[45] In 2 multigenerational multiplex families, a deletion of 635 kb in *IL1RAPL1* c.83_779del, p.28_259del was observed in 5 affected males. This deletion is predicted to produce a truncated 464 amino acid protein, devoid of the extracellular Ig domains. The variant has been shown to cause depression, seizures, oppositional behaviour and impulsivity along with the intellectual disability.[46]

As expected for an X-linked recessive inherited disorder, almost all cases have been reported in males. An exception is a 373 kb interstitial deletion in *IL1RAPL1* involving exon 4–6 in an 8-year-old girl associated with intellectual disability, developmental regression, autism spectrum disorder, epilepsy and a behavioural disorder. It was hypothesized that the manifestation of the disorder in a female patient might be the result of unfavourable X-linked inactivation pattern or the effects of polygenic modifiers on the brain.[44]

IL1RAPL1 (OMIM: 300206) — also known as *MRX10*, *MRX21* and *TIGIRR-2* — is located on chromosome Xp21 and encodes a protein consisting of 696 amino acids. *IL1RAPL1* (interleukin 1 receptor accessory protein like-1) is a member of the Toll/interleukin 1 receptor family. IL1RAPL1 expression is higher in the hippocampus of the brain, with roles in synapse formation and modulation, dendritic spine formation, presynaptic secretion,[47] presynaptic differentiation, dendrite complexity and neuronal maturation.[48] IL1RAPL1 interacts with neuronal calcium sensor 1 (NCS1), a dopamine receptor interacting protein, which has also been implicated in schizophrenia and bipolar disorder that often feature psychosis.[49] The Ig domains of IL1RAPL1 interact with the Ig domains of PTP-δ, increasing excitation along TIR and PDZ binding domains.[48] The PDZ binding motif interacts with the postsynaptic scaffolding protein (PSD-95), which plays an essential role in synapse maturation and differentiation by accumulating synaptic proteins.[50] The substitution Thr234Ala might reduce the protein interaction with polar solvent substrates: threonine is a polar, uncharged amino acid, and alanine is a nonpolar aliphatic amino acid. Interestingly, *Il1rapl1* knockout mice have behavioural deficits. This mouse model has disturbed neuronal physiology leading to inhibitory–excitatory imbalance,[51] decreased synaptic spine density in the hippocampal region of the brain, disturbed synapses excitation, hyperactivity,[52] elevated locomotor activities, altered dendrite morphology, learning difficulties,[53] profound anxiety and disturbed behavioural flexibility.[54]

Studies in model organisms reveal that *Rgs3* and *Il1rapl1* loss of function cause brain or behavioural defects in animals. Our work supports the involvement of variants of the respective orthologous genes in behavioural problems in humans as well. Our findings for *RGS3* and *IL1RAPL1* in families with schizophrenia, together with a previous description of recessively inherited missense variant *USP53* p.(Cys228Arg) in 2 patients with psychosis (doi: 10.22541/au.162626102.28489874/v1), provides the first direct evidence that variants in proteins of the neurotransmitter interactome can directly produce the psychosis phenotype with a very high effect.

Limitations

Although our studies suggest that *RGS3* and *IL1RAPL1* variants are implicated in schizophrenia, definitive proof requires the identification of rare deleterious variants in the same genes in additional patients with psychiatric disorders. There is also a possibility that 1 of these variants was a false positive, but it is unlikely that 3 independent families would each implicate a single variant affecting a gene related to brain function by chance, especially with pre-existing suspicions of associated variants related to neurotransmission.

Conclusion

We have identified 2 rare variants — c.649C > T:p.(Arg217Cys) in *RGS3* and c.700A > G:p.(Thr234Ala) in *IL1RAPL1* — in Pakistani consanguineous families with multiple affected individuals. Functional studies involving variant-specific animal models or cell lines will broaden our knowledge of genetic variants in psychiatric disorders. These discoveries may identify additional proteins necessary for brain function, which could lead to the development of drugs for treatment of this devastating disorder.

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Competing interests: None declared.

Contributors: J. Pardo and S. Naz designed the study. A. Kanwal and J. Pardo acquired the data, which A. Kanwal, J. Pardo and S. Naz analyzed. A. Kanwal, J. Pardo and S. Naz wrote the article, which J. Pardo reviewed. All authors approved the final version to be published, agree to be accountable for all aspects of the work and can certify that no other individuals not listed as authors have made substantial contributions to the paper.

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