

# Support for the N-Methyl-D-Aspartate Receptor Hypofunction Hypothesis of Schizophrenia From Exome Sequencing in Multiplex Families

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**Importance:** Schizophrenia is a complex genetic disorder demonstrating considerable heritability. Genetic studies have implicated many different genes and pathways, but much of the genetic liability remains unaccounted for. Investigation of genetic forms of schizophrenia will lead to a better understanding of the underlying molecular pathways, which will then enable targeted approaches for disease prevention and treatment.

**Objective:** To identify new genetic factors strongly predisposing to schizophrenia in families with multiple affected individuals with schizophrenia.

**Design:** We performed genome-wide array comparative genomic hybridization, linkage analysis, and exome sequencing in multiplex families with schizophrenia.

**Setting:** Probands and their family members were recruited from academic medical centers.

**Participants:** We intended to identify rare disease-causing mutations in 5 large families where schizophrenia transmission appears consistent with single-gene inheritance.

**Intervention:** Array comparative genomic hybridization was used to identify copy number variants, while exome sequencing was used to identify variants shared in all affected individuals and linkage analysis was used to further filter shared variants of interest. Analysis of select variants was performed in cultured cells to assess their functional consequences.

**Main Outcome Measures:** Rare inherited disease-related genetic mutations.

**Results:** No segregating rare copy number variants were detected by array comparative genomic hybridization. However, in all 5 families, exome sequencing detected rare protein-altering variants in 1 of 3 genes associated with the N-methyl-D-aspartate (NMDA) receptor. One pedigree shared a missense and frameshift substitution of *GRM5*, encoding the metabotropic glutamate receptor subtype 5 (mGluR5), which is coupled to the NMDA receptor and potentiates its signaling; the frameshift disrupts binding to the scaffolding protein tamalin and increases mGluR5 internalization. Another pedigree transmitted a missense substitution in *PPEF2*, encoding a calmodulin-binding protein phosphatase, which we show influences mGluR5 levels. Three pedigrees demonstrated different missense substitutions within *LRP1B*, encoding a low-density lipoprotein receptor-related protein tied to both the NMDA receptor and located in a chromosome 2q22 region previously strongly linked to schizophrenia.

**Conclusions and Relevance:** Exome sequencing of multiplex pedigrees uncovers new genes associated with risk for developing schizophrenia and suggests potential novel therapeutic targets.

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**S**CHIZOPHRENIA ENCOMPASSES disturbances of thought, behavior, and emotion.<sup>1</sup> It is a complex disease with multiple genes and environmental factors contributing to its risk.<sup>2,3</sup> Multiple genetic loci have been reported through genetic linkage<sup>4</sup> and association<sup>5,6</sup> studies.

Two models have been proposed to explain the genetic architecture of complex neuropsychiatric diseases such as schizo-

phrenia. The common disease-common variant model posits that multiple common variants of relatively small effect contribute interactively to disease susceptibility. Genome-wide association studies offer an approach for discovering such common variants. The Schizophrenia Psychiatric Genome-wide Association Study Consortium recently assembled and conducted a mega-analysis of genome-wide association studies in a 2-stage analysis of 51 695 individuals. They replicated 2 pre-

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viously implicated loci (6p21.32-p22.1 and 18q21.2) and found genome-wide significance for 5 novel loci (1p21.3, 2q32.3, 8p23.2, 8q21.3, and 10q24.32-q24.33) for schizophrenia.<sup>6</sup> However, the odds ratios for these single-nucleotide polymorphisms (SNPs) were modest, and many were intragenic. Since genome-wide association studies rely on the detection of common polymorphisms that are rarely responsible for disease but are in linkage disequilibrium with causative variants, alternative approaches such as resequencing and functional assays are necessary to identify the functionally significant variants within each associated haplotype.

Alternatively, under the common disease–rare variant model, rare variants of large effect are highly penetrant and subjected to strong purifying selection and their frequency in the population is essentially maintained by de novo occurrence. Recent high-throughput genomic studies including copy number variant (CNV) analyses for detecting deletions and duplications<sup>7</sup> and exome sequencing have identified several rare variants in the etiology of schizophrenia.<sup>8,9</sup> In addition, serendipitous discovery of rare chromosomal translocations has implicated several candidate genes including, for example, disrupted in schizophrenia (*DISC1*) in a large Scottish pedigree.<sup>10</sup>

Because of the need for larger sample sizes to achieve sufficient statistical power to derive any disease association, recent genetic studies have shifted away from family-based to large case-control studies. While contribution of common variants toward schizophrenia risk has been modest, rare variant discovery has suggested extreme locus heterogeneity, largely based on the random mutation model for single-nucleotide change or CNV.<sup>7-9,11-14</sup> In fact, recent studies have determined that de novo mutations involving predisposing chromosomal segments and single genes, respectively, play major roles in sporadic cases with schizophrenia.<sup>15</sup> Since there are many new genetic mutations in each generation,<sup>16</sup> it is not always possible to assign causative roles to specific mutations in sporadic cases. In addition, de novo mutations may predominate among severe cases with early onset and affect reproductive fitness. Thus, genetic models accounting for new mutations do not sufficiently explain the heritable risk of schizophrenia in the general population. Therefore, in the current study, we focused on multiplex families with several affected individuals using whole-genome methods to identify private, highly penetrant mutations that cosegregate with disease. In all the 5 pedigrees studied, we identified rare variants cosegregating with schizophrenia in genes that can be broadly tied to *N*-methyl-D-aspartate (NMDA) receptor function.

## METHODS

### SUBJECTS

Families were selected from the Genetics Initiative of the National Institute of Mental Health for a schizophrenia multisite study.<sup>17</sup> The background of the initial National Institute of Mental Health study, ascertainment strategies, data collection, pedigree extension, diagnosis, and data analyses were previously described.<sup>17</sup> Briefly, trained clinicians established *DSM-III-R* diagnoses of schizophrenia and schizoaffective disorder, depressed for sub-

jects 18 years and older using the semistructured Diagnostic Interview for Genetics Studies.<sup>1,18</sup> A family informant was also interviewed about family psychiatric history using the Family Interview for Genetic Studies.<sup>19</sup> Demographics, ethnicity, and phenotypic information were obtained from National Institute of Mental Health Distribution 8.0.

The majority of individuals in each pedigree had schizophrenia. All study protocols were approved by the respective institutional review boards from the original institutions. All subjects consented to research participation as well as sharing of DNA and deidentified clinical information to qualified investigators.

### FAMILIES

We selected 5 large families (**Figure 1**) with multiple affected subjects with schizophrenia in which its genetic transmission appeared most consistent with single-gene inheritance. Two pedigrees were of African American ancestry (pedigrees 1 and 3), and 3 were of European American ancestry (pedigrees 2, 4, and 5) per information collected by the original study sites. Ethnicity is listed for comparison with the control population. The mean age at onset of schizophrenia was 23.4 years. DNA was available from a total of 41 subjects, consisting of 24 affected (*DSM-III-R* criteria for schizophrenia [n=18] or schizoaffective disorder, depressed [n=4] or psychosis not otherwise specified [n=2]) and 17 unaffected individuals.

### CNV ANALYSIS

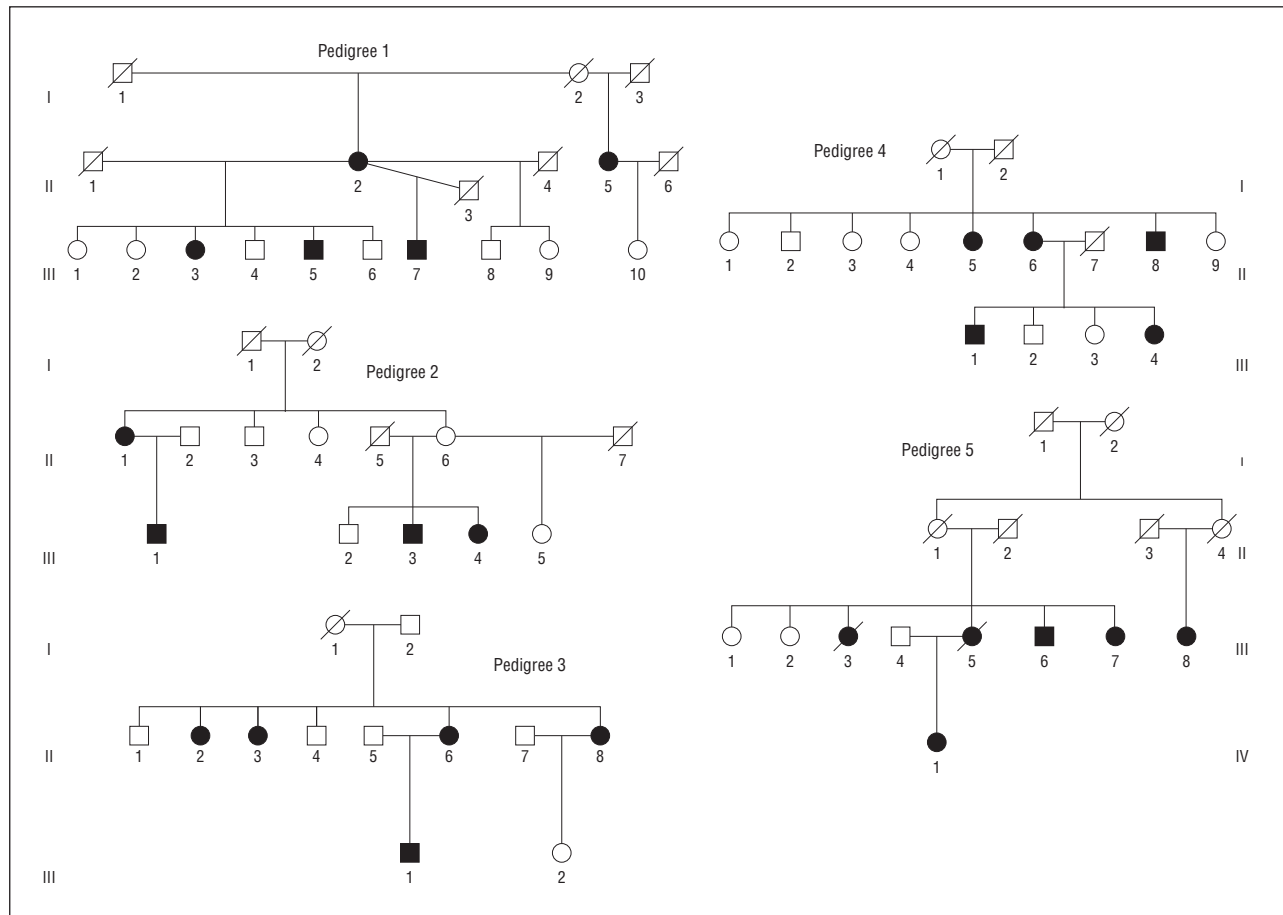
All affected individuals with available DNA were subjected to CNV analysis (24 in total) (eTable 1, <http://www.jamapsych.com>). The CNVs were investigated using a custom-designed hot-spot chip targeted to recurrent regions of rearrangements with known disease associations<sup>20</sup> with additional low-resolution coverage over the entire genome (termed *genomic backbone*) to identify novel large CNVs (>350 kilobase pairs [kbp])<sup>20</sup> (described in detail in the eAppendix eMethods section). Overall, our method was designed to detect variants more than 50 kbp in the targeted regions and more than 350 kbp in the genomic backbone at a high sensitivity (>99%).<sup>20</sup>

### GENETIC LINKAGE ANALYSIS AND HAPLOTYPING

All pedigree members with DNA available from the 5 pedigrees (41 total, 24 affected and 17 unaffected) (eTable 1) were genotyped using the Illumina HumanLinkage-24 BeadChip, detecting 6090 SNP markers. If 10% or more of the samples failed genotyping for an individual SNP, they were discounted from subsequent analyses. Mendelian pedigree inconsistencies were identified using PedCheck (<http://watson.hgen.pitt.edu/register/docs/pedcheck.html>) and non-mendelian errors were investigated by MERLIN (<http://www.sph.umich.edu/csg/abecasis/Merlin/index.html>), with all probable errors being removed. Multipoint nonparametric linkage analysis was performed using the ALL statistics of MERLIN. MERLIN was used to phase genotypes into haplotypes.

### EXOME SEQUENCING

We chose 2 or 3 of the most distantly related schizophrenic subjects from each of the 5 pedigrees to perform exome sequencing (12 subjects total) with the rationale that these affected individuals within families would share the same potentially pathogenic variant and those who are the most distantly related (because they share the smallest proportion of their ge-



**Figure 1.** Schizophrenia pedigrees studied by array comparative genomic hybridization and whole-exome sequencing. Filled-in symbols indicate affected individuals.

nomes) would narrow the number of putative causative variants. Whole-exome enrichment was completed using the SureSelect Human All Exon kit (Agilent) optimized for use with SOLiD sequencing. Three micrograms of genomic DNA were fragmented by sonication to approximately 150 to 200 base pairs (bp) and prepared for library construction using the manufacturer's protocol.

Sequences were hybridized to SureSelect Biotinylated RNA baits (Agilent) for target enrichment, followed by washing, elution, and additional amplification. Each captured library was subsequently bar coded allowing multiplex pools of 12 individuals. The bar-coded libraries were sequenced using the standard protocols for the SOLiD version 4 sequencer (Applied Biosystems). Sequencing produced either 50-bp fragment or 75-bp paired-end reads.

BioScope version 1.3 (Applied Biosystems) was used for aligning sequencing reads to human reference genome HG18 (<http://hgdownload.cse.ucsc.edu/goldenPath/hg18/chromosomes>). Mapping generated BAM files (<http://samtools.sourceforge.net/>) used to identify variants within each sample. Exome coverage was assessed by aligning mapped reads with exonic nucleotides using the consensus coding sequence (CCDS) database from May 2010 (<http://hgdownload.cse.ucsc.edu/goldenPath/hg18/database/>). Single-nucleotide polymorphism calling used the Find SNPs tool within BioScope using medium call stringency, with the "skip high coverage" option turned off. Indels were assigned using default parameters of the Find Small InDels tool.

To identify rare disease-associated mutations, we discriminated between variants that were protein altering (missense,

nonsense, coding indel, or disrupting splicing) (SeattleSeq Annotation [<http://snp.gs.washington.edu/SeattleSeqAnnotation134/>] based on the National Center for Biotechnology Information and CCDS databases), not found among 23 in-house sequenced exomes of individuals without known psychiatric disorders, and also not present or very rare in the publically available database dbSNP135 (<http://www.ncbi.nlm.nih.gov/projects/SNP>) (allele frequency  $\leq 1\%$ ) and the National Heart, Lung, and Blood Institute (National Heart, Lung, and Blood Institute Exome Sequencing Project, Seattle, Washington [<http://evs.gs.washington.edu/EVS>] [ESP5400, May 2012]) and National Institute of Environmental Health Sciences (National Institute of Environmental Health Sciences Environmental Genome Project, Seattle, Washington [<http://evs.gs.washington.edu/niehsExome>] [May 2012]) (allele frequency  $\leq 0.1\%$ ) exome variant servers. Genomic positions were converted from hg18 to hg19 using the Lift Genome Annotations tool (<http://genome.ucsc.edu/cgi-bin/hgLiftOver>). PolyPhen-2 was used to predict the possible impact of an amino acid substitution on the structure and function of a human protein (<http://genetics.bwh.harvard.edu/pph2/>). The Genomic Evolutionary Rate Profiling score was used as a measure of nucleotide-specific evolutionary constraint.<sup>21</sup>

## DNA CONSTRUCTS

The *GRM5b* isoform (OriGene) was cloned into pCI-neo (Promega). Multistep polymerase chain reaction was used to insert a FLAG-tag between metabotropic glutamate subtype 5

(mGluR5) residues 22 and 23. Variants were introduced with the Agilent QuikChange site-directed mutagenesis kit; sequences were confirmed by Sanger sequencing. Protein expression and proper membrane distribution of FLAG-tagged constructs were confirmed by transfection and immunoblotting of soluble and insoluble cell fractions.

## CELLS

HEK293T cells (ATCC) were maintained in Dulbecco modified Eagle medium; fetal bovine serum, 10%; L-glutamine, 1%; and gentamicin, 0.1%. Primary hippocampal neurons were prepared from E18 Sprague-Dawley rats and cultured as described.<sup>22</sup> Use and care of animals followed National Institutes of Health Animal Research Advisory Committee guidelines. Cells were transfected using Lipofectamine (Invitrogen).

## IMMUNOBLOTTING AND IMMUNOPRECIPITATION

Transiently transfected HEK293 cells were lysed with Triton X-100, 1%, in TRIS-buffered saline. Lysates were incubated with antibodies at 4°C overnight, then with protein A/G agarose beads for 2 hours. After washing, beads were incubated with sample buffer and eluted proteins were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and analyzed by Western blot. Monoclonal rabbit anti-mGluR5 antibody was purchased from Abcam. Monoclonal mouse anti-FLAG antibody was purchased from Sigma. Monoclonal rabbit anti-HA antibody was purchased from Cell Signaling Technology.

## MEMBRANE PROTEIN ISOLATION

To assay potential differential membrane distribution of mGluR5 in the presence of PPEF2, cell compartments were separated posttransfection using the ProteoExtract Native Membrane Protein Extraction Kit (Calbiochem). Prior to cell lysis and membrane extraction, 100 μM mGluR5 agonist 3,5-dihydroxyphenylglycine was added to culture media for 30 minutes.

## IMMUNOFLUORESCENCE ASSAY OF RECEPTOR TRAFFICKING

The fluorescence-based antibody uptake internalization assay in the presence of agonist (50 μM 3,5-dihydroxyphenylglycine, 30 minutes, 37°C) was performed as described.<sup>22</sup> Images were analyzed on an LSM 510 confocal microscope (Zeiss). Optical sections were collected, and figures show maximum projections. For quantification, internalization was measured with MetaMorph 6.0 (Universal Imaging). Values represent means and standard errors of the mean. Significance was determined with an unpaired *t* test.

# RESULTS

## CNV ANALYSIS

The CNVs are listed in eTable 2. After comparison with CNV data from 8329 controls,<sup>23</sup> several common copy number polymorphisms were identified; however, no rare CNVs (ie, observed in <8 of 8329 controls) were detected in any affected individuals in a pedigree. Further, no previously reported pathogenic large rare CNVs were identified.<sup>7,24</sup> Therefore, we conclude that rare CNVs

are not responsible for schizophrenia in these families.

## EXOME SEQUENCING

Exome sequencing yielded an average mapped 2.48 Gb per sample; within coding regions, there was an average of 44 reads/nucleotide, with 87% of exonic nucleotides covered by 5 or more reads (eTable 3 and eTable 4). An average of 42 738 variants (42 050 SNPs and 688 indels) were seen per individual, of which 14 851 (14 779 SNPs and 73 indels) were in coding regions (eTable 5).

To identify disease-causing mutations, we filtered for protein-altering rare mutations (<0.1%) that were present in all affected pedigree members and also excluded variants falling outside regions not shared within a pedigree as determined by genetic linkage analysis (eTable 6). Variants passing all of these filtering steps were confirmed by Sanger DNA sequence analysis of all affected individuals in each pedigree. All variants within a given shared haplotype, as defined by linkage, will be present in all family members.

Twenty-two unique variants in 21 different genes (Table and eTable 7) were seen in all affected members of a pedigree (10 segregating variants in pedigree 2; 4 apiece in pedigrees 1, 3, and 4; and none in pedigree 5).

Among these genes, only *GRM5*, encoding the mGluR5 receptor, has been previously implicated in schizophrenia.<sup>25,26</sup> Novel *GRM5* missense substitution G369V was present in all 4 affected members of pedigree 2 for whom DNA was available and was validated by Sanger DNA sequencing. We rescreened all the pedigrees by Sanger sequencing of the entire *GRM5* coding sequence and found an additional novel frameshift (P1148fs) in pedigree 2 (eFigure 1). The frameshift had been detected in sequenced exomes from only 1 of 3 sequenced individuals from this pedigree but actually occurred in all 4 affected pedigree members. (Because P1148fs and G369V were always present together in pedigree 2, even in individuals with an unrelated parent, we concluded that they reside in *cis* on the same allele.) The frameshift adds 17 novel residues before terminating prematurely in the ninth and final exon and is therefore expected to produce a truncated polypeptide not subject to nonsense-mediated decay.

In 2 other pedigrees (pedigrees 1 and 4), variants were found in all affected individuals in the same gene, *LRP1B*, encoding a low-density lipoprotein receptor–related protein expressed predominantly in the brain<sup>27,28</sup> (Table and eFigure 1). Missense substitutions G3458K and A924G were observed in all affected individuals in pedigrees 1 and 4, respectively.

Pedigree 5 lacked variants shared in all affected pedigree members; we examined all linkage regions by haplotype analysis but did not observe any genomic region shared by all 5 affected individuals (eTable 6). These data exclude a single-locus model in this pedigree and suggest an alternative oligogenic model for schizophrenia where more than 1 phenotypically relevant gene is necessary for the manifestation of the disorder among the different cases in this pedigree.<sup>29</sup> We then determined if any of the individuals in pedigree 5 who had undergone



**Table. Rare Protein-Altering Variants Segregating in All Affected Individuals Within a Pedigree<sup>a</sup>**

| Pedigree | Gene                     | Genomic Position (hg19) (Chromosome: Nucleotide) | Nucleotide Change | NHLBI EVS Frequency (Alleles) |                  | NIEHS EVS Frequency (Alleles) | Amino Acid Change        | GERP Score | PolyPhen-2 Prediction |                   |
|----------|--------------------------|--|-------------------|-------------------------------|------------------|-------------------------------|--------------------------|------------|-----------------------|-------------------|
|          |                          |  |                   | European American             | African American |                               |                          |            | HumVar                | HumDiv            |
| 1        | <i>LRP1B</i>             | 2:141200115                                      | C/T               | 0/7020                        | 3/3738           | 0/190                         | G3458K                   | 4.46       | Possibly damaging     | Benign            |
|          | <i>ANKRD26</i>           | 10:27352995                                      | A/G               |                               |                  | 0/190                         | F429L                    | -5.72      | Benign                | Benign            |
|          | <i>ZNF438</i>            | 10:31134185                                      | T/C               | 0/7020                        | 2/3738           | 0/190                         | H721R                    | -11        | Benign                | Benign            |
| 2        | <i>COL18A1</i>           | 21:46907409                                      | G/A               | 0/6762                        | 6/3384           | 0/190                         | A628T                    | -2.83      | Benign                | Benign            |
|          | <i>RPS6KC1</i>           | 1:213415395                                      | C/A               | 0/7020                        | 2/3738           | 0/190                         | T859N                    | 3.62       | Benign                | Benign            |
|          | <i>WDR17</i>             | 4:177046444                                      | C/T               | 9/7020                        | 0/3738           | 1/190                         | A267V                    | 2.63       | Possibly damaging     | Benign            |
|          | <i>EZH2</i>              | 7:148515151                                      | T/C               | 0/7020                        | 0/3738           | 0/190                         | K348R                    | 5.78       | Possibly damaging     | Benign            |
|          | <i>SFTPC</i>             | 8:22020201                                       | G/A               | 1/6882                        | 0/3574           | 0/190                         | A53T                     | 4.41       | Probably damaging     | Probably damaging |
|          | <i>SSRP1</i>             | 11:57102013                                      | C/T               | 0/7020                        | 0/3738           | 0/190                         | R55H                     | 5.87       | Probably damaging     | Probably damaging |
|          | <i>GRM5</i>              | 11:88386377                                      | C/A               | 0/7200                        | 0/3738           | 0/190                         | G369V                    | 5.77       | Probably damaging     | Probably damaging |
|          | <i>GRM5<sup>b</sup></i>  | 11:88241863                                      | insG              | NA                            | NA               | 0/190                         | 1148fs                   | 3.95       | NA                    | NA                |
|          | <i>NAALAD2</i>           | 11:89868771                                      | T/C               | 0/7200                        | 0/3738           | 0/190                         | Y43H                     | -5.98      | Benign                | Benign            |
|          | <i>OSBPL8</i>            | 12:76767190                                      | A/C               | 0/7016                        | 0/3736           | 0/190                         | C617W                    | 5.22       | Benign                | Benign            |
|          | <i>MYO1C</i>             | 17:1375255                                       | C/T               | 0/7200                        | 0/3738           | 0/190                         | R621H                    | 5.19       | Probably damaging     | Probably damaging |
| 3        | <i>LAMA3</i>             | 18:21407372                                      | G/A               | 1/6760                        | 0/3354           | 0/190                         | V922M                    | -4.98      | Possibly damaging     | Benign            |
|          | <i>PPEF2</i>             | 4:76811270                                       | C/T               | 0/7020                        | 6/3738           | 0/190                         | R86H                     | 3.33       | Benign                | Benign            |
|          | <i>RAB11FIP1</i>         | 8:37732714                                       | C/A               | 0/7020                        | 0/3738           | 0/190                         | R314L                    | 4.37       | Probably damaging     | Possibly damaging |
|          | <i>WHSC1L1</i>           | 8:38133184                                       | T/C               | 0/6646                        | 0/3072           | 0/190                         | D1430G                   | 4.38       | Benign                | Benign            |
|          | <i>ZC3H7A</i>            | 16:11855766                                      | G/A               | 0/7014                        | 2/3736           | 0/190                         | S738L                    | 3.67       | Benign                | Benign            |
| 4        | <i>GBP3</i>              | 1:89478867                                       | C/T               | 0/7020                        | 1/3738           | 0/190                         | G/A 5' splice donor site | 3.85       | NA                    | NA                |
|          | <i>LRP1B</i>             | 2:141739845                                      | G/C               | 2/7020                        | 0/3738           | 0/190                         | A924G                    | 4.55       | Possibly damaging     | Benign            |
| 5        | <i>DNAH7</i>             | 2:196852859                                      | T/A               | 0/6598                        | 0/2994           | 0/190                         | G483V                    | 5.81       | Possibly damaging     | Benign            |
|          | <i>NUP160</i>            | 11:47810074                                      | G/A               | 8/7020                        | 2/3738           | 0/190                         | P1201S                   | 3.64       | Benign                | Benign            |
|          | <i>LRP1B<sup>c</sup></i> | 2:140992440                                      | C/T               | 3/7017                        | 0/3738           | 0/190                         | G4525E                   | 3.39       | Probably damaging     | Probably damaging |

Abbreviations: EVS, exome variant server; GERP, Genomic Evolutionary Rate Profiling; HumDiv, a data set used to train PolyPhen-2 that was compiled from all damaging alleles with known effects on the molecular function causing human mendelian diseases, present in the UniProtKB database, together with differences between human proteins and their closely related mammalian homologs, assumed to be nondamaging; HumVar, a data set used to train PolyPhen-2 that consists of all human disease-causing mutations from UniProtKB, together with common human nonsynonymous single-nucleotide polymorphisms (minor allele frequency >1%) without annotated involvement in disease, which were treated as nondamaging; NA, not applicable; NHLBI, National Heart, Lung, and Blood Institute Exome Sequencing Project; NIEHS, National Institute of Environmental Health Sciences Environmental Genome Project.

<sup>a</sup>Genes assigned causative roles are in bold.

<sup>b</sup>Variants detected by exome sequencing (but not in all affected pedigree members) confirmed to be in all affected members of pedigree 2 by Sanger sequencing.

<sup>c</sup>Variant only found in 2 of 2 screened affected individuals of pedigree 5, as noted in the text.

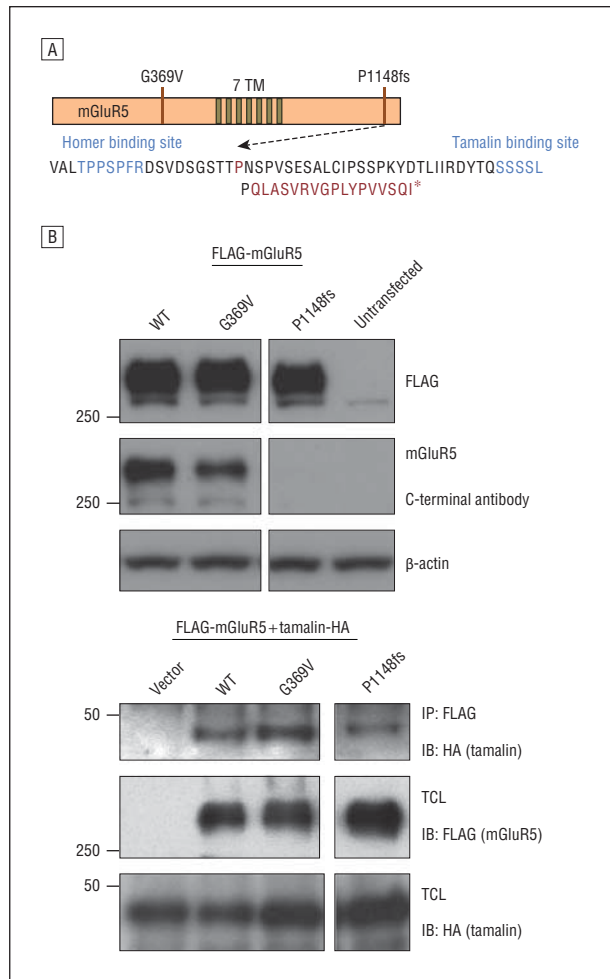
exome sequencing exhibited variants in the same genes as found in the other pedigrees. In fact, we observed a third *LRP1B* missense substitution, G4525E, confirmed by Sanger DNA sequencing of the pedigree (eFigure 1), in 2 of our affected individuals (III:3 and III:7). Although this *LRP1B* variant was not observed in all affected individuals in this pedigree, in light of the presence of variations in this gene in 2 other pedigrees, it may contribute to disease in these 2 pedigree members or phenocopies may exist in this pedigree.

### FUNCTIONAL EVALUATION OF mGluR5 AND PPEF2

One measure of genetic causality is whether mutations in the same gene are observed recurrently. Because only 1 pedigree was found to have alteration of *GRM5*, we performed molecular biological studies to determine if the observed variants are functionally consequential. Pedigree 2 transmits both a missense and frameshift substitution occurring in *cis* in *GRM5*, encoding mGluR5. G369 resides within mGluR5's extracellular domain (Figure 2A), and mutation there could alter sensitivity for glutamate. The *cis* P1148fs frameshift mutation locates to the distal carboxyl-terminal region and leaves intact the homer binding domain but deletes residues gov-

erning interaction with tamalin (Figure 2A). Homer and tamalin are scaffolding proteins regulating mGluR5 trafficking and signaling, and *HOMER* gene family polymorphisms have been associated with schizophrenia.<sup>30,31</sup> Given potentially disparate effects, we examined each variant separately. Neither disrupted interaction of mGluR5 with homer in coimmunoprecipitation assays (eFigure 2). P1148fs did, however, reduce coimmunoprecipitation with tamalin when compared with wild-type mGluR5 in HEK293 cells (Figure 2B). Additionally, using a standard receptor trafficking assay,<sup>22</sup> we observed that P1148fs, but not G369V, decreased surface expression (data not shown) and increased agonist-induced mGluR5 internalization in transfected hippocampal neurons (Figure 3). We conclude that *GRM5* P1148fs leads to reduced agonist-induced activation of mGluR5 in neurons through disruption of tamalin binding.

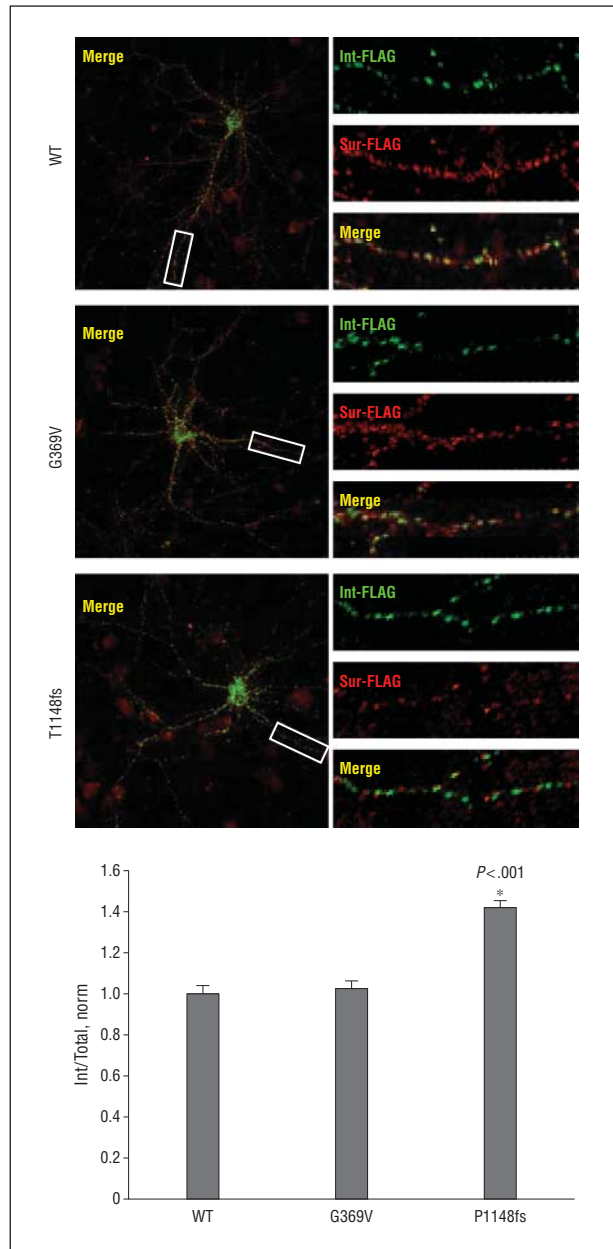
Intriguingly, all affected individuals in pedigree 3 shared a missense variant (R86H) in *PPEF2*. *PPEF2* encodes a neuronally expressed protein phosphatase that binds calmodulin.<sup>32</sup> Given that mGluR5 and *PPEF2* both bind calmodulin, we tested whether *PPEF2* expression influences mGluR5 levels. HEK293 cells transfected with *PPEF2* demonstrated increased levels of mGluR5 in the presence of the mGluR5 agonist (Figure 4), suggesting that disturbance of *PPEF2* could lower mGluR5 membrane levels.



**Figure 2.** Functional studies of metabotropic glutamate receptor subtype 5 (mGluR5). A, Schematic of mGluR5, showing location of variants. TM indicates transmembrane. B, Coimmunoprecipitation of cotransfected FLAG-tagged mGluR5 with HA-tagged tamalin in HEK293 cells. Upper panel documents equal expression levels and need for FLAG-tagged construct, given that the frameshift deletes the C-terminal epitope recognized by the native antibody. Lower panel represents cotransfection and coimmunoprecipitation with HA-tagged tamalin. (Images are cut from same film, same exposure to delete irrelevant lanes.) IB indicates immunoblot; IP, immunoprecipitation; TCL, total cell lysates; and WT, wild type.

## COMMENT

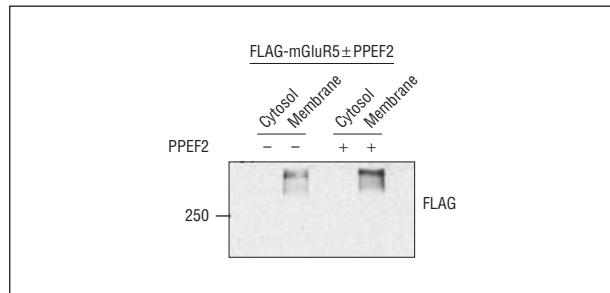
In 4 of 5 families where schizophrenia appears to be transmitted with single-gene inheritance, we identified novel or rare variants that were present in all affected individuals in 1 of 3 genes in the glutamatergic pathway (*GRM5*, *PPEF2*, and *LRP1B*). In the fifth pedigree, where linkage and haplotype analysis indicated possible oligogenic inheritance of schizophrenia, an additional variant of *LRP1B* was found in a subset of affected individuals (2 of 5 cases). We did not detect pathogenic CNVs in these families, as has been reported to occur de novo in sporadic cases of schizophrenia.<sup>7,8,11-13</sup> One possible explanation is that large rare CNVs confer risk for a more severe form of the disease (childhood onset or with associated comorbid intellectual disability), limiting reproductive fitness and subsequent familial transmission.



**Figure 3.** Effects of GRM5 variants on metabotropic glutamate receptor subtype 5 (mGluR5) trafficking. Agonist-induced internalization assays of mGluR5 variants on transfection of GRM5 of primary rat hippocampal neurons with 3,5 dihydroxyphenylglycine treatment. Int indicates internalized; norm, normalized ratio to WT; Sur, surface; and WT, wild type. \*Significant value.

Frameshift mutation (occurring in *cis* with missense substitution) of *GRM5* was present in pedigree 2 and found to reduce binding to the scaffolding protein tamalin, thereby increasing the internalization of its product, mGluR5, on agonist stimulation.

Glutamate is the major excitatory neurotransmitter in mammalian brains. Activation of mGluR5 enhances NMDA receptor signaling,<sup>25</sup> so its disruption is consistent with the “NMDA glutamate receptor hypofunction hypothesis” of schizophrenia.<sup>33</sup> *GRM5* does map near a translocation that segregates with schizophrenia and related affective disorders in 1 large family.<sup>34</sup> Intronic polymorphisms have also been associated with schizophre-



**Figure 4.** Immunoblot showing influence of PPEF2 on FLAG-tagged metabotropic glutamate receptor subtype 5 (mGluR5) on cotransfection in the presence of the 3,5 dihydroxyphenylglycine agonist in HEK293 cells.

nia in a case-control series.<sup>35</sup> Copy number variants or other mutations affecting *GRM5* have not been reported in schizophrenia,<sup>7,8,12,13</sup> but CNVs spanning the locus were recently reported in other psychiatric disorders (eg, attention-deficit/hyperactivity disorder<sup>36</sup>). Evidence implicating *GRM5* in schizophrenia has largely come from other types of studies.<sup>25</sup> *GRM5* exhibits altered expression in postmortem schizophrenic brains.<sup>37</sup> Its deficiency in gene-targeted mice produces behavioral changes characteristic of schizophrenia,<sup>38</sup> and mGluR5 agonists appear therapeutic in animal models.<sup>39</sup>

Missense substitution of *PPEF2* was observed in pedigree 3, and we demonstrate that *PPEF2* expression can modulate mGluR5 levels, presumably because of competition for binding of calmodulin. *PPEF2* has not been widely studied, but it is known to regulate hippocampal neuron morphology,<sup>40</sup> which has been reported as disturbed in schizophrenia.<sup>41</sup>

Three of 5 families demonstrated different missense substitutions in *LRP1B*. Significantly, *LRP1B*'s location on chromosome 2q coincides with the top-scoring peak identified using 1 of 2 strategies in meta-analysis of schizophrenia genetic linkage studies.<sup>4</sup> Recently, *LRP1B* was found to undergo strong genetic selection in modern humans.<sup>42</sup> *LRP1B* is homologous to another low-density lipoprotein receptor family member *LRP1*, which was recently found to undergo de novo mutation in schizophrenia.<sup>9</sup> Mouse gene knockout studies indicate that *LRP1B* and *LRP1* may functionally compensate for one another in the nervous system.<sup>28</sup> The residues of *LRP1B* substituted in pedigrees 1 and 4, G3458 and A924, respectively, fall within low-density lipoprotein receptor "ectodomains" involved in binding its ligands, which include apoE-carrying lipoproteins.<sup>27</sup> The variant observed in the 2 members of pedigree 5 locates to a position, G4525, within an alternatively spliced exon (exon 90) of *LRP1B*, which largely distinguishes *LRP1B* from *LRP1*. *LRP1B* binds to scaffolding protein PSD95 through the latter's PDZ domains,<sup>43</sup> which are also responsible for PSD95's binding of the NR2 subunit of the NMDA receptor.<sup>44</sup> Mice deficient for *LRP1B* exhibit subtle alterations in paired-pulse facilitation,<sup>28</sup> a proxy for schizophrenia in animal models.<sup>45</sup> Alteration of *LRP1B* could disrupt NMDA receptors through competition for PSD95. Tamalin also binds PSD95,<sup>46</sup> so *LRP1B* could possibly also regulate mGluR5 levels via sequestration of tamalin. Both *LRP1* and *LRP1B* modulate processing of  $\beta$ -amyloid precursor protein and are implicated in Alzheimer dis-

ease.<sup>47</sup> These observations may be significant given that psychosis is a feature of dementia associated with aging and that other genes implicated in schizophrenia,<sup>48</sup> including *NRG1*, encoding neuregulin 1, are also involved with  $\beta$ -amyloid precursor protein processing.

Taken together, our findings support a recent convergent functional genomics approach that evaluated a whole host of gene expression studies in humans and animal models to identify and prioritize genes.<sup>26</sup> Summing all existing studies, glutamate receptor signaling pathways was one of the key pathophysiological pathways in schizophrenia.

Limitations to this study include bias in the exome capture technique, mapping and variant calling programs, or filtering methods. However, since these rare variants reported herein were discovered using complementary genome-wide screening methods, validated via traditional sequencing and rarely found in large sequencing databases of controls, we believe that they play a role in schizophrenia in these families. Confirmation of their impact will require additional large case-control studies to estimate the frequency of these variants and confirm statistical evidence for the identified variants. Nonpenetrance of individual variants as evident in 1 of our families remains a challenge and inclusion of broader phenotypic assessments (eg, negative symptoms or schizophrenia-related endophenotypes) might be necessary to further evaluate the presence of subclinical phenotypes.

## CONCLUSIONS

Although schizophrenia remains genetically complex, our findings implicate genes involved with glutamatergic neurotransmission in rare multiplex families, which is likely to contribute to its etiology. mGluR5 agonists are already under study,<sup>33,39</sup> and *PPEF2* and *LRP1B* may point to further targets for schizophrenia drug therapy. Furthermore, additional pharmacogenomics studies that stratify patients based on the affected genetic pathways may better guide the most effective pharmacotherapy.

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